SINGLE MOLECULE FLUORESCENCE MICROSCOPY OF CARBON NANOTUBES

BY

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DISSERTATION

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Abstract

Single molecule microscopy has been extensively used in the past decade to study individual biomolecules with excellent spatial and temporal resolution. Meanwhile, characterization of nanomaterials and their development towards a variety of biological applications has progressed rapidly. Despite the comparable size of nanoparticles and biomolecules, no microscopy platform and technique currently exists to study the interactions of single biomolecules with nanoparticles.

Here, we have successfully developed a set of experimental tools to study the interactions of DNA and proteins on a nanomaterial surface. We observe nucleic acid-encapsulated carbon nanotubes by using fluorophore-labeled complementary DNA to explore the sequence-specific affinity of DNA to the nano-surface. Our results demonstrate cooperative exfoliation of the oligonucleotides from the nanomaterial surface and sequence-dependent bioavailability of nanotube-adsorbed DNA for hybridization. The platform is employed in conjunction with a super resolution algorithm to pinpoint sites of DNA hybridization along the length of the nanotube.

The ability of a microfluidic channel to easily exchange solution is used to study specific and non-specific nuclease activity on nanotube-adsorbed DNA. Protein function is mapped to local DNA topology on the nanomaterial and distance-dependent arrest of protein activity is observed, resulting in a nanotube-induced arrest of 60% protein activity within 1 nm from the nanoparticle. Accessibility of different points of contact between the DNA and nanotube are assayed for nuclease resistance and range from 5% to 50%.
To my dear family, friends and those who have believed in me
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Chapter 1

Introduction

Single molecule microscopy is an experimental technique in which the photons emitted by a fluorophore are collected and analyzed to understand the immediate environment of that molecule. We start by introducing fluorescence and develop the concept of fluorescence resonance energy transfer (FRET), a distance-dependent interaction between two fluorescent molecules that finds use as a nanoscale molecular ruler. FRET is then applied to study the intramolecular dynamics that occur when human telomeric DNA is treated with synthetic ligands designed as potential anticancer drugs. Switching from molecular biology to nanotechnology, we discuss nanomaterials and the need for understanding how biomolecules interact with them. We conclude by focusing on carbon nanotubes and motivate the development of an experimental platform that brings single molecule fluorescence microscopy to the study of nucleic acids and proteins on a nanomaterial surface.

1.1 Fluorescence

Fluorescence is a form of luminescence, the emission of a photon (with wavelength between 300 to 1500 nm) from an electronically excited state of a molecule [1]. Photophysical processes for an isolated molecule occur due to transitions between the electronic states with different
energy levels and are represented the Perrin-Jablonski diagram (Figure 1.1) [2].

![Figure 1.1 Perrin-Jablonski diagram of a general fluorescent molecule. The ground state $S_0$, the singlet electronic states $S_1$ and $S_2$, and the triplet state $T_1$ are represented by thick black horizontal lines while the thinner horizontal black lines depict the vibrational states associated with each electronic state. Photophysical processes (at different rates) occur due to transitions between different states are represented by color-coded vertical arrows.]

A molecule in solution at room temperature is most likely to be in its lowest energy ground state, $S_0$. On absorbing a photon (of wavelength $\lambda_1$), an electron in the $S_0$ state transitions into one of the vibrational states of $S_1$ or $S_2$ almost instantaneously in about $10^{-15}$ s. As this is faster than almost any intra- or inter-molecular process, the absorption spectrum of a molecule does
not change unless a modification occurs in its molecular structure itself. A molecule excited into one of the higher vibrational levels of $S_1$ loses energy through collisions with the environment and vibrational relaxation leads it into the lowest level of $S_1$ in $10^{-12}$ seconds. If the molecule was excited into the $S_2$ state, it undergoes an internal conversion into the $S_1$ state in $10^{-12}$ s before undergoing further vibrational relaxation down to the lowest level of $S_1$. The transition from $S_1$ to $S_0$ is called fluorescence and is accompanied by the emission of a less energetic photon (with a longer wavelength $\lambda_2$); this difference in wavelength is known as the Stokes shift. An alternative pathway for a molecule in the lowest energy $S_1$ state involves an intersystem crossing (at rates that can compete with fluorescence) by the molecule into a triplet state $T_1$. From $T_1$, the molecule can undergo radiative de-excitation via a much slower process (called phosphorescence), or return to $S_1$ (reverse intersystem crossing) due to numerous collisions with the solvent molecules.

By quantitatively detecting the photons emitted by a fluorophore, certain changes in the environment of the fluorophore can be detected by the resulting change in emitted fluorescence intensity. In particular, any physical or chemical process that competes with the fluorescent de-excitation pathway from $S_1$ to $S_0$ will affect the number of fluorescent photons emitted for fixed excitation intensity. When biomolecules are chemically conjugated with fluorophores, observing the number of fluorophores present and measuring their intensities can provide highly detailed information about the system being studied. However, if intra-molecular or inter-molecular interactions are being studied that require the detection of sub-nanometer distance changes [3], we need to introduce the concept of FRET.
1.2 Fluorescence Resonance Energy Transfer

Resonance Energy Transfer (RET) is a distance-dependent non-radiative energy transfer that occurs due to a dipole-dipole interaction between two weakly coupled molecules [4]. When both these molecules are fluorophores and the energy transfer results in fluorescence emission from both, it is termed Fluorescent or Forster Resonance Energy Transfer (FRET).

![Perrin-Jablonski diagram for a donor and acceptor fluorophore undergoing FRET.](image)

**Figure 1.2** (A) Perrin-Jablonski diagram for a donor and acceptor fluorophore undergoing FRET. (B) Distance dependence of FRET efficiency.

As seen from the combined Perrin-Jablonski diagram for the combined donor-acceptor system (Figure 1.2A), non-radiative energy transfer or FRET is an intermolecular process that competes with other S$_1$ to S$_0$ photophysical transitions [5]. If the fluorescence lifetime of the donor in the
absence of acceptor is $\tau_D^0$ (i.e. the time spend in the $S_1$ excited state before returning to the $S_0$ ground state), then the energy transfer rate from the excited donor to the acceptor is given by:

$$k_T = \frac{1}{\tau_D^0} \left[ \frac{R_0}{r} \right]^6$$

(1.1)

where $r$ is the distance between the donor and the acceptor and $R_0$ is the Forster radius i.e. the distance at which transfer and spontaneous decay of the excited donor are equally likely. This means a reduction in the photons emitted by the donor via fluorescence due to energy transfer to the acceptor. Non-radiative energy transfer excites one of the acceptor electrons from the ground state into a higher energy state, from which the electron can follow any of the de-excitation pathways described in Figure 1.1. Though the energy transfer from the donor to the acceptor does not involve a photon, the $S_1$ to $S_0$ transition for the acceptor can occur with the emission of a photon via fluorescence. The net result is the emission of fluorescence from both the donor and acceptor molecule, with the relative intensity of the two emissions depending on the energy transfer efficiency between the two.

The Forster radius depends on the dye pair used and is approximately 65 Å for TMR-Cy5 and 54 Å for Cy3-Cy5. More generally, it is calculated as:

$$R_0 = 0.2108 \left[ \kappa^2 \Phi_D n^{-4} \int_0^\infty I_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \right]^{1/6}$$

(1.2)

where $\kappa^2$ is the orientation factor between the two transition dipoles (and is approximated as $2/3$ when the molecules are freely rotate or as 0.476 if the acceptors are randomly distributed
but fixed in time with respect to the donors), $\Phi_D$ is the fluorescence quantum yield of the donor, $n$ is the average refractive index of the medium, $I_D(\lambda)$ is the fluorescence spectrum of the donor normalized so that $\int_0^\infty I_D(\lambda) d\lambda = 1$, $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor in M$^{-1}$ cm$^{-1}$, $R_0$ is in Å, and $\lambda$ in nm.

Thus, the FRET efficiency is the probability of a donor transferring its electronic energy to the acceptor and is defined as:

$$E = \frac{k_r}{k_r + 1/\tau_D^0} \quad (1.3)$$

In terms of the emitted intensity from a donor ($I_{\text{donor}}$) and acceptor ($I_{\text{acceptor}}$) molecule [6]:

$$E = \frac{I_{\text{acceptor}}}{I_{\text{acceptor}} + I_{\text{donor}}} = \frac{1}{1 + (r/R_0)^6} \quad (1.4)$$

The great utility of FRET is its dependence on the 6$\text{th}$ power of ($r/R_0$). A small change in $r$ near $R_0$ leads to a much larger change in FRET and for the dye pair shown in Figure 1.2B, a 3 nm distance change between the two molecules (from 4 to 7 nm) changes FRET from 0.8 to 0.2.

Thus, experimentally determining the donor and acceptor intensity at a given time gives the FRET efficiency $E$ and the strong dependence of $E$ on the inter-dye distance $r$ ensures that a change in $r$ results in a large, observable change in FRET. Single molecule FRET thus acts as a spectroscopic ruler and has been used to measure intra-molecular DNA [7], DNA-protein [8] as well as protein-protein [9] interactions in vitro and in vivo [10].
1.3 Telomeric G-Quadruplexes and Ligands

Telomeres are the DNA regions at the ends of linear eukaryotic chromosomes [11]. Human telomeric DNA is composed of (GGGTTA)_n repeats and terminates in a 3’ single-strand overhang 100-200 nucleotides in length [12]. Though single strand DNA usually adopts a random coil conformation in solution, telomeric DNA is capable of forming a higher order four-stranded helical structures, known as the G-quadruplex [13]. This DNA-GQ conformation forms due to the stacking of multiple G•G•G•G tetrads, can be inter or intra-molecular and is stabilized by cations like K⁺ and Na⁺. A large number of intra-molecular quadruplex structures have been solved as solutions with NMR or in crystal form [14]. The G-quadruplex conformation formed is highly dependent on the exact telomeric sequence, the cation used for stabilization [15], its concentration, sample temperature and the extent of molecular crowding present [16, 17].

![G-Quadruplex Structure](image)

Figure 1.3 Four planar guanine molecules form one quadruplex plane and a G-tetrad core.

The existence of these quadruplexes in vivo has been confirmed for primitive ciliates [18] and it is hypothesized that the folded quadruplex structure evolved to protect the single strand
chromosome end (formed after DNA replication) from being detected and processed as a single-strand break by DNA repair machinery [19]. It is not clear whether telomeric DNA in human cells exists at least transiently as a single strand or if it is always in a complex with multiple proteins surrounding it. However, the telomere is a very attractive target for anti-cancer drug design and is being thoroughly studied for the following reason [20].

Figure 1.4 The human telomere with the G-strand (blue) and the C-strand (red) is covered by the proteins that constitute the shelterin complex. Telomeric RNA (dashed black) is transcribed by DNA-dependent RNA polymerase II. In vitro, both telomeric DNA and telomeric RNA can form G-quadruplex structures.

In normal cells, telomeres gradually shorten with cell division until a certain replicative limit is reached (typically between 60 and 70), at which point the cell loses its ability to divide and often progresses into senescence or undergoes apoptosis [21]. Germ line cells avoid telomere shortening by having a low level of telomerase, a reverse transcriptase that synthesizes
GGGTTA repeats onto the G-strand, expressed to maintain a stable telomere length. In contrast, cancer cells avoid senescence by undergoing a number of transformations and one common trait shared by over 90% of cancer cells is the expression of telomerase. This immediately indicates a number of approaches to targeting telomeric DNA in cancer cells. If the G-quadruplex conformation exists in vivo, it provides a distinct recognizable three-dimensional structure for potential drugs to bind. Alternately, a ligand targeting the (GGGTTA)n sequence to induce quadruplex formation could better disrupt functioning in cancer cells.

We shall explore the changes induced in the intrinsic dynamics of a small telomeric DNA molecule when treated with a quadruplex-targeting ligand [22]. We use a single molecule FRET pair to observe the small conformational changes that occur on quadruplex folding and unfolding [23]. The first drug studied is a fixed-conformation macrocyclic ligand that can induce quadruplex formation. A different approach to ligand synthesis is inspired by the ability of simple chains of naturally occurring amino-acids to fold into complex three-dimensional protein structures [24]. The synthetic analog of this design philosophy are foldamers, repeating oligomers fold into specific three-dimensional conformations to interact with the target of choice. We test the interaction of this ligand with telomeric DNA as well.

1.4 Biological Interactions with Nanomaterials

Nanomaterials are materials possessing at least one physical dimension in the nanometer regime [25]. Nanomaterials have novel physical and chemical properties that have led to their use for various biomedical applications including imaging [26], in vivo sensing [27] and drug delivery [28]. These applications involve interactions between nanoparticles and cellular
components [29], many of which result from the recognition and functioning of proteins and nucleic acids on a nanomaterial surface. How biomolecules function and interact in the immediate environment of a nanomaterial surface is not well understood [30]. For an organic biomolecule optimized to interact with cellular machinery in vivo, an encounter with an engineered nanoparticle can be highly unpredictable [31]. While the physical parameters of nanoparticles including the size, shape, surface groups and charge have been correlated with biological activity and toxicity at the cellular, organelle and organismal level, the overall conclusions are contradictory [32].

Understanding these interactions is crucial for designing efficient biocompatible devices while reducing their potential toxicity. Although many tools exist to characterize both biomolecules and nanomaterials, these methods are currently unable to give a detailed picture of biomolecular structure at the nano-bio interface [33]. As a result, the molecular basis for local electronic properties, bioavailability, toxicological effects, and basic molecular structure and conformation of biomolecules on nanoparticles remain unclear [34]. We focus specifically on single-walled carbon nanotubes (SWNT) and motivate the requirement for developing a microscopy platform to answer questions of structure, molecular recognition and toxicity mechanisms for DNA and protein interactions on a nanotube surface.

1.5 Single Walled Carbon Nanotubes

Single walled carbon nanotube-based devices have various potential uses in biological applications due to their unique optical, chemical and mechanical properties [25, 35, 36],
including their successful application as biosensors [37-43], imaging agents [26, 44-47] and drug carriers [28, 48-51]. We describe what single walled nanotubes are and their relevant properties below [35, 52].

Single walled carbon nanotubes (SWNT) are hollow cylindrical nanostructures with a diameter close to 1 nm and lengths ranging from 100 to over 2000 nm. Composed entirely of carbon atoms, a SWNT can be visualized as an infinite sheet of graphene rolled into a cylinder along a well-defined vector. In the graphene coordinate plane shown in Figure 1.5, every carbon atom can be connected to the origin with the chiral vector $\vec{C}$, where

$$\vec{C} = n\vec{a}_1 + m\vec{a}_2 \text{ (where } n,m \text{ are integers and } 0 \leq m \leq n)$$

Figure 1.5 Graphene structure and chiral vector of a nanotube. Each $(n,m)$ coordinate defines a unique nanotube species and rolling the nanotube along the chiral vector (with P1 and Q1 superimposing on P2 and Q2 respectively) results in a cylindrical nanostructure.
The coordinate vectors \((n,m)\) uniquely specify every possible nanotube species and determine the diameter and electronic structure of each species. From purely geometric calculations, the diameter \(d\) of a \((n,m)\) nanotube is given by:

\[
d = \frac{r_{CC} \sqrt{3 \left[ n^2 + nm + m^2 \right]}}{\pi}
\]  

(1.2)

where \(r_{CC}\) is the carbon-carbon bond distance, 0.144 nm. The nanotubes used in our work have diameters ranging from 0.8 to 1.2 nm. The electrical properties of a SWNT also depend on the \((n,m)\) indices and in general, if:

\[
(n-m = 3q)
\]  

(1.3)

where \(q\) is an integer, the nanotube is metallic, while otherwise it will be semiconducting.

Though the unique physical, chemical and electronic properties of carbon nanotubes are being studied and developed for a vast number of applications, we shall focus on the use of DNA-wrapped single-walled carbon nanotubes (DNA-SWNT) as biologically relevant optical sensors.

Nanotubes display photoluminescence, i.e. on the absorption of a photon, an electron is excited into a higher energy state which leads to the formation of exciton, an electron-hole pair. Internal transitions lead to the electron returning to its initial state in in \(10^{-10}\) seconds and is accompanied by the emission of a photon. The emission wavelengths are in the near-IR, ranging from 950 to 1500 nm and this range overlaps the 900 – 1400 nm window where living tissues are most transparent. Additionally, SWNT as chromophores display no irreversible photobleaching, blinking or other photophysical artifacts present in conventional fluorophores.

Two challenges prevent the use of SWNT as biomarkers in vivo: (1) nanotubes are hydrophobic
and are not soluble in water, and (2) nanotubes form tight bundles due to π-π interactions between the surface electrons and this resulting nanotube aggregate is not photoluminescent.

DNA-assisted dispersion and separation of carbon nanotubes is one solution to both these problems [53, 54]. Every DNA nucleotide contains an aromatic base with π electrons present. When single strand DNA is sonicated in solution with a bundle of aggregated nanotubes, the cavitation bubbles created temporarily separate the individual SWNT and ssDNA molecules can bind to the nanotube sidewalls via π-π stacking interactions. DNA bases stack on the nanotube surface while the sugar-phosphate backbone is exposed to the solution (as shown in Figure 1.6), and this non-covalently modified DNA-wrapped nanotube (DNA-SWNT) is soluble and does not interact with other nanotubes. While several of the physico-chemical properties of the nanotube are altered due to the DNA interaction, individual DNA-SWNT are photoluminescent and can function as chromophores in the near-IR.

![Diagram of DNA-SWNT complex](image)

**Figure 1.6 Adopted from [55]** – A single strand DNA molecule self-assembles as a monolayer on single walled carbon nanotubes. The π electrons in the aromatic DNA bases stack onto the π electron system on the SWNT sidewall. This DNA-SWNT complex is soluble in aqueous solution.
DNA-SWNT have been developed for use as label-free in vitro and in vivo sensors. A nanotube can sense its immediate chemical environment for the presence of other molecules and a change in emission intensity and spectrum can be correlated with that particular molecule.

In Chapter 2, we describe the sample preparation, fluorophore labeling, experimental schemes, data acquisition and data analysis methods used in the rest of the work. Chapter 3 lays the groundwork visible TIRF microscopy of nanotubes by using near-IR emission to develop an immobilization and imaging platform. Chapter 4 introduces the single molecule total-internal-reflection (TIRF) microscopy technique used to study fluorophore-labeled nanotubes. In Chapter 5, we apply our platform to study the interactions of proteins and DNA on the nanotube surface. Chapter 6 focuses on telomeric DNA interactions with ligands, as measured with single molecule FRET.

1.6 References

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Chapter 2:

Single Molecule Fluorescence Microscopy: Instrumentation and Analysis

In this chapter, we shall describe the optical setup used for acquiring single molecule data, the data itself and the different analysis methods used. We shall also discuss some of the standard proteins and reagents used in the experiments described in the later chapters.

2.1 Instrumentation

The optical pathway described below is represented in Figure 2.1A [1]. A single mode laser with one emission mode is used as the light source for fluorophore excitation. Both the green and red laser light passes through a half-wave plate. This half-wave plate changes the polarization of the incoming beam by half a wavelength. The two beams are either transmitted or reflected by a dichroic mirror and combine at a polarizing beam splitter (PBS). By tuning the polarization of each excitation laser with the half-wave plate, the intensity of each laser excitation that passes through the PBS can be individually controlled. The laser light then enters a Pellin-Broca quartz prism at an angle controlled by a mirror (M1) and passes unhindered through the index-matching oil and the quartz slide surface. If this incident angle is sufficiently high, the beam undergoes total internal reflection on encountering the lower index-of-refraction medium inside the sample chamber. An exponentially decaying evanescent field is generated at the
quartz-buffer interface and penetrates ~ 100 nm into the sample chamber. Only the fluorophores located near the surface are excited by this field, guaranteeing a high signal to noise ratio in the fluorescence emission from this surface.

**Figure 2.1** (A) Optics layout for dual laser prism-type total-internal-reflection excitation and single molecule detection with an EM-CCD. (B) Spectral separation of two distinct emission wavelengths into spatially separated regions on one CCD, and the dual emission from Cy3 and Cy5 present on the same molecule.
The emission first passes through a notch filter that reflects the 532 nm and 633 nm excitation lines while transmitting the emissions from the fluorophores themselves. A mechanical slit is used to shape the emission into a 2:1 sided rectangle and a pair of doublet lenses expand the (75 µm x 75 µm) image to fill the (8.2 mm x 8.2 mm) CCD sensor. A dichroic mirror spectrally separates the green and red emission wavelengths that are reflected using mirrors M2 and M3 into the CCD. As seen in Figure 2.1B, both the green and red images are spatially separated on the CCD itself and are treated as two distinct images (channels) by our analysis software. The sample shown is DNA containing one Cy3 and Cy5 each that is simultaneously excited by both 532 nm and 633 nm excitation. A mapping algorithm is used to transform the locations of molecules in one channel into the corresponding locations in another. For the zoomed in molecule in Figure 2.1B, the Cy5 (red) emission is one pixel to the right and two pixels lower than the corresponding molecule in the Cy3 channel. In this way, by using an evanescent field to excite only the first 100 nm of the 100 µm sample chamber and a highly sensitive EM-CCD camera, single fluorescent molecules in solution can be reliably detected.

The FRET dye pair used for telomeric DNA studies in Chapter 6 is described here. The tetramethylrhodamine (TMR) and Cyanine 5 (Cy5) molecules shown (Figure 2.2A) have different structures but both contain aromatic groups with delocalized aromatic structures that make them fluorescent [2]. Their absorption and emission spectrum (Figure 2.5B) overlap significantly, which is a requirement for both radiative and non-radiative energy transfer. Experimentally, the absorption and emission peaks make it straightforward to excite TMR at 532 nm, then efficiently reject the excitation light and collect TMR and Cy5 emission.
Figure 2.2 (A) Molecular structure of Tetramethylrhodamine (TMR) and Cyanine 5 (Cy5) fluorophores. (B) The absorption and emission spectra for TMR and Cy5.
2.2 Data Acquisition

Figure 2.3 (A) A 16 x 16 pixel image of a Cy5 molecule obtained at 100 ms resolution and the corresponding contour plot of the intensity. (B) The same molecule imaged for 20 frames and the mean intensity image.
The data acquired from our sm-TIRF experimental setup is a series of 512 x 512 pixel 8-bit TIFF images and each pixel represents an intensity value ranging from 0 to 255. Because there is intrinsic noise associated with the measurements, we take the mean of 20 frames to create a composite image that is used to detect and identify single molecules. As seen in Figure 2.3A, the intensity profile becomes markedly more Gaussian for the 20-frame mean image and a Gaussian fit for the intensity profile in Figure 2.3B gives the width along the x-axis as 2.6 pixels and along the y-axis as 2.2 pixels. Each pixel represents ~ 140 nm in real-space and so a single fluorophore point source that is imaged as a 2 x 2 pixel Gaussian confirms that our imaging system is diffraction-limited. By identifying the location of a molecule and designating the pixel region that represents its total emission, we can extract the intensity of each molecule as a function of time from the 512x256 pixel stack that can contain hundreds of molecules.

2.3 Microfluidic Chamber Preparation

The microfluidic chambers used for prism-type TIRF microscopy are constructed of a quartz slide and a No.1 glass coverslip [3]. A diamond-bit drill tip is used with a high speed Dremel to
Figure 2.4 Physical construction of the microfluidic chamber and the BSA-Biotin or PEG preparation used to passivate the surface for single molecule experiments.

Drill ~1-2 mm holes into the quartz surface. Double-sided tape is cut into strips that then form individual channels on the slide surface of approximately 100 µm depth. To prevent non-specific interactions of the surface with other molecules, the inner chambers can be coated with either Bovine Serum Albumin (BSA) or Polyethylene Glycol (PEG). While BSA suffices in repelling DNA molecules from adhering to the surface, PEG is superior at passivating the surface from both DNA and proteins and is therefore preferred whenever possible. The BSA used is conjugated with biotin to form a surface with biotin sites available for binding to Neutravidin. When a PEG surface is used, 1% of the PEG is doped with biotinylated-PEG to offer biotin binding sites to Neutravidin.
2.4 Reagents and Proteins Used

Tris buffer: tris(hydroxymethyl)aminomethane buffer is the standard buffer used for the experiments described in this work. Tris buffer can maintain pH from 7.1 to 9.0. The ability of a PEG surface to minimize non-specific interactions with nucleic acids is significantly degraded below pH 7. Additionally, the imaging buffer used in our systems forms gluconic acid and leads to a decrease in pH. We therefore use between 10 to 50 mM Tris to maintain a stable pH over the entire length of the experiment.

BSA-Biotin: Bovine Serum Albumin (BSA) is a serum protein that efficiently coats the glass and quartz surfaces in the microfluidic sample chamber. By using a biotinylated form of BSA, non-specific interactions between nucleic acids and the slide chamber is minimized while Neutravidin binding to the biotin molecule on BSA is possible for downstream immobilization. We use 1 mg/mL BSA-Biotin to saturate any binding surfaces in less than a minute of protein incubation.

Neutravidin: Neutravidin is a tetrameric biotin binding protein that minimizes non-specific interactions with nucleic acids and proteins while binding very tightly to biotin ($K_d \sim 10^{-15}$ M). Binding of Neutravidin to surface immobilized BSA-Biotin or biotinylated PEG is significantly decreased in a salt-free buffer. We thus use 0.2 mg/mL Neutravidin in a buffer containing 50 mM or higher Na$^+$ to saturate biotin sites in less than a minute. Neutravidin can be easily
deactivated by vortexing and must be used without being subject to harsh mechanical agitation.

**Glucose Oxidase + Catalase:** Our imaging buffer is designed to reduce the concentration of molecular O$_2$ in the buffer. Glucose oxidase accomplishes this by converting glucose into gluconic acid and hydrogen peroxide. Catalase converts hydrogen peroxide into water and oxygen, with the net result of the coupled reaction being a decrease in the concentration of free molecular oxygen. Glucose oxidase requires a monovalent cation to function and works efficiently between pH 6.0 and 8.0.

### 2.5 Single Molecule Intensity Data

The data obtained for each single molecule in a TIR movie is an intensity-time trace (as shown in Figure 2.5A). The intensity of each step itself can be obtained for quantified and represented as an intensity step-size histogram (Figure 2.5B). The number of photobleaching steps can be obtained (Figure 2.5C) along with the photobleaching lifetime of each fluorophore (Figure 2.5D). These are the three standard methods used to analyze the intensity-time traces obtained from single molecules.
Figure 2.5 (A) Intensity time trace of a DNA-SWNT molecule labeled with multiple Cy3 fluorophores. (B) Histogram of the intensity of each photobleaching step obtained over all molecules in a movie. (C) Histogram of the number of photobleaching steps per molecules obtained over multiple movies in an experimental condition and (D) photobleaching lifetime for each Cy3 molecule obtained for all molecules in a single movie.
2.6 Single Molecule FRET Data

Figure 2.6 (A) Donor (green) and acceptor (red) emission from a single molecule showing anti-correlated dynamics. (B) FRET as calculated from Eq. 2.4 as a function of time. (C) The FRET histogram for the molecule in (A) and (B), binned over the entire intensity-time trace. (D) Dwell Time (survival) distribution for all the molecules in the experimental condition that this particular molecule was selected from. (E) FRET distribution for all the molecules in the experimental condition, showing the donor-only peak at 0.2 and three peaks in total.

The raw data obtained from single-molecule FRET is a pair of donor and acceptor intensities for each image frame (Figure 2.6A), identical to the method described in Section 2.6. FRET for each
time frame is calculated according to Equation 2.4 and represents the intermolecular distance between the donor and acceptor molecule (Figure 2.6B). A histogram of the FRET displayed by this molecule before donor or acceptor photobleaching displays a two state behavior with (uncorrected) FRET values at 0.4 and 0.8 (Figure 2.6C). The dwell times (time spent) in either of the two states shown (higher FRET state in this case) can be extracted from the FRET trace and plotted to reveal the dwell time distribution, double exponential in the case shown in Figure 2.6D. The FRET histogram for all of the active single molecules observed (Figure 2.6E) shows three FRET states at 0.4, 0.65 and 0.8 along with a donor-only peak at 0.18. This histogram displays the behavior of all the molecules present while the single-molecule histogram focuses on the behavior of a single molecule. As evident, single molecule data when averaged over a large number of molecules (> 10,000 in Figure 2.6E) can represent the ensemble behavior observed in bulk studies

### 2.7 Super Resolution Algorithm

The data acquired from the EM-CCD is processed as a stack of 8-bit grayscale TIFF images. A thresholding and fitting algorithm defines a contour along the perimeter of the fluorescent object (Figure 1A). The total intensity emitted by all the pixels within the contour is plotted as a function of time. Steps corresponding to the photobleaching of individual Cy3 molecules are clearly observable (Figure 2A). For each photobleaching step, 3 to 8 frames are defined as the “photobleaching event”. Three frames immediately before \([-3, -2, -1]\) and after \([+3, +2, \ldots]\),
(+1)] the photobleaching event are selected. By choosing one pre-bleaching and one post-bleaching frame, we generate 9 pairs of “image differences” [4]:

\((-3,+3), (-3,+2), (-3,+1) \mid (-2,+3), (-2,+2), (-2, +1) \mid (-1,+3), (-1,+2), (-1, +1)\)

Figure 2.7 (A) Diffraction-limited spot of a sub-500 nm with multiple fluorophores. (B) Intensity-time trace showing the photobleaching of multiple Cy3 molecules. (C) A 2D-Gaussian representing the intensity profile of a single fluorophore. (D) The location of every fluorophore along a nanotube surface.

Each image difference is fit to a 2D Gaussian to obtain the centroid coordinates and standard deviation in both x and y axis: \((x_i, y_j)\) and \((\sigma x_i, \sigma y_j)\) (Figure 2.7C). The set of 9 \((x_i, y_j)\) coordinates combined gives a \((x_{\text{mean}}, y_{\text{mean}})\) and a significantly smaller \((\sigma x_{\text{mean}}, \sigma y_{\text{mean}})\) that results in a
computed localization of the fluorophore to sub-diffraction limit spot. Repeating this procedure for each photobleaching step reveals the location of every fluorophore. As fluorophores can reversibly blink (temporarily appear dark), some of these locations correspond to the same fluorophore. On selecting the unique locations, the position of individual fluorophores is observed to lie along a linear object.

2.8 References


Chapter 3

Near-Infrared Microscopy of Single Walled Carbon Nanotubes†

Single walled carbon nanotubes (SWNT) form insoluble bundles that can be suspended in solution by various sequences of single and double stranded DNA [1, 2]. The near-IR photoluminescence emission from a nanotube is highly sensitive to changes in its environment [3] and was used to detect the conformational rearrangement of a DNA molecule adsorbed on its surface [4]. However, it was unclear whether the DNA on the nanotube was single or double stranded. The model proposed a divalent-cation induced B to Z transition of DNA on the nanotube, which requires double-stranded DNA but the (GT)_{15} sequence used does not form a Watson-Crick basepair. We answered this question using ensemble FRET to show that (GT)_{15} does not form a parallel or anti-parallel duplex in solution and hence cannot bind to a nanotube in a double stranded configuration [5].

† This work in this chapter has been published as:

(1) Divalent Ion and Thermally Induced DNA Conformational Polymorphism on Single-walled Carbon Nanotubes,


(2) Multimodal optical sensing and analyte specificity using single-walled carbon nanotubes,

With the understanding that single-strand (GT)$_{15}$ DNA tightly wraps around the nanotube with a periodic pitch, we developed the use of DNA-nanotube sensors for highly specific analyte detection with single-molecule sensitivity [6]. Molecular adsorption on the DNA-SWNT surface perturbs the electronic structure of the nanotube and modulates its photoluminescence emission [7]. We adopted the surface-immobilization chemistry previously only used to study biomolecules [8] to prepare and fix biotinylated DNA-SWNT onto a microfluidic chamber surface. By measuring the photoluminescence from a single surface-immobilized nanotube, we identified the reaction of individual hydrogen peroxide molecules with the sensor by observing discrete stepwise quenching in the near-IR intensity time traces.

### 3.1 Sample Preparation

Single-walled HiPCO carbon nanotubes (Rice University) were suspended with 30-base d(GT)$_{15}$ (Integrated DNA Technologies, Inc.), in a 1:1 mass ratio in 0.1 M NaCl in distilled water and bath sonicated for 1 h. The mixture was centrifuged for 90 min at 16,300 g and the pellet discarded. The supernatant was then dialyzed against 10 mM Tris Buffer at pH 7.4, using 100 kDa molecular weight cutoff dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA) for 3-5 days, to remove the free DNA, producing the DNA-SWNT samples used in the experiments. The fact that nanotubes were encapsulated in d(GT)$_{15}$ and individually dispersed in solution was confirmed by performing air and wet AFM on (GT)$_{15}$-SWNT.
3.2 AFM Imaging of (GT)$_{15}$-SWNT

Atomic Force microscope (AFM) samples were prepared by depositing (GT)$_{15}$-SWNT on freshly cleaved mica. Dry AFM measurements were made by first exposing the mica surface to 1 M MgCl$_2$ and drying with ultrapure nitrogen before depositing (GT)$_{15}$-SWNT solution at a concentration of 5-10 mg/L. Samples imaged in fluid were prepared by depositing the (GT)$_{15}$-SWNT solution onto the mica and adding 1 M MgCl$_2$ into a Veeco Fluid Cell. A Digital Instruments Multimode AFM in tapping mode was used for all measurements.

Figure 3.1 AFM images of DNA-SWNT complexes. (a) AFM height image of (GT)$_{15}$-SWNT on freshly cleaved mica conducted in air showing regular banding pattern of the d(GT)$_{15}$ oligonucleotide extending up to 0.8 nm above the surface of the nanotube. (b) Wet AFM of (GT)$_{15}$-SWNT in water shows diminished bands. (d-e) 3D renderings of the micrographs.
As seen in Figure 3.1, \( \text{d(GT)}_{15} \) wraps around the nanotube surface with a regular banding pattern. While \( \text{d(GT)}_{15} \) does not form a standard base pair, G-G and T-T pairs can hydrogen bond to form pseudo-base pairs [9]. Additionally, G-rich sequences are capable of forming higher order structures like G-quadruplexes due to hydrogen-stacking interactions between the guanine residues [10]. To test for the existence of any non-single strand structure formation by \( \text{d(GT)}_{15} \), we performed an ensemble FRET assay as described.

### 3.3 Ensemble FRET Assay for \( \text{d(GT)}_{15} \) Dimer Formation

Modified oligonucleotide sequences labeled with the Cy3 fluorophore, biotin, and NH\(_2\) were purchased (HPCL purified) from Integrated DNA Technologies Inc. (Coralville, IA). The sequences were 5'-Cy5-(GT)\(_{15}\)-Biotin-3', 5'-(GT)\(_{15}\)-Cy3-3' and 5'-Cy3-(GT)\(_4\)-G-(X)(GT)\(_{10}\)-3' where X is an amine bound to a C\(_6\) on a thymine. Oligonucleotide pairs were chosen to allow the formation of either a parallel strand duplex or an antiparallel strand duplex. Sequences were annealed in a 1:1 ratio of 2-4 \( \mu \)M concentrations in 10 mM Tris pH 8 with 100 mM Na or 1 M Na. They were heated to 85 °C in a PX2 Thermal Cycler (Thermo Electron Corporation, Milford MA) for 10 min, then gradually cooled down to 4 °C over a period of 6 h. The annealed solution at 4 °C was diluted in the 10 mM Tris pH 8.0 buffer and kept at 4 °C in a chilled quartz cuvette. Fluorescence measurements were performed on the diluted sample by exciting at 535 nm and measuring the emission with a Varian Cary Eclipse Fluorescence spectrophotometer. The temperature was controlled by a Varian Cary Single Cell Peltier accessory (Palo Alto, CA).
Figure 3.2 Fluorescence emission from the annealed DNA samples and the control of separate Cy3-ssDNA and Cy5-ssDNA on 535 nm excitation. The lack of Cy5 emission implies that no parallel or antiparallel duplex was formed, and hence no FRET between Cy3 and Cy5 occurred.

Based on the emission spectrum observed that, we found that the d(GT)$_{15}$ oligonucleotide does not form a duplex. An annealed d(GT)$_{15}$ oligonucleotide labeled with Cy5 at the 5’ end and an identical strand with Cy3 labeled at the 3’ were observed in ensemble. On exciting the annealed sample at the Cy3 absorption maximum of 535 nm, we expect to see a strong Cy5 emission at 664 nm due to FRET between the dye pair if double stranded DNA is formed. The GT annealed product did not produce an emission peak at 664 nm for either parallel or antiparallel duplex formation at 100 mM Na and at 1 M Na (Figure 3.2). The small peak visible at 660 nm is
understood to be Cy5 emission on indirect absorption of the 535 nm incident light. This can be seen in the dotted curve which is the sum of the emission obtained on separately excited Cy3-ssDNA and Cy5-ssDNA. Hence, we conclude GT does not form a parallel or antiparallel duplex in free solution.

3.4 Near-IR Microscopy of Surface Immobilized DNA-SWNT

Having confirmed that the coating around the nanotube in DNA-SWNT is a monolayer of single strand DNA, we used a biotinylated DNA strand to prepare DNA-nanotubes. We immobilized these nanotubes on a microfluidic slide surface using biotin-Neutravidin chemistry and performed near-infrared microscopy to measure photoluminescence from single nanotubes. DNA-SWNT formed with d(GT)₁₅ and HiPCO nanotubes and the single molecule experimental chamber for near-IR microscopy was prepared as follows:

Nanotubes were encapsulated with DNA via probe-tip sonication in a 4:1 DNA:HiPCO SWNT mass ratio for 2 min. A ratio of 1:4 biotinylated: non-biotinylated d(GT)₁₅ DNA was used to produce complexes with multiple biotinylated oligonucleotides per SWNT. Solutions were centrifuged at 16,000 g for 90 minutes and the pellet was discarded. A sample chamber for single-molecule experiments was created as described in Section 2.3. The surface was successively treated with 1 mg/ml biotinylated-BSA in T100 (10 mM Tris pH 8.0 and 0.1 M NaCl) and 0.2 mg/ml Neutravidin in T100. Biotinylated DNA-SWNT (concentration approximately 1 mg/L) in T100 was added to the sample chamber and incubated for at least 30 minutes before imaging (Fig. 3.3a). Channels were flushed with deionized water before imaging. An aliquot of
10 μM H₂O₂ was dropped on the inlet hole of the slide and allowed to diffuse into the sample chamber during data acquisition.

Figure 3.3 Single-molecule H₂O₂ detection. a) Scheme of biotinylated DNA–SWNT binding to a glass surface with BSA–biotin and Neutravidin. b) Single near-infrared movie frame showing photoluminescence from several DNA–SWNT complexes (scale bar, 10 µm). c) Fitted traces from a movie showing single-step SWNT emission quenching upon perfusion of H₂O₂. d) Histogram of fitted step sizes from five traces taken from one near-infrared movie.

The near-infrared photoluminescence spectra were acquired using 633 nm excitation and a Carl Zeiss Axiovert 200 fluorescence microscope coupled to a Princeton Instruments 2D-OMA V InGaAs camera with a 256 x 320 pixel array and Acton spectrograph. Visible fluorescence
images were acquired with an AxioCam MRm CCD camera. Spectra were processed by fitting to a Gaussian lineshape to determine the peak center wavelength and were captured at 1 frame/s.

Immobilized DNA–SWNT complexes were imaged using their near-infrared photoluminescence signal upon laser excitation (Fig. 3.3b). Time traces of SWNT quenching (Fig. 3.3c) were obtained by measuring the intensity of four pixel spots in movies recorded at 1 frame per second, resulting in multiple traces that exhibit single-step attenuation upon perfusion of H₂O₂. The traces yield a narrow histogram of normalized quantized intensity changes after regression with a stochastic step-fitting algorithm [11], confirming the discrete nature of the interaction (Fig. 3.3d). The average normalized step height of 0.05–0.1 is consistent with a 90-nm exciton excursion range [12] and spot size of 900 nm, confirming a quantized single-step magnitude of 0.1.

These measurements demonstrated single-molecule detection of H₂O₂, providing promise for a new classes of biosensors with this level of sensitivity i.e. single peroxide detection by a single carbon nanotube. Furthermore, it suggested the possibility of transporting the scheme shown in Figure 3.3A to a TIR microscope by using fluorophore-conjugated DNA to prepare and image single nanotubes in the visible spectrum.
3.5 References

Chapter 4:

Single-Molecule Platform for Visible Fluorescence
DNA-SWNT Microscopy†

Nanotubes can be unmodified, modified with synthetic chemical moieties or biofunctionalized with naturally occurring molecules such as nucleic acids, peptides and enzymes [1, 2]. The motivation for functionalization can be to tune the sensitivity of the nanotube [3] towards a particular application, to increase uptake and bioavailability of the nanoparticle into the cell [4-7] and to minimize cytotoxic outcomes that can result from multiple different pathways in vivo [8-10]. When a nanoparticle enters a biological fluid, its surface is immediately coated with a variety of proteins with different binding affinities [11-14]. Hence, there are two nano-bio interfaces that are of important. The controlled preparation of a functionalized nanotube with a DNA or peptide coating, and the interaction of this functionalized nanotube with biomolecules present in vivo. In this chapter, we shall focus on the interface between a nanotube surface and the single strand DNA molecules that wrap around it.

† The work in Chapter 4 is a manuscript in preparation:

Visualizing Biomolecular Structure, Allostery, and Toxicity Mechanisms on a Carbon Nanotube,

Jena PV, Landry MP, Heller DA, Jain A, Chemla YR, Strano MS, Ha T.
Current techniques to study biomolecules on the nanotube surface have been unable to provide a detailed picture of DNA on the nanotube surface [15]. The photoluminescence of nanotubes is modulated by a variety of different environmental stimuli and elucidating the cause of near-IR emission change is more difficult than merely detecting it. Any process on the nanomaterial surface that does not lead to a change in photoluminescence cannot be spectroscopically detected. Unfortunately, the near-complete quenching of most fluorophores in close proximity to the strongly absorbing π–rich carbon nanotube surface has hampered visible microscopy of carbon nanotubes. We extend the single-molecule platform demonstrated in Chapter 3 for near-IR detection to detecting visible emission from DNA-SWNT conjugated with organic fluorophores. In this chapter, we describe a surface immobilized TIR-based platform to observe individual DNA molecules on a nanotube and uncover details governing the interaction between DNA bases and planar aromatic fluorophores that strongly stack on the nanotube surface [16-18].

4.1 Preparation of DNA-SWNT

Single-walled carbon nanotubes produced via the HiPCO process were obtained from Rice University. Approximately 0.2 mg DNA was mixed with a w/w excess of SWNT in 800 µL 0.1 N NaCl and ultra-sonicated via probe tip sonicator (Fisher Scientific, Sonic Dismembrator Model 500, Pittsburg, PA, USA) for two minutes at 10 W in an ice bath. Samples were then centrifuged at 16,000 g for 90 minutes and the supernatant was purified with a Micron YM-100 filter
(Millipore, Billerica, MA, USA) to obtain dispersed DNA-SWNT. Samples were resuspended in 50 - 200 µL 20 mM Tris-HCl, 50 mM NaCl, pH 8.0 (T50 buffer) and stored at 4°C. Suspended fluorophore-labeled DNA-SWNT form a stable solution (> 5 years).

**4.2 Absorption Spectrum of DNA-SWNT**

![Absorption Spectrum of DNA-SWNT](image)

**Figure 4.1 Absorption Spectrum of DNA-Nanotube complexes.** (A) Supernatant obtained after sonicating (GT)$_{15}$+Cy3 DNA with HiPCO nanotubes and centrifuging for 90 minutes at 16,000 g. (B) Filtrate removed by Micron YM-100 filter showing only DNA and Cy3 absorption peaks. (C) Resuspended (GT)$_{15}$+Cy3-SWNT showing multiple nanotube absorption peaks and Cy3.
After probe tip sonication, the resulting mixture contains suspended and individually dispersed DNA-SWNT, free DNA, and unsuspended SWNT bundles. To purify the individually dispersed DNA-wrapped SWNT from this mixture, we centrifuged the sample and the absorption spectrum of the supernatant is dominated by the free ssDNA (Figure 4.1A). After the supernatant is filtered to separate free ssDNA (Figure 4.1B) from the suspended DNA-SWNT, the purified DNA-SWNT sample (Figure 4.1C) shows multiple absorption peaks corresponding to different nanotube species and the Cy3 peak resulting from Cy3-labeled DNA adsorbed onto the nanotube. We use the presence of multiple absorption peaks from ~350 to 800 nm as the spectroscopic signature of individually suspended carbon nanotubes.

![Absorption spectrum of unlabeled and Cy3-labeled (GT)$_{15}$--SWNT.](image)

**Figure 4.2 Absorption spectrum of unlabeled and Cy3-labeled (GT)$_{15}$--SWNT.**

Preparing DNA-SWNT with unlabeled (GT)$_{15}$ and (GT)$_{15}$-Cy3 ssDNA results in spectra that is identical except for the additional contribution between 500 and 600 nm by the Cy3 molecule.
(Figure 4.2), indicating that the (a) the incorporation of a fluorophore onto the DNA-SWNT does not affect the electronic states of the nanotubes and that (b) the process of sonication followed by coupling of Cy3 to the nanotube does not change the molecular structure of Cy3 in any way. For the single molecule experiments now described, we use between 100 to a 1000 fold dilution of the stock DNA-SWNT solution.

4.3 TIRF Imaging of Fluorophore-Labeled DNA-SWNT

Figure 4.3 (A) Experimental scheme for immobilizing and imaging fluorophore-labeled DNA-SWNT. (B) TIRF image showing multiple fluorescent objects (scale bar is 10 µm). Close-up shows linear objects corresponding to SWNT > 300 nm in length and circular spots for shorter SWNT. (C) The number of detected fluorescent molecules in the absence of Cy3-DNA-SWNT due to background alone is significantly less.

Previously, we had immobilized DNA-SWNT onto a Neutravidin-coated surface by using biotinylated DNA to suspend the nanotube. Any free biotinylated single-strand DNA that was not removed by filtration or dialysis would not affect the a near-IR measurement as that emission is from the nanotube alone. When using fluorophore-labeled DNA, the presence of
fluorescent biotinylated DNA would make differentiating the emission from DNA and from DNA-SWNT difficult and hence we rely on a non-specific interaction between Neutravidin and the DNA-SWNT surface for surface immobilization. The Cy3-labeled DNA-SWNT in Figure 4.3A was prepared using (GT)$_{15}$+Rnd5 DNA, a 50 base ssDNA composed of (GT)$_{15}$ followed by a 20 base sequence:

5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGT GCG AAT TCA CGG CTA AGC G Cy3 3’

and immobilized on the surface. Incubating nanotubes in solution for longer than 15 minutes does not lead to more surface immobilization.

The TIRF image in Figure 4.3B shows multiple Cy3-emitting DNA-SWNT in the imaging area (scale bar = 10 µm) that range from circular spots of ~ 4 pixel diameter to linear objects over 15 pixels in length. This variation in length is consistent with the length distribution of the nanotubes obtained by AFM and reflects the diffraction-limited spot size image that is formed by emission from a molecule less than 300 nm in length. The PEG-Neutravidin slide surface imaged (in Tris 20 mM pH 8.0 Na 50 mM buffer) also presents some background fluorescence emission that can be detected. These unidentified fluorescent objects can result from the autofluorescence of Neutravidin, impurities in the sample buffer and non-uniform coverage of the slide surface by the PEG coating. By exciting a nanotube-free channel as control and detecting the emission from it with the same experimental settings used as for the sample channel, we can compare the number of detected objects. As seen from the histogram in Figure 4.3C, we find 5 times more fluorescent objects in the sample channel than in the control, thus confirming that the fluorescence observed is from the (GT)$_{15}$+Rnd5+Cy3-SWNT.
Figure 4.4 (A) TIRF image from a surface control and a Cy3-(GT)$_{15}$-SWNT sample, (B) along with a histogram of the number and length of the detected objects.

To differentiate background fluorescence from the Cy3-DNA-SWNT sample by more than just the number of detected objects, we compared the fluorescence profile of the two by obtaining a series of TIRF images (as shown in Figure 4.4 A) and performing a thresholding analysis to detect the individual objects. A polygon outline for each object was drawn and to compare objects that vary in shape from linear to circular, we chose the longest dimension (Feret’s diameter) and plotted its histogram (Figure 4.4B). As evident, the majority of fluorescent objects detected in the nanotube channel are longer on the average than in the control channel and only the fluorescent nanotubes have molecules longer than 6 pixels in any dimension. This further differentiates surface-immobilized fluorophore-labeled DNA-SWNT from the background fluorescence detected in the microfluidic chamber.

In addition to the fluorescence resulting from the background, stray Cy3-(GT)$_{15}$ DNA that could non-specifically bind to the surface can also be a source of fluorescence. In this case, the
emission is from an actual fluorophore and will have comparable or even greater brightness than the emission from nanotube-adsorbed (GT)\textsubscript{15}+Cy3 ssDNA. We test this by immobilizing biotinylated (GT)\textsubscript{15}+Cy3 ssDNA on a surface and comparing the emission profile from individual fluorophores with the emission profile from (GT)\textsubscript{15}+Cy3-SWNT (Figure 4.5). In this case, the heterogeneity in shape and presence of longer molecules distinguishes a Cy3-labeled DNA-SWNT from Cy3-labeled ssDNA.

![Diagram](image)

**Figure 4.5 Emission profile difference between (GT)\textsubscript{15}+Cy3-SWNT and biotinylated (GT)\textsubscript{15}+Cy3-ssDNA** (A) TIRF images show the heterogeneity in the shapes of the nanotube sample whereas the DNA-only image contains homogeneous diffraction-limited spots. (B) Length-distribution histogram of the samples showing the homogeneous distribution (gray) of the DNA-only sample in contrast to the longer-tailed (green) DNA-SWNT.

To optimize the immobilization efficiency of (GT)\textsubscript{15}+Rnd5+Cy3-SWNT without using biotinylated DNA, we compared the number of DNA-SWNT detected on a PEG surface with and without Neutravidin. We found (Figure 4.6) an 8-fold increase in the number of immobilized
fluorophore-labeled DNA-SWNT when a Neutravidin binding layer was present above the PEG surface and for the rest of the DNA-nanotube experiments described, we continue to immobilize molecules this way. We next describe nanotube samples with different fluorophore-labeled nucleic acid sequences.

**Figure 4.6** Approximately 8 times more (GT)\textsubscript{15}+Rnd5+Cy3-SWNT bind to a 0.2 µg/mL Neutravidin coated PEG layer than to the PEG layer itself.

### 4.4 Fluorophore-Labeled DNA-SWNT Constructs

The following fluorophore-labeled ssDNA sequences were used to suspend nanotubes. (GT)\textsubscript{15} is a thoroughly studied sequence that yields the highest fraction of suspended nanotubes.

(GT)\textsubscript{15}+Cy3: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GTG 3’

We used a 20 base non-GT sequence appended onto (GT)\textsubscript{15} to form (GT)\textsubscript{15}+Rnd5+Cy3:

5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCG AAT TCA CGG CTA AGC G Cy3 3’
To test the interactions of an internally-labeled fluorophore-DNA sequence to suspend a nanotube and to learn of its interactions with the nanotube, we prepared \((GT)_{15}+\text{Cy5}+\text{Rnd1}: 5' \text{GTGTGTGTGTGTGTGTGTGTGT} \text{GCG /iCy5/ TGT CTA AGA T 3'}\)

A visual representation of the constructs is presented in Figure 4.7:

\[(GT)_{15}+\text{Cy3-SWNT} \quad (GT)_{15}+\text{Rnd5}+\text{Cy3-SWNT} \quad (GT)_{15}+\text{Cy5}+\text{Rnd1-SWNT}\]

**Figure 4.7 A visual representation of the fluorophore-labeled DNA-SWNT constructs**

Free fluorophore-labeled DNA was removed by filtration as described and the final total DNA concentration (both free and on nanotube combined) was below 10 nM in our sample preparation. This concentration is insufficiently low to bind non-specifically to the PEG-Neutravidin surface.

### 4.5 Quantifying Emitting Cy3 on DNA-SWNT

Approximately 170 fluorescent nanotubes per imaging area (75 µm x 37.5 µm) are detected when \((GT)_{15}+\text{Rnd5-Cy3 SWNT} \) is immobilized on the slide surface. We obtained intensity time traces as shown in Figure 4.8A that were taken at 100 ms time resolution. As the photobleaching of a single Cy3 molecule on each DNA-SWNT is detectable as the sharp intensity drop that occurs within one frame, we quantified the number of photobleaching steps.
observed per nanotube (Figure 4.8B) as $1.75 \pm 0.99$ ($n = 215$ DNA-SWNT).

![Graph A](image1.png)

**Figure 4.8 (A)** Three traces recorded at 100 ms time resolution showing multiple Cy3 fluorophores photobleaching on a single $(GT)_{15}+\text{Rnd5}+\text{Cy3-SWNT}$. (B) Distribution of the number of Cy3 detected per nanotube.

This number represents the number of active Cy3 molecules and is much less than the 15 – 30 DNA strands expected on a 500 nm nanotube. This indicates that between 50 to 90% of fluorophores are completely quenched by the nanotube surface and are hence undetectable.

To further study this quenching, we used $(GT)_{15}+\text{Cy3}$, a sequence that should bind even more tightly to the nanotube.

### 4.6 Characterization of Quenching by the Nanotube Surface

We immobilized $(GT)_{15}+\text{Cy3-SWNT}$ onto the surface and though fluorescent objects were visible on the CCD, they were not bright or distinct enough from the background counts to be
accepted by the spot-picking algorithm. As cyanine fluorophores are aromatic and can stack onto adjacent DNA bases in single and double strand DNA, we hypothesized that the quenching was due to the planar rings in the fluorophore stacking onto the nanotube surface. We attempted to mitigate this by adding unlabeled single strand (AC)$_6$ DNA as duplex formation with (GT)$_{15}$+Cy3 could separate Cy3 from the nanotube surface and lead to dequenching. On addition of (AC)$_6$ to (GT)$_{15}$+Cy3-SWNT, the number of visible fluorescent nanotubes increased dramatically (Figure 4.9 A). The fluorescent sample density increased from ~ 34 to over 211, a 5-fold increase (Figure 4.9B), indicating that most of the fluorophores on each nanotube are completely quenched. The (AC)$_6$ hybridized (GT)$_{15}$+Cy3-SWNT were over 3 times brighter (Figure 4.9C), signifying that the detected molecules before hybridization were also partially quenched. Thus, the planar structure of Cy3 allows it to stack onto the nanotube surface with complete quenching of the fluorophores present on a nanotube and partial quenching of the remaining visible fluorophores.

The schematic in Figure. 4.10A indicates how addition of unlabeled (AC)$_6$ can lead to dequenching of Cy3 off the nanotube surface. We count the number of detected molecules from 25 different imaging areas and plot the histogram in Figure. 4.10B, thus confirming that the increase in fluorescent molecules from 85.95 ± 5.34 (s.e.m.) to 296.1 ± 13.9 (s.e.m.) occurs over the entire slide surface area and is not some localized artifact. In both pre- and post-hybridization condition, we next obtained single molecule traces to calculate the number of photobleaching steps observed from each of the fluorescent nanotubes.
Figure 4.9 (A) Appearance of visible molecules in the TIRF image on addition of (AC)$_6$ to (GT)$_{15}$+Cy3-SWNT. (B) Increase in the number of detected nanotubes on hybridizing with (AC)$_6$ and (C) the significantly brighter molecules resulting from hybridization.

Initially, the number of emitting Cy3 per nanotube (Figure 4.8C) is strongly peaked at 1 with an average of 1.21 ±0.47 (s.d.). After hybridization with (AC)$_6$ and subsequent dequenching of Cy3, we detect 1.79 ±0.85 Cy3 per nanotube and the distribution is broader. Combining the increase in the number of fluorescent nanotubes with the increase in the average Cy3 per nanotube
indicates that approximately 200 DNA-SWNT became visible due to hybridization. Thus, over 80% of the nanotubes completely quench the fluorescence from all Cy3 molecules present on their surface and on the average, hybridization only recovers the fluorescence of two Cy3 molecules per nanotube.

We conclude that single fluorophore-labeled DNA-SWNT can be successfully imaged with visible TIRF microscopy. Using the excitation and emission optics now routinely available in biology research labs can make studying molecular biology on carbon nanotubes far more accessible. We determined the number of duplexes formed per nanotube to be low due to the strong interaction between the DNA bases and the nanotube surface. This would explain the inability of previous ensemble assays to detect hybridization of a fluorophore-labeled DNA strand to and off the nanotube. We find that planar organic fluorophores like Cy3 and Cy5 can stack on the nanotube surface and are completely or partially quenched. Dequenching via hybridization to a complementary strand can form between 1 to 2 duplexes per nanotube on the average and this rescues fluorescence in more than 80% of the nanotubes. Now, we shall apply this platform to study the interactions of incoming DNA and protein molecules with DNA-SWNT at the nanometer level.
Figure 4.10 (A) Schematic of (GT)\textsubscript{15}+Cy3-SWNT hybridized with a dark complementary strand, (B) the increase in sample density per imaging area along with (C) the change in the number of visible Cy3 per nanotube.
4.7 References


Chapter 5:

DNA and Protein Activity on a Carbon Nanotube Surface

We now have a working platform to image fluorophore-labeled DNA-SWNT with single fluorophore detection capability. With the surface-immobilization, imaging conditions and experimental controls in place, we are ready to study the biological interactions that DNA-SWNT would undergo when encountering proteins and nucleic acids. By applying the experimental techniques described in Chapter 2 to the surface immobilized DNA-SWNT platform developed in Chapter 4, we demonstrate the power of this approach to resolve biomolecular structure and activity on a nanomaterial surface.

Recent reports probing the immediate environment of a nanomaterial surface and the biomolecular reactions that occur within it indicate an incomplete understanding. For example, in vitro translation of a gold nanoparticle-DNA is enhanced due to non-specific adsorption of translation-related proteins to the nanoparticle [1], while a polyacrylic acid-functionalized gold nanoparticle can bind and induce unfolding of fibrinogen [2]. Hybridization of

† The work in Chapter 5 is a manuscript in preparation:

Visualizing Biomolecular Structure, Allostery, and Toxicity Mechanisms on a Carbon Nanotube,

Jena PV, Landry MP, Heller DA, Jain A, Chemla YR, Strano MS, Ha T.

**5.1 DNA Hybridizing to DNA-SWNT**

We apply the same procedure to immobilize unlabeled DNA-SWNT as previously developed to immobilize labeled DNA-SWNT, with the obvious difference that the DNA-SWNT molecules are now not visible. Though hybridization to the wrapping DNA with a complementary strand has been attempted by various groups, hybridization was not detected in earlier assays, likely due to the strong affinity of DNA nucleotides to the nanotube surface. DNA non-specifically adsorbed onto the nanotube surface can be hybridized to in bulk solution with the subsequent removal of the hybridized duplex. As we could detect single molecules of Cy3 from Cy3-labeled DNA-SWNT, we attempted to again exploit the high signal-to-noise afforded by TIRF excitation to hybridize to surface-immobilized unlabeled DNA-SWNT with fluorophore-labeled complementary DNA strands. We experimentally determined that flowing in 10 nM of a complementary single-strand DNA (the prefix c shall be utilized to denote a complementary strand, i.e. cDNA) and allowing it to incubate for 10 minutes saturates the binding capacity of a DNA-SWNT sample and incubation with a higher DNA concentration or for longer time period does not increase the number of visible nanotubes. Thus, a section of ssDNA dissociates off the nanotube surface and hybridizes with a complementary strand as shown in Figure 5.1A. In the TIRF image, these Cy3-cRnd1:(GT)15+Rnd1-SWNT hybridized DNA:DNA-SWNT complexes are
visible (Figure 5.1 B) either as linear extended objects longer than ~ 8 pixels in length or as
diffraction-limited spots of ~ 4 pixel diameter.

![Figure 5.1 Single-molecule TIRF microscopy of DNA-carbon nanotube complexes.](image)

**Figure 5.1 Single-molecule TIRF microscopy of DNA-carbon nanotube complexes.** (A) Nanotubes encapsulated with the (GT)$_{15}$+Rnd1 sequence are surface-immobilized and hybridized in situ with a Cy3-labeled Rnd1 complement (cRnd1). (B) Cy3-conjugated nanotube-DNA complexes visualized via TIR excitation appear as multiple fluorescent objects in a field of view (scale bar 10 µm). TIRF images show sub-300 nm length nanotubes as diffraction-limited spots and longer nanotubes as linear extended objects.

To confirm that the fluorescence objects observed in Figure 5.1B are due to Watson-Crick base-paired duplexes formed on the nanotube surface, we compare this count with the number of fluorescent spots resulting from the surface itself, the immobilized nanotubes, 10 nM Cy3 cDNA in the absence of nanotubes, and a nanotube sample hybridized with 10 nM of a non-complementary Cy3-xDNA strand in Figure 5.2A. Nearly 7 times more fluorescent objects form when DNA-SWNT is hybridized with its complementary strand, thus confirming the DNA-SWNT schematic shown in Figure 5.1A to be correct.
Figure 5.2 (A) Observed fluorescence is due to Cy3-cRnd1 specifically hybridized to Rnd1 adsorbed on a nanotube (n>20 for all controls, error bar is s.d.) (B) Intensity time trace (100 ms time resolution) of a (GT)$_{15}$+Rnd1-SWNT hybridized with Cy3-cRnd1 shows 6 photobleaching steps of similar amplitudes (243.0 ± 27.62 a.u). (C) A histogram quantifying the number of duplexes formed per nanotube for all DNA-SWNT in the sample (mean = 2.36 ± 0.09, s.e.m.) reflects the length distribution of the nanotubes.

Nanotube-immobilized molecules can be observed for extended periods and we obtain the emitted fluorescence from DNA-nanotube complexes as a function of time. The intensity-time trace of a particular single nanotube shows discrete steps corresponding to the photobleaching of 6 Cy3 molecules on the nanotube surface (Figure 5.2B). The intensity of each fluorophore is comparable, indicating that all six duplexes have relatively similar orientations with respect to the nanotube surface (Figure 5.22). Because each duplex contains a single fluorophore, the number of photobleaching steps observed per molecule represents the number of duplexes formed per functionalized nanotube. Nanotubes ranging in length from 100 to 400 nm can form between 1 to 11 duplexes with a median of 2 (Figure 5.2C). The distribution of quenching steps per nanotube is consistent with the nanotube length distribution, as measured by AFM (Figure 5.4A), and a Poisson fit indicates 0.46 duplexes per 100 nm length (Figure 5.4B). Correlating this
with AFM data and modeling assays suggests that approximately one in ten DNA strands on the nanotube are available for duplex formation.

For the duplex formed on the nanotube to be stable, it must have a minimum length that depends on its sequence, the salt concentration in the buffer and the temperature of the sample. The Tm as a function of duplex length for the (GT)$_{15}$+Rnd1 sequence

\begin{verbatim}
(GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT CTA AGG ATG CGT GTA)
\end{verbatim}

is calculated at room temperature and 200 mM Na$^+$ concentration (Figure 5.3A). As we do not observe any dissociation of the Cy3 cRnd1 in the experiment, stability of the duplex formed suggests a duplex length between 12 to 16 bp. Thus, a duplex between 4 to 6 nm in length projects out of the nanotube on the surface. To generalize the consistent intensity step size obtained for the molecule shown in Figure 5.2B, we calculated the normalized intensity step size for all the molecules present in the imaging area shown in Figure 5.1B. A histogram of the normalized step size (Figure 5.3B) is fit with a Gaussian distribution (over 90% of the intensities fall within the curve shown) and confirms the orientation of the duplexes formed per nanotube to be relatively similar. We left the sample in solution at 4°C and imaged it after 96 hours. As seen in Figure 5.2C, the duplex formed and the nanotube-Neutravidin interaction are highly stable with minimum reduction in sample density observed after four days.
Figure 5.3 (A) T_m calculations for the Rnd1 duplex formation as a function of total base-pair length. (B) Intensity step size normalized for each molecule as described, showing a relatively consistent step size of (0.2 ± 0.2) for over 90% of the molecules. (C) Fluorophore-DNA duplexed surface immobilized DNA-SWNT is stable for over 96 hours in the microfluidic slide chamber.

The absolute length of the DNA-SWNT sample is determined by AFM (Figure 5.4A) and follows a distribution that depends on the initial length of the nanotube sample and the overall sonication power and time. The length of fluorophore-labeled DNA-SWNT imaged with TIRF is shown in Figure 5.4B and though the pixel-length scale is not convertible and directly comparable with the AFM data, the two distributions are compatible. Modeling the number of duplexes formed on hybridizing to a DNA-SWNT as a Poissonian process is consistent with a nanotube sample of heterogeneous length and a constant probability of duplex-formation per unit length.
Figure 5.4 (A) AFM images of (GT)15+Rnd1-SWNT were analyzed and reflect the length distribution of the nanotube sample (B) Cy3 cRnd1 hybridized DNA-SWNT length distribution as imaged with TIRF. (C) Histogram of the number of Cy3 cRnd1 duplexes formed per nanotube can be fit as a Poisson distribution with the probability of 2.33 duplexes per nanotube.

5.2 Super Resolution Imaging to Resolve Duplex Locations

To map the spatial arrangement of Cy3-labeled duplex DNA on the nanotube surface, we developed a super-resolution algorithm capable of localizing the position of the oligonucleotides with sub-50 nm accuracy as described in Section 2.7. An extended and linear molecule as shown in Figure 5.5A appears to visually shorten in length as irreversible photobleaching of multiple Cy3 molecules takes place. The intensity emitted from the fluorescent molecule shows clear downward steps corresponding to Cy3 photobleaching. The emission profile corresponding to the loss of fluorescence from a single Cy3 molecule is fit with a two-dimensional Gaussian to localize the position of the dye at the centroid coordinates, forming a super-resolution image of dye positions along the nanotube [7]. We find a linear arrangement of fluorescent duplexes and observe their distribution along the surface of a 900 nm-long and a 300 nm-long nanotube with 10 nm resolution (Figure 5.5 B).
Figure 5.5 (A) Image and intensity time trace of a Cy3-cRnd1:((GT)$_{15}$+Rnd1-SWNT complex showing the decrease in observed nanoparticle length as Cy3 molecules photobleach at distinct time points. (B) Sub-diffraction limit distribution of fluorophores superimposed on the far-field image of 6 Cy3-Rnd1 duplexes along a 900 nm long nanotube and 8 Cy3-duplexes on a 300 nm long nanotube. (Error bars represent the standard error of the mean dye position, < 10nm).

Application of the super resolution algorithm for a molecule displaying three photobleaching steps is as follows. The emission intensity profile (3D surface plot) of the molecule is a convolution of multiple Airy disks originating from each Cy3 present and the intensity time trace defines regions where all three Cy3 are present (I) and when the first (II) and second Cy3 photobleach (III) in Figure 5.6A. By subtracting the images in Figure 5.6B as shown, we obtain the intensity emission corresponding to a single fluorophore (Figure 5.6C) and fit this with a 2D Gaussian to obtain the positions of the fluorophores in Figure 5.6D. This particular DNA-SWNT had three Cy3 molecules at a minimum distance of 434 nm along the nanotube surface.

A more refined version of this uses multiple frames from regions I,II and III to obtain multiple sets of fluorophore locations. The mean and standard error of the mean after 3 iterations can
localize the fluorophores to within 30 nm, to obtain the images shown in Figure 5.5B. We conclude that the fluorophores are not clustered on any specific region of the nanotube.

Figure 5.6 (A) an intensity profile of the selected fluorophore-labeled DNA-SWNT as each Cy3 bleaches. (B) The intensity time trace for this SWNT labeled with 3 Cy3 dyes. (C) The Gaussian
obtained from the difference in the intensity profile due to photobleaching occurring shows two distinct fluorophores in (A-B) and one in (B-C). (D) Two-dimensional Gaussian representation of the peaks in Fig. 5.6. By subtracting the relevant intensity projections to form a Gaussian representing one fluorophore, we fit it to a 2D Gaussian to obtain the location of each fluorophore detected.

5.3 Sequence Dependent Activity of Nanotube-Adsorbed DNA

Does the availability of DNA on the nanotube to hybridization depend on the specific sequence of the DNA? We use dual-color colocalization to compare the sequence-specific bioavailability of nanosurface-bound DNA. By hybridizing to GT$_{15}$-Rnd1-SWNT with a Cy3-labeled complement to Rnd1 (Cy3-cRnd1) and a Cy5-labeled complement to GT$_{15}$ (Cy5-cGT$_{15}$), we tag each nanotube with both fluorophores. A sequential imaging approach was used to measure Cy3 emission with a 532-nm laser and Cy5 emission with a 633-nm laser. We observe colocalized Cy3 and Cy5 emission from the same nanotubes (Figure 5.7A). As each GT$_{15}$-Rnd1 oligonucleotide offers a potential binding substrate to both complementary strands, the number of Cy3 and Cy5-labeled nanotubes reflects the relative accessibility of GT$_{15}$ and Rnd1 for hybridization. We detect nearly twice as many Rnd1-hybridized nanotubes to GT$_{15}$-hybridized nanotubes (~252 vs. 150), indicating that the GT$_{15}$ sequence binds approximately 67% more strongly to the nanotube as it is less available for hybridization than the Rnd1 sequence (Figure 5.7A). Preferential hybridization to different sections of the same oligonucleotide occurs due to unequal binding affinity of each section to the nanotube. Two additional variants of the random 16 base sequence were generated via cyclic permutation of a 5 nucleotide subsection of “Rnd” as
follows:

\[(\text{GT})_{15} + \text{Rnd2}: \ 5' \ \text{GTGTGTGTGTGTGTGTGTGTGTGTGT} \ \text{GAT} \ \text{GCG} \ \text{CTA} \ \text{AGT} \ \text{GTA} \ \text{T} \ 3'\]

\[(\text{GT})_{15} + \text{Rnd3}: \ 5' \ \text{GTGTGTGTGTGTGTGTGTGTGTGTGT} \ \text{GAT} \ \text{CTA} \ \text{AGG} \ \text{CGT} \ \text{GTA} \ \text{T} \ 3'\]

After hybridizing with cGT\(_{15}\) and the proper complementary sequence cRnd, the fraction of duplexes formed with Rnd was consistently two-fold higher (Figure 5.7B). In contrast, hybridizing the two complements to a GT\(_{15}\)+Rnd strand immobilized on a glass surface, in the absence of nanotubes, forms an equal number of GT\(_{15}\) and Rnd duplexes (Figure 5.8). Our single-molecule results demonstrate sequence-specific availability of nanoparticle-bound DNA to hybridization, are consistent with surfactant-based ensemble assays, and quantify the ratio of availability of each strand. Extending this to four color emission [8] can further probe the sequence-specific availability and arrangement of multiple distinct DNA sequences.

![Figure 5.7. Activity of nanotube-adsorbed DNA. (A) The nanotube-bound (GT)\(_{15}\)+Rnd1 oligonucleotide is hybridized with a 1:1 mixture of complementary Cy3-cRnd1 and Cy5-c(GT)\(_{15}\) forming multiple Rnd1 (cyan) and GT\(_{15}\) (magenta) duplexes on the same nanotube (scale bar 10 \(\mu\)m). A histogram comparing the number of (GT)\(_{15}\) duplexes and Rnd1 duplexes per](image)
imaging area (B) More duplexes form with the Rnd1 portion than with the (GT)$_{15}$ section of the oligonucleotide on the nanotube.

For the DNA control shown in Figure 5.8, hybridizing Biotin-GT$_{15}$+Rnd1 ssDNA with Cy3 cGT$_{15}$ (green) and Cy5 cRnd1 (magenta) simultaneously results in a DNA duplex that contains both Cy3 and Cy5 molecules. By sequentially detecting the Cy3 and Cy5 emission, we observe a similar number of fluorophores from the same imaging area i.e. an equal number of cGT$_{15}$ and cRnd1 duplexes are formed. In contrast, twice as many duplexes with cRnd1 were observed on the nanotube. Thus, preferential hybridization with the Rnd section over the GT$_{15}$ section only occurs on the nanotube surface.

Figure 5.8 Sequence-dependent hybridization to DNA in the absence of nanotubes. Hybridizing with cGT$_{15}$ and cRnd1 to (GT)$_{15}$-Rnd1 DNA results in an identical number of Cy3 and Cy5 labeled DNA molecules.
The reliability of these results depends on the detection efficiency of Cy3 and Cy5 emission and the accurate colocalization of emission from the same molecule. For the fluorophore detection control shown in Figure 5.9, hybridizing to GT15+Rnd1-SWNT with Cy3 cGT15 and Cy5 cGT15 gives identical number of Cy3 and Cy5 labeled nanotubes, as does hybridizing to GT15+Rnd-SWNT with Cy3 cRnd1 and Cy5 cRnd1. As the number of Cy3 and Cy5 duplexes is expected to be identical in both cases, this indicates the lack of bias in the detection of Cy3 and Cy5 molecules by the optical system.

Figure 5.9 Detection as a function of fluorophore. Hybridizing to (GT)15+Rnd1-SWNT with (i) Cy3 cRnd1 and Cy5 cRnd1 and with (ii) Cy3 c(GT)15 + Cy5 c(GT)15.
The second set of controls tests the colocalization accuracy of the mapping algorithm coupled with the standard set for colocalization. A DNA duplex with both Cy3 and Cy5 fluorophores is immobilized onto the surface (Figure 5.10A). The locations of Cy3 molecules after Cy3 excitation and Cy5 molecules after Cy5 excitation are colocalized if they lie within 6 pixels and approximately 63% colocalization is observed. In contrast, when two ssDNA samples, containing either Cy3 or Cy5 are immobilized on the surface at a comparable concentration, the degree of colocalization due to stochastically neighboring DNA molecules is significantly less (Figure 5.10B).

The emission from Cy3 and Cy5 labeled molecules is spatially separated in the detection optics via a dichroic mirror, thus creating two 512 (l) x 256 (w) pixels windows on the 512 x 512 pixel EM-CCD detector. The x and y position of a molecule in the Cy3 channel is mapped to a x and y position in the Cy5 channel using a polynomial morphing of the Cy3 and Cy5 image with a bead sample that emits in both detection channels. To prevent multiple molecules from being identified by the image analysis algorithm as one molecule, we control the sample density on the surface such that ~ 6 pixels separate each fluorescent molecule from the next. Therefore, the difference in number of Cy3 and Cy5 molecules observed from the same imaging area when hybridizing to a nanotube sample is due to a difference in the accessibility to hybridization of the different DNA sequences on the nanotube.
Figure 5.10 Controls for colocalizing emission from Cy3 and Cy5 present on the same molecule. (a) Approximately 63% colocalization between a Cy3 and Cy5 DNA construct and less than 12% colocalization for a similar sample density with the fluorophores present on separate DNA strands.

5.4 Difference in Hybridization to (GT)$_{15}$ and (GT)$_{30}$-SWNT

The (GT) motif binds tightly to a SWNT surface and has been the subject of various experimental and computational studies. The structure of (GT)$_{15}$ and (GT)$_{30}$ wrapped SWNT as observed with AFM was found to be very similar [9]. When hybridizing (GT)$_{15}$ or (GT)$_{30}$ with Cy3-T$_6$-(CA)$_5$-C (Cy3 cGT$_{15}$), the duplex formed is only 12 bp in length and hence multiple cGT$_{15}$ can hybridize to the same (GT)$_{15}$ sequence. Naturally, more duplexes can form when hybridizing to (GT)$_{30}$. If a section of ssDNA must desorb off the nanotube to be accessible for hybridization with a complementary strand, then (GT)$_{30}$-SWNT might project a longer ssDNA substrate into...
solution than (GT)\textsubscript{15}. Finally, a study of DNA-functionalized gold nanoparticles found binding strength of oligonucleotides to the nanoparticle to be length-dependent. To test the effect of (GT) length, we prepared (GT)\textsubscript{15}-SWNT and (GT)\textsubscript{30}-SWNT and hybridized both with Cy3 cGT\textsubscript{15} (Figure 5.11). On analyzing the number of Cy3 duplexes formed per nanotube, we saw no difference with a mean of 1.66 duplexes for (GT)\textsubscript{15}-SWNT and 1.84 for (GT)\textsubscript{30}-SWNT. In contrast, hybridizing to biotinylated single strand (GT)\textsubscript{15} and (GT)\textsubscript{30} revealed significantly more duplexes (Figure 5.12) formed with (GT)\textsubscript{30}. Indeed, even though each nanotube has between 10 – 30 ssDNA strands adsorbed on it, the number of available binding sites for all these strands combined is still less than the number of duplexes formed with just one molecule of (GT)\textsubscript{30}. We conclude that the duplex forming potential of (GT)\textsubscript{15} and (GT)\textsubscript{30}-SWNT is statistically identical. The duplex formed does not project out in solution from the SWNT surface by a significant length of the single strand DNA section preceding it because (GT)\textsubscript{30} did not display any more binding than (GT)\textsubscript{15}.
Figure 5.11 (GT)$_{15}$-SWNT and (GT)$_{30}$-SWNT display a similar number of sites for hybridization to a Cy3-labeled complementary DNA strand.

Figure 5.12 Hybridizing to (GT)$_{15}$ and (GT)$_{30}$ ssDNA without the nanotube shows nearly twice as many duplexes formed for (GT)$_{30}$ than for (GT)$_{15}$. 
5.5 Hybridizing to the 5’ and 3’ end of (GT)$_{15}$-SWNT

![Diagram of (GT)$_{15}$-SWNT hybridization](image)

Figure 5.13 (GT)$_{15}$-SWNT hybridized with (A) 5’ Cy3 – T$_6$ – (CA)$_5$-C i.e. (5’ Cy3 cGT$_{15}$) has a similar probability of duplex formation per unit nanotube length as (B) (CA)$_5$-C-T$_6$-Cy3 3’.

It is not known if the nanotube surface binds more tightly to the 5’ or 3’ end of DNA, or if the directionality of the DNA single strand is not relevant in terms of binding affinity. We tested this by hybridizing to surface-immobilized (GT)$_{15}$-SWNT with (Figure 5.13A) a 5’-Cy3 cGT$_{15}$ that would hybridize at the 3’ end of (GT)$_{15}$ and with (Figure 5.13B) a 3’-Cy3-cGT$_{15}$ that would
hybridize to the 5’ end of (GT)$_{15}$. As seen in the histograms, $1.81 \pm 0.04$ duplexes per nanotube were formed with 5’ Cy3 cGT$_{15}$ and $1.88 \pm 0.04$ with 3’ Cy3 cGT$_{15}$. Hence, the nanotube does not bind with different affinity to the 5’ or 3’ end of the (GT)$_{15}$ sequence adsorbed on it.

5.6 Allostery during Hybridization to DNA-SWNT

Figure 5.14 Cooperativity observed during DNA hybridization to nanotube. (A) The nanotube-bound oligonucleotide is hybridized with complementary Cy3-labeled strand (cY), which can form cY:Y duplexes at multiple Y sites (green) along the nanotube. (B) For DNA sequence (GT)$_{15}$+Rnd, the number of cRnd duplexes formed per nanotube is $2.3 \pm 0.2$ and for DNA sequence (GT)$_{15}$, the number of duplexes formed on hybridizing to the 5’ end of (GT)$_{15}$ is $1.5 \pm 0.3$. (C) The probability of duplex formation at Y (blue) by cY increases when hybridizing at X (purple) by cX is also taking place. (D) The number of cRnd duplexes per nanotube hybridized to (GT)$_{15}$+Rnd in the presence of c(GT)$_{15}$ increases by 32% to $3.1 \pm 0.2$ while the number of
duplexes formed at the 5’ end of (GT)₁₅ in the presence of 3’ c(GT)₁₅ increases by 60% to 2.4 ± 0.1. Positive cooperativity is not observed in the absence of the nanotube.

The current model for nanotube-adsorbed DNA predicts complete surface coverage of the nanotube surface by multiple DNA strands. The equilibrium conformation of DNA molecules and the nanotube is disturbed by transient defects that temporarily make the surface at the defect location accessible to outside chemicals, like surfactant molecules [10]. We hypothesized that stability of the DNA-nanotube system might be significantly perturbed across more than just the immediate location of the defect because the different DNA strands on a nanotube are still one conjugated π-electron system. The question being posed here is, could hybridization of a complementary DNA strand to a specific section of the nanotube-adsorbed DNA strand change the probability for duplex formation at a different section on another strand? We tested this possibility with two assays.

For a nanotube-adsorbed DNA sequence with distinct 5’ (X) and 3’ (Y) regions, duplex formation with a Cy3-labeled complementary strand to Y (cY) can occur at multiple Y sites along a nanotube (Figure 5.14A, green): Hybridizing to Rnd on GT₁₅+Rnd forms 2.3 duplexes per nanotube while hybridizing to the 5’ end of GT₁₅ forms 1.5 duplexes per nanotube (Figure 5.14B). However, if duplex formation is possible at both ends of the DNA strand, then the presence of cX hybridizing to the 5’ end at X (purple in Figure 5.14C) significantly increases the probability of duplex formation by cY at the 3’ end, Y (blue in Figure 5.14C). We detect a 32% increase in the number of Rnd duplexes when cGT₁₅ is hybridizing to nanotube-adsorbed GT₁₅+Rnd and a 60% increase in the number of duplexes formed on the nanotube at the 5’ end of (GT)₁₅ when duplex formation at the 3’ end can also take place (Figure 5.14D). This
cooperativity is not observed in the absence of the nanotube (Figure 5.15A), is not due to intrinsic differences in the 5’ and 3’ end of ssDNA (Figure 5.13) or the specific oligonucleotide sequence used (Figure 5.15B). Thus, we observe the nanotube to induce allosteric effects during DNA hybridization, illustrating how biological processes can be fundamentally altered by interactions with a nanoparticle surface.

Surface immobilized (GT)$_{15}$+Rnd1-SWNT was hybridized with Cy3 cRnd1 and with a 1:1 ratio of Cy3 cRnd1 and cGT$_{15}$. Hybridization of the Rnd1 complement (cRnd1) to nanotube-bound GT$_{15}$-Rnd1 is increased by 43% from 0.46 duplexes to 0.61 every 100 nm in the presence of the cGT$_{15}$ complement, while no such effect is observed in the absence of the nanotube. Thus, the cGT$_{15}$ duplex formation on one strand increases the probability of cRnd1 duplex formation on another strand. Both duplexes are unlikely to have formed on the same strand as we never observe FRET when Cy3 cRnd1 and Cy5 cGT$_{15}$ are used. This property holds for multiple variations of the Rnd sequence and controls confirm the result to be independent of fluorophore positioning and detection artifacts.

We conclude that hybridization of multiple short oligos onto different target regions on a longer DNA substrate adsorbed on a nanoparticle is cooperative. As the increase in duplex formation occurs on a different DNA strand than the strand being hybridized to, the cooperativity observed is a form of allosteric activation due to long-range destabilization of the DNA-nanotube system.
Figure 5.15 (A) Number of duplexes formed when Cy3 cRnd1 is hybridized with surface-immobilized 5'-Biotin-(GT)_{15}+Rnd1 ssDNA in the presence of c(GT)_{15} and on its own shows no cooperativity. (B) Two variations of the 16-nucleotide Rnd sequence were generated to form (GT)_{15}+Rnd1/2/3-SWNT, to ensure that cooperativity was not specific to the (GT)_{15}+Rnd1 sequence but to Rnd2 and Rnd3 as well. When (GT)_{15}+Rnd-SWNT was hybridized with cRnd alone and with a 1:1 equimolar ratio of cRnd and cGT_{15}, more cRnd duplexes were formed in the presence of the cGT_{15} strand.
5.7 Interaction of DNA-SWNT with a Single-Strand Nuclease S1

When nanoparticles enter a biological fluid like blood or plasma, the nanomaterial surface is coated with proteins that may interact with other proteins or undergo a conformational change and display unexpected downstream behavior. The classes of biomolecules that a DNA-SWNT is likely to encounter in vivo are nucleases that are designed to recognize and act on accessible DNA strands. We proceeded to characterize the interactions of DNA-SWNT with a non-specific nuclease S1 and with a restriction endonuclease DdeI.

The bioavailability profile of nanotube-bound DNA was mapped using S1, a small monomeric nuclease that non-specifically cleaves ssDNA [11, 12]. We used the three ssDNA sequences with Cy3 fluorophore conjugated to an oligonucleotide shown in Figure 5.5, namely (GT)_{15}+Cy3-SWNT, (GT)_{15}+Rnd5+Cy3-SWNT and (GT)_{15}+Cy5+Rnd1-SWNT to prepare fluorophore-labeled DNA-SWNT and immobilized it on the slide surface (Figure 5.16A). We define the accessibility of a nucleotide position by the fractional decrease in fluorescent nanotube density upon oligonucleotide cleavage and observe a 47% reduction due to cleavage of (GT)_{15}+Rnd5+Cy3-SWNT (Figure 5.16B). In contrast, the accessibility of the Cy3-labeled 3’ terminal nucleotide on (GT)_{15}+SWNT is 18% (Figure 5.16C), indicating that the terminal base is less accessible on (GT)_{15} than on (GT)_{15}+Rnd5. The accessibility of an internal nucleotide positioned 7 bases from the 3’ end of (GT)_{15}+Rnd1-SWNT is significantly attenuated at 4.8% (Figure 5.16D), indicating a much stronger protective influence of the nanotube on internally-positioned bases. The distance between the fluorophore and the nanotube, combined with the size of S1 suggests that the nuclease makes physical contact with the nanotube-DNA interface. Combining these results
into an accessibility map (Figure 5.16E) indicates that nuclease-induced cleavage of a functionalized nanoparticle can be modulated with the appropriate choice of nucleotide sequence and position observed for DNA hybridization.

Figure 5.16. Mapping the accessibility of nanotube-bound DNA to nuclease activity. (A) Nanotube-immobilized, Cy3 labeled (GT)$_{15}$-Rnd5 oligonucleotide is treated with S1 nuclease, resulting in a reduction of fluorescent spots (scale bar 10 µm). (B) A decrease in observed sample density due to cleavage of the fluorophore illustrates the fraction of substrate...
accessible to the nuclease (C) Cy3-labeled, nanotube-bound (GT)₁₅ sequence and (D) internally-Cy3 labeled DNA exhibit significantly diminished accessibility to S1 nuclease. (E) Accessibility map of different regions of DNA-encapsulated nanotubes to nuclease activity (n>100 for all samples).

As a control to confirm the functioning of S1 nuclease, we prepared a biotinylated (GT)₁₅-Cy3 single strand DNA and immobilized it on a Neutravidin-PEG surface (Figure 5.17A). On reacting the DNA substrate with S1 N at 1X reaction condition, we observe over 95% cleavage (Figure 5.17B), thus indicating that S1N activity on the DNA-SWNT is indeed modulated due to the presence on the nanomaterial surface.

Figure 5.17 (A) Surface immobilized biotinylated GT15-Cy3 ssDNA is reacted with S1 nuclease. (B) Over 95% degradation occurs and the fluorescent emission from the DNA substrate is reduced to background levels.

These results have clear implications for nano-biomolecule interactions, as the function and activity of DNA adsorbed onto the surface of a nanoparticle are shown to vary in their accessibility to biomolecules. Both the rate at which DNA on the nanotube is degraded and the
bioavailability of a chemical moiety carried by the DNA-SWNT will depend on the relative position on the nanotube surface.

5.8 Interaction of DNA-SWNT with an Endonuclease DdeI

The structure adopted by a protein in the immediate vicinity of a nanomaterial surface, and the modulation of a proteins activity as a function of distance from the nanomaterial are important to understand when designing novel nanoparticles intended for in vivo targets. To construct an assay that can report on the activity of a protein at a well-defined distance from the nanomaterial surface, we used the hybridization method described in section 4.1 to form a duplex with a recognition site for a restriction endonuclease at a fixed distance from the nanotube. The effects of nanoparticle proximity on protein functioning was then probed with nanometer resolution as follows.

Nanotubes were encapsulated with an oligonucleotide containing a Ddel endonuclease restriction site(Figure 5.18A). In situ hybridization to the Ddel-9bp sequence with a Cy3-labeled complementary strand (Cy3-cDdel-9bp) forms a duplex containing the Ddel restriction site 9 basepairs away from the nanotube. Upon reaction with Ddel under standard conditions, we observe a 70% decrease in the fluorescent nanotube density due to cleavage of the Cy3-labeled section of the duplex by Ddel (Figure 5.18B). The nanotubes themselves are still present on the surface and we confirm this by hybridizing the post-reaction complex with a Cy5-labeled complement to (GT)15 to recover the initial fluorophore density (Figure 5.19). Upon preparing nanotube samples with DNA sequences of the form (GT)15+Ddel-nbp, with the recognition site at n=0, 3 and 6 bp downstream from the (GT)15 section (Figure 5.18C), we find that the fraction
of substrate remaining decreases monotonically as a function of increasing distance from the nanotube surface (Figure 5.18 D-E). As this distance dependence is not observed with DNA-only controls (Figure 5.20 and 5.21), we confirm a nanoparticle-induced arrest of protein activity. These results have clear implications for nanoparticle toxicity, as the function and activity of a biomolecule adsorbed at the surface are shown to depend precisely on confirmation of the biomolecule with respect to the nanoparticle.

**Figure 5.18 Arrest of protein activity on a nanosurface.** (A) Nanotubes encapsulated by the (GT)$_{15}$+Ddel 9 bp oligonucleotide, containing a Ddel restriction site, hybridized in situ with a Cy3-labeled complementary strand (cDdel-9), were introduced to the Ddel endonuclease, resulting in a decrease of fluorescence due to removal of the Cy3-labeled portion. (B) The fractional change in the number of fluorescent duplexes per imaging area is quantified and
defined as the accessibility to Ddel at a cutting site 9bp from the (GT)$_{15}$-Nanotube interface

(C) A family of duplexes (GT$_{15}$-Ddel-n) containing the CTAAG Ddel recognition site (red) at increasing distances (n bp) from the (GT)$_{15}$-sequence (D) Decrease in sample density is quantified for n=6 bp, 3 bp and 0 bp, and is represented as the fraction of (GT)$_{15}$+Ddel n bp-SWNT remaining after treatment with Ddel and (E) the activity of Ddel as a function of distance from the (GT)$_{15}$-nanotube surface in 1 nm steps increases monotonically (n>100 for each sample).

![Diagram](image)

**Figure 5.19** Control for (GT)$_{15}$+Ddel n bp-SWNT remaining on the surface (A) (GT)$_{15}$+Ddel n bp-SWNT sample is immobilized on the surface. (B) (GT)$_{15}$+Ddel n bp-SWNT hybridized in situ with Cy3 cDdel-n is reacted with Ddel, and (C) a decrease in fluorescent sample density due to cleavage activity of Ddel (scale bar 10 µm). (D) Hybridization of the post-reaction product with Cy5 c(GT)$_{15}$ leads to the appearance of Cy5-emitting DNA-SWNT. (E) The Cy5 sample density is comparable to the initial Cy3 density (scale bar 10 µm).

To confirm that the nanotubes themselves are still present in the imaging area, we hybridize the post-Ddel reaction product with Cy5 cGT$_{15}$ (Figure 5.19). The Cy5-emitting sample density is comparable with the starting Cy3-emitting sample density, indicating that only the Cy3-ssDNA segment has been removed while the rest of the DNA-SWNT is still present on the surface.
We tested the activity of Ddel on Biotinylated (GT)\textsubscript{15}+Ddel-n-bp ssDNA on a surface (Figure 5.20). No systematic distance dependence of the fraction of sample cut on the position of the cutting sequence was observed. To simulate a situation where the Ddel duplex is directly on the nanotube surface with no single-strand GT\textsubscript{15} tail projecting in solution, we tested the activity of Ddel on Biotinylated Ddel-n-bp ssDNA (Figure 5.21). Here, no cleavage of the product was observed for any of the Ddel substrates.

![Figure 5.20](image.png)

**Figure 5.20** No distance dependence observed for Ddel activity on Biotinylated GT15-Ddel substrates.
In conclusion, we have successfully developed a set of experimental microscopy techniques to study the interactions of DNA and proteins on a nanomaterial surface. We observed nucleic acid-encapsulated carbon nanotubes, designed to explore DNA affinity to the nano-surface, demonstrated cooperative exfoliation of the oligonucleotides from the nanomaterial and sequence-dependent bioavailability for hybridization. We employed our platform in conjunction with a super resolution algorithm to pinpoint sites of DNA hybridization along the length of the nanotube. Protein function was mapped to local DNA topology on the nanomaterial and distance-dependent arrest of protein activity was observed, resulting in a nanotube-induced arrest of 60% protein activity within 1 nm from the nanoparticle. Validated by examples of biomolecule interactions on the nanotube surface, we present these techniques as generally applicable for nanoparticle systems to answer questions of toxicity mechanisms, structure, and molecular recognition.
Figure 5.22 Duplex formation on the nanotube can be heterogeneous or homogeneous with respect to the angle formed by each duplex with the nanotube axis. Fluorophores at different distances from the nanotube would experience unequal excitation due to the exponentially-decaying evanescent field. As we observe similar intensity from all fluorophores on a given nanotube, the average distance (time averaged over the 100 ms data acquisition exposure window) of each fluorophore from the nanotube surface is comparable, we conclude that the duplexes have similar orientations.
5.9 Materials and Methods

DNA sequence information

DNA oligonucleotides used were purchased from Integrated DNA Technologies (IDT, Coralville, IA).

For DNA-DNA interaction experiments the DNA name and sequences are as follows:

(GT)$_{15}$+Rnd1: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT CTA AGG ATG CGT GTA T 3’

(GT)$_{15}$+Rnd2: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT GCG TGT CTA AGA T 3’

(GT)$_{15}$+Rnd3: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT GCG CTA AGT GTA T 3’

(GT)$_{15}$+Rnd4: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT CTA AGG CGT GTA T 3’

Cy3-cRnd1: 5’ Cy3 ATA CAC GCA TCC TTA G 3’

Cy3-cRnd2: 5’ Cy3 ATC TTA GAC ACG CAT C 3’

Cy3-cRnd3: 5’ Cy3 ATA CAC TTA GCG CAT C 3’

Cy3-cRnd4: 5’ Cy3 ATA CAC GCC TTA GAT C 3’

(GT)$_{15}$: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT 3’

(GT)$_{30}$: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT 3’

Cy5 c(GT)$_{15}$: 5’ Cy5 TTT TTT CAC ACA CAC AC 3’

Cy3 c(GT)$_{15}$: 5’ Cy3 TTT TTT CAC ACA CAC AC 3’

For DNA-SWNT and S1 Nuclease studies:

(GT)$_{15}$+Rnd5+Cy3: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GCG AAT TCA CGG CTA AGC G Cy3 3’

(GT)$_{15}$+Cy3: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT GCG TGT CTA AGA T 3’

(GT)$_{15}$+Cy3+Rnd1: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT GCG /iCy3/ TGT CTA AGA T 3’

For DNA-SWNT and Ddel Restriction Endonuclease studies:

(GT)$_{15}$+Ddel-9-bp: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GAT GCG TGT CTA AGA T 3’
Cy3-cDdeI-9-bp: 5’ Cy3 ATC TTA GAC ACG CAT C 3’

(GT)$_{15}$+DdeI-6-bp: 5’ GTGTGTGTGTGTGTGTGTGT GT GAT GCG CTA AGT GTA T 3’

Cy3-cDdeI-6-bp: 5’ Cy3 ATA CAC TTA GCG CAT C 3’

(GT)$_{15}$+DdeI-3-bp: 5’ GTGTGTGTGTGTGTGTGTGTGT GT GAT CTA AGG CGT GTA T 3’

Cy3-cDdeI-3-bp: 5’ Cy3 ATA CAC GCC TTA GAT C 3’

(GT)$_{15}$+DdeI-0-bp: 5’ GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT CTA AGG ATG CGT GTA T 3’

Cy3 cDdeI-0-bp: 5’ Cy3 ATA CAC GCA TCC TTA G 3’

Control DNA: 5’ Biotin GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT CTA AGG ATG CGT GTA T 3’

Cy3 cDdeI-0-bp: 5’ Cy3 ATA CAC GCA TCC TTA G 3’

S1 Nuclease and Ddel Restriction Endonuclease Assay

S1 nuclease (Fermentas, EN0321, Waltham, MA, USA) in 20 mM Tris HCl (pH 7.5), 50 mM NaCl, 0.1 mM ZnCl$_2$ and 50% (v/v) glycerol was diluted 300 times with 6 µL 5X Reaction buffer 200 mM sodium acetate (pH 4.5 at 25°C, 1.5 M NaCl and 10 mM ZnSO$_4$) in 30 µL water for each reaction chamber. Nuclease was left to react with the sample for 15 minutes at room temperature. The reaction was ended by flushing the channel twice with 100 µL T50 buffer.

Ddel enzyme (New England Biolabs, R0175S, Ipswich, MA, USA) was diluted 100 fold in NEBuffer 3 and 50 µL of 1X Ddel was incubated for 15 minutes at room temperature in each flow chamber. The reaction was ended by flushing the channel twice with 100 µL T50 buffer to remove Ddel enzyme.
5.10 References


Chapter 6:

DNA G-Quadruplex Interactions with Synthetic Ligands†

The single-strand ends of mammalian chromosomes terminate in guanine-rich repeats that can fold into higher-order non-canonical DNA structures [1]. The G-quadruplex formed by the human telomeric DNA sequence has been of particular interest, owing to the importance of telomere maintenance for cellular proliferation [2]. Small molecules that bind and stabilize the G-quadruplex can inhibit cell growth via mechanisms that may involve disruption of the telomere and/or the prevention of telomere extension [3-7]. Consequently, the G-quadruplex formed by telomeric DNA is under investigation as a potential molecular target for anti-cancer drugs [8]. Human telomeric DNA is intrinsically dynamic [9] and the effect of quadruplex-binding ligands on these dynamics is not known.

6.1 FRET to Study Human Telomeric G-Quadruplex

We studied the effect of quinolinecarboxamide macrocycle 1 (Figure 6.1A), a potent quadruplex stabilizing ligand with promising anti-cancer properties [10], on the structural dynamics of human telomeric DNA using single molecule (FRET) spectroscopy.

† This work in Chapter 6 has been published as:

G-Quadruplex DNA Bound by a Synthetic Ligand is Highly Dynamic, Jena et al, JACS, 2009, 131, 12522-12523
As explained in the FRET section of the introduction, single molecule FRET can report on the conformations and dynamics of DNA [11]. In the experimental scheme illustrated in Figure 6.1B, a donor (tetramethylrhodamine, TMR) and acceptor (Cy5) labeled human telomeric DNA sequence (GGGTAA)_3-GGG, h-telo, was tethered to the BSA-Biotin coated quartz slide surface via a biotin-Neutravidin interaction and illuminated using total internal reflection excitation [12]. FRET, which is experimentally defined as the ratio of acceptor emission to the combined donor and acceptor emission, was calculated for each single molecule. This experimental configuration was chosen as it has been shown to allow resolution of the intrinsic structural dynamics between the various conformations adopted by h-telo [9, 13, 14].

Figure 6.1 (A) Macrocycle 1 (B) The (GGGTAA)_3-GGG oligonucleotide is hybridized to the complementary stem strand and immobilized via a biotin-Neutravidin interaction to a bovine serum albumin-biotin coated quartz surface. TMR (green) and Cy5 (red) are the donor and acceptor fluorophores, respectively.
6.2 Structural Conformations Adopted by *h-telo* due to 1

Figure 6.2 (A) FRET histogram of *h-telo* with 2 mM K⁺ showing unfolded (U) and two distinct intramolecular folded states (F1 and F2). (B) *h-telo* with 100 mM K⁺, folded into F2. (C) *h-telo/1* (1 µM) complex, primarily in F1. (D) *h-telo/1* complex in 100 mM K⁺, 4 h after removal of free 1 (1 µM). (All measurements at 25° C)

An unfolded *h-telo* molecule results in the greatest distance between the fluorophores and should display a low FRET value while the decreased distance between the fluorophores in a folded G-quadruplex should result in higher FRET. Monovalent cations, especially potassium, are efficient at inducing and stabilizing folded G-quadruplex structures [15], and consistent
with previous single molecule FRET studies, unfolded *h-telo* (U) can be folded into two
resolvable conformations, F1 and F2 by 2 mM K⁺ (E = 0.43, 0.63 and 0.80 respectively, Figure
6.2A). At 100 mM K⁺ concentration, only F2 is observed (Figure 6.2B). In contrast, at the
saturating concentration of 1 (1 µM) and without K⁺, *h-telo* is found primarily in F1 (Figure
6.2C). Therefore, 1 drives the human telomeric DNA to a conformation that is not well
populated under physiological K⁺ concentrations.

At intermediate concentrations of 1 (Figure 6.3), we find that 100 nM 1 induces quadruplex
formation (as evident by the presence of F1 and F2 peaks in the absence of K⁺) into both F1 and
F2 states. Transitions between both F1 and F2 states are observed, indicating that ligand-bound
*h-telo* can form both folded conformations. The ligand 1 was designed to stabilize the G-
quadruplex structure formed and previously tested with ensemble FRET on a single strand
construct. To ensure that the interaction being measured in our TMR and Cy5 labeled partial
duplex construct is specific to the quadruplex structure formed, we performed a control with a
mutant *h-telo* sequence.
Figure 6.3 h-telo + 1 shows both F1 and F2 folded states at intermediate 1 concentration and preferential stabilization of the F1 state at higher concentration.

We designed an h-telo mutant sequence that contained 4 point mutations. This sequence was labeled with the same fluorophores as h-telo and assembled into an identical partial duplex construct. We treated this sample with varying concentrations of the ligand and obtained the data in Figure 6.4. It appears evident from the histograms that the ligand interacts specifically with the G-quadruplex structure formed by the h-telo sequence and not with either of the fluorescent probes or the partial duplex junction.
Figure 6.4 Titration of an h-telo mutant with 1 shows that 1 only interacts specifically with the G-quadruplex structure formed and not with either of the fluorescent probes used. The structure formed at ~ 0.3 FRET does not correspond to the folded or unfolded peaks observed for h-telo stabilized with 1.

6.3 Association and Dissociation of 1 and K⁺ from h-telo

The K⁺ stabilized h-telo can be completely unfolded in less than one minute by flushing the sample chamber with a buffer without K⁺ (Figure 6.5). In contrast, binding of 1 µM 1 to h-telo was essentially irreversible. After incubating h-telo with 1 µM 1 and obtaining a FRET histogram (Figure 6.2C), we removed free 1 by repeated flushing of the sample chamber. Four hours after removal of free 1, we imaged h-telo molecules in a buffer with no 1 or K⁺ and observed no
change in the FRET histogram. We then flowed in 100 mM K⁺ in an attempt to facilitate the release of 1 and change the h-telo conformation to F2, but did not detect any change (Figure 6.2D). The 1 associated with h-telo was resistant to removal via buffer exchange at all tested concentrations of 1 – 10 nM, 100 nM and 1 μM (Figure 6.6).

![Graphs showing FRET values before and after K⁺ flow](image)

**Figure 6.5 (A) h-telo stabilized by 10 mM K⁺. (B) 1 minute after removal of K⁺ by flushing the channel with 0 mM K⁺ buffer.**

Given the tight binding of 1 to h-telo, we expected each h-telo/1 complex to display a constant FRET value, representing a fixed folded conformation. Instead, we found the h-telo/1 complex (formed by incubating h-telo with 1 μM 1) in a solution with no free 1 to be dynamic (Figure 6.7A). A histogram of the fraction of time spent folded (Figure 6.7Bb, obtained from 10 minute time traces of 110 h-telo/1 (1 μM 1) complexes) showed that over 30% of the complexes spent at least 10% of the time in the unfolded conformation. We observed similar dynamics with 100 nM and 10 nM 1 as well (Figure 6.8).
As the concentration of immobilized \textit{h-telo} and bound 1 is in the picomolar range, potential rebinding of dissociated 1 in the absence of free 1 in solution is extremely unlikely and hence, 1 must remain associated with \textit{h-telo} during the folding and unfolding transitions. That is, \textit{h-telo} can undergo conformational changes as dramatic as unfolding and refolding without complete dissociation of 1. Our result here demonstrates that \textit{h-telo} can be completely unfolded for an extended period of time while still bound to a ligand.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.6.png}
\caption{(A) \textit{h-telo} stabilized by 100 nM 1 and 60 minutes after removal of free 1. 
(B) \textit{h-telo} stabilized by 10 nM 1 and 60 minutes after removal of free 1.}
\end{figure}
Figure 6.7 (A) Single molecule FRET (with no free 1 in solution) showing a h-telo/1 (1 μM 1) complexes (i) primarily in F1 (ii) transitioning between U, F1 and F2 (iii) primarily unfolded with transitions to both F1 and F2. (B) A histogram representing the fraction of time spent folded by h-telo/1 complexes.

Figure 6.8 (A): (h-telo/1) complexes formed by incubating h-telo with 10 nM 1 were imaged 20 minutes after removal of free 1 from the channel. These three complexes U, F1 and F2 states.
Figure 6.8 (B): (h-telo/1) complexes formed by incubating h-telo with 100 nM 1 were imaged 20 minutes after removal of free 1 from the channel. These three complexes U, F1 and F2 states.

6.4 Conformational Sub-Species Formed by h-telo/1 Complex

It was previously observed that h-telo in the presence of 2 mM K+ displays six conformations: long-lived and short-lived species of U, F1 and F2 that differed in their lifetimes by an order of magnitude. To test if the h-telo/1 complex also possessed conformational sub-species, we incubated h-telo with 100 nM 1 (h-telo/1 at this concentration of 1 shows both folded states, Figure 6.9A inset), and washed away the free ligand. The FRET time traces showed that h-telo/1 complexes can access the same molecular conformations as K+ stabilized h-telo – long and short-lived U, F1 and F2 (Figure 6.9B).
Figure 6.9 (A) Fraction of h-telo/1 complexes in F2 remaining in F2 after time t. (inset) h-telo stabilized with 100 nM 1 showing U, F1 and F2. (B) h-telo/1 complexes showing both short (<100 s) and long (>100 s) U, F1 and F2 states in the absence of free 1.

We quantified this by measuring the dwell times of F2 (from 110 individual complexes over 758 transitions) to obtain the survival probability of a complex in F2 remaining in F2 after time t (Figure 6.9A). Fitting the curve with a double exponential decay function gave decay times of 99.5 (± 1.3) s and 8.5 (± 0.11) s. These decay times differ by a factor of 12 and show that F2 of the h-telo/1 complex is composed of at least two species with significantly different stabilities. The decay times for F2 stabilized with 2 mM K⁺ differed by a factor of 9 (188 (± 2) s and 20 (± 0.1) s) and the comparable ratio of the F2 decay times for both 1 and K⁺ stabilized h-telo
suggests that K⁺ and 1 induce h-telo conformational sub-species that are similar in their relative stabilities.

In conclusion, our measurements indicate that the binding of the cationic trimeric quinoline carboxamide macrocycle 1 to human telomeric DNA is essentially irreversible and promotes the formation of a quadruplex conformation not favored under physiological conditions. Strikingly, the ligand-quadruplex complex can show extended unfolded and folded states in the absence of ligand association and dissociation, demonstrating that the ligand remains associated with the quadruplex during the folding and unfolding transition states. Our analysis showed that the ligand-quadruplex complex and the potassium stabilized quadruplex contain conformational sub-species with relative stabilities that are very similar. Thus, this ligand can tightly bind and stabilize its target quadruplex without eliminating the intrinsic dynamics of the human telomeric G-quadruplex.

We propose that the ability of a ligand to stabilize the G-quadruplex without altering the underlying dynamics between conformational sub-species is a fundamental property that can be used to divide quadruplex targeting ligands into two broad classes. These findings could be of immediate interest to the community of scientists that synthesize quadruplex specific ligands, as well as researchers studying telomeric sequences.
6.5 Foldamers Targeting Human Telomeric G-Quadruplexes†

Foldamers are polymers that fold into a conformationally ordered state in solution due to non-covalent interactions between spatially separated monomers [16]. The idea is to adopt the ability of naturally occurring molecular machinery that are chemically composed of simple repeating subunits that fold into complex three dimensional structures with highly specific properties and functions [17]. In contrast to the fixed shape of the cationic trimeric quinoline carboxamide macrocycle 1 studied in the first part of this chapter, we now focus on the ligand shown in Figure 6.10, IKLR-4-27 (here on referred to as 4), a linear chain of four monomers that can self-organize in solution and is designed to target the G-quadruplex structure formed by the human telomeric sequence (GGTGA).

To probe the interaction of 4 with h-telo, we use the same h-telo construct previously used (with the DNA sequences listed at the end of the chapter) on a PEG surface instead of BSA-Biotin. As shown in the experimental scheme (Figure 6.11), the rest of the experimental parameters and hence experimental procedures followed are identical.

† This work in this section has been prepared as a manuscript:
Helical ligands with high affinity for different G-quadruplex sequences,
Katta Laxmi-Reddy, Raphaël Rodriguez, Prakrit V. Jena, Benoît Baptiste, Zeyuan Dong, Frédéric Godde, Taekjip Ha, Shankar Balasubramanian and Ivan Huc
Figure 6.10 Structure of Foldamer IKLR-4-27, referred to as 4 from now on.

The Cy5-TMR FRET pair used can detect two folded conformation and one unfolded conformation for h-telo and the histogram peaks in Figure 6.12 representing h-telo folding by K⁺ are the baseline to which the folding induced by 4 is compared. The unfolded peak U corresponding to a random-coil configuration of h-telo is at ~ 0.35 while F1 and F2 are at ~ 0.5
and 0.7 respectively. The peak at 0 is due to the donor-only molecules with photobleached Cy5, and leakage from the donor channel into the acceptor channel has been corrected as described in section 2.5.

Figure 6.12 The FRET peaks seen when h-telo is stabilized with K⁺ are seen at 0 for donor only, ~ 0.3 for the unfolded state U, ~ 0.5 for F1 and ~ 0.7 for F2.
We first tested the ability of 4 to induce folding of h-telo from the unfolded state. An h-telo sample was confirmed to be completely unfolded in a 0 mM K\(^+\) buffer (Figure 6.13) and addition of 100 nM 4 did not induce folding into either F1 or F2. Hence, unlike 1 that could induce folding of h-telo into the F2 state at even 10 nM concentration, 4 can potentially stabilize a folded Quadruplex-conformation but cannot induce it. This removes the need to use a mutant sequence for testing the specificity of the structure that 4 binds to, i.e. Figure 6.13 shows that 4 does not interact with the unfolded single strand DNA, either of the fluorophores or the partial duplex formed at junction between the duplex DNA stem and the h-telo single strand.

![Graph](image)

**Figure 6.13** Foldamer F4 does not induce folding of unfolded h-telo in the absence of K\(^+\), as seen by the absence of any FRET peak higher than the unfolded state at ~ 0.3.
Now we induce folding of \( h\text{-}telo \) (Figure 6.14) into the F1 and F2 state with 10 mM \( K^+ \).

Introducing 100 nM \( 4 \) in a 10 mM \( K^+ \) buffer shows the F2 peak decreasing as the F1 peak increases, indicating that \( K^+ \)-stabilized \( h\text{-}telo \) in the F1 state is being further stabilized by \( 4 \), leading to \( K^+ \)-stabilized \( h\text{-}telo \) in the F2 state to transition into the F1 state. After 20 minutes, the transition approaches equilibrium and 80% of the \( h\text{-}telo \) population is folded in the F1 conformation. The dashed vertical lines indicate that F1 and F2 peak positions remain unchanged for \( K^+ \) and \( 4 \) stabilized \( h\text{-}telo \), i.e. the \( h\text{-}telo \) structures representing F1 and F2 do not change under the influence of \( 4 \).

To study the dissociation of \( 4 \) from \( h\text{-}telo \), we allowed \( h\text{-}telo \) incubated with 10 mM \( K^+ \) and 100 nM \( 4 \) to reach equilibrium. By flushing the channel twice with a buffer containing no \( K^+ \) or \( 4 \), we attempted to dissociate \( 4 \) from \( h\text{-}telo \) and observed over 50% dissociation after 30 minutes (Figure 6.15). This indicates that \( 4 \) binds tightly to \( h\text{-}telo \) and though \( K^+ \) can be removed from folded \( h\text{-}telo \) immediately, \( 4 \) cannot. Given enough time, significant dissociation is observed, thus confirming the interaction of \( 4 \) with \( h\text{-}telo \) to be reversible.
Figure 6.14 Unfolded *h-telo* is stabilized into the F2 state by 10 mM K⁺. Addition of 100 nM 4 indicates the binding of 4 to folded *h-telo*, the conversion of F2 to F1 (50% within 2 minutes) and the complete conversion of F2 to F1 in 20 minutes at 100 nM 4.
Figure 6.15 Dissociation of 4. 100 nM 4 bound to folded h-telo can be removed by flushing the channel with a 0 mM K⁺ 0 mM 4 buffer. Over 50% unfolding of h-telo is observed after 30 minutes.

We conclude that the foldamer-approach to synthesizing ligands targeting G-quadruplex structures is a promising, especially since 4 binds to a naturally folded quadruplex structure instead of inducing folding from a random coil. Single molecule FRET applied to both macrocyclic and foldamer-based ligands can successfully report on the stability of the folded structure, the conformation stabilized and kinetic, non-thermodynamic dissociation from the bound target.
6.6 Materials and Methods

The DNA sequences used in the experiments were custom-synthesized oligonucleotides by Integrated DNA Technologies, (Coralville, Iowa):

h-telo:
5’Cy5-(GGG TTA GGG TTA GGG TTA GGG) AGA GGT AAA AGG ATA ATG GCC ACG GTG CG-
3’Biotin

Complement forming DNA stem:
5’ - CGC ACC GTG GCC ATT ATC CTT (amino-C6 dT)TA CCT CT-3’

h-telo: 5’ Cy5 – GGG TTA GGG TTA GGG TTA GGG

mutant: 5’ Cy5 – GTG TTA GTG TTA GTG TTA GTG

The amino-modified C6 dT was labeled with tetramethylrhodamine and annealed with the telomeric strand, resulting in the construct shown in Scheme 1b: a 21-base long 4 repeat telomeric sequence attached to a 29-basepair partial duplex stem. The annealed DNA was stored in 20 mM Tris 50 mM NaCl pH 8.0 (T50). For immobilizing the DNA, each channel was first treated with 50 µL 1 mg/ml Bovine Serum Albumin-Biotin (Sigma-Aldrich) for 5 minutes, and then incubated for 1 minute with 50 µL 0.2 mg/ml Neutravidin (Pierce). DNA was diluted in T50 to 50 – 200 pM final concentration and 50 µL was added to the channel and allowed to bind to immobilized Neutravidin for 5 minutes. Free DNA was removed by flushing the channel with 100 µL T50. This results in approximately 250 molecules in each 100 µm by 50 µm imaging area on the slide and coverslip surfaces. We then incubated the molecules in 50 µL of 20 mM
Tris pH 8.0 (T0) buffer for five minutes to allow complete unfolding of the quadruplex. This was confirmed by observing h-telo molecules in the imaging buffer (10 mM Tris pH 8.0, 0.8% (wt/wt) Glucose, 0.1 g/ml glucose oxidase, 0.02 mg/ml Catalase (Sigma) and 1 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) to be completely unfolded.

To change the salt concentration of the h-telo sample, a 100 mM and 1 M KCl stock was diluted by direct addition into the imaging buffer. The trimeric quinoline carboxamide macrocycle 1 ligand was stored at 4°C and added to the imaging buffer for h-telo/ligand experiments, as was foldamer 4.

6.7 References


