On Single-Molecule DNA Sequencing with Atomic Force Microscopy using Functionalized Carbon Nanotube Probes

by

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Submitted to the Department of Mechanical Engineering in partial fulfillment of the requirements for the degree of Master of Science in Mechanical Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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BARKER
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Abstract

A novel DNA sequencing method is proposed based on the specific binding nature of nucleotides and measured by an atomic force microscope (AFM). A single molecule of DNA is denatured and immobilized on an atomically flat surface, and a force probe functionalized with a nucleotide is scanned along the molecule to detect locations of the probe nucleotide’s complement.

To increase the spatial resolution of the atomic force microscope so that individual bases can be distinguished, a single-walled carbon nanotube is grown from the AFM probe and functionalized with a single nucleotide. The carbon nanotube diameter is of the order as the nucleotide base spacing—providing the necessary spatial resolution for single molecule sequencing. The absolute force detection limit of the microscope is thermal noise-limited and derived herein from the equipartition theorem. The calculated minimum detectable force is less than experimentally obtained nucleotide binding forces, indicating that the AFM is capable of directly measuring single nucleotide interactions.

A model of the oscillating AFM probe dynamics is developed, allowing a methodical approach to determining attractive forces with a chemically-specific sensor. This attractive force detection is performed by measuring the phase lag of the oscillating probe near the sample surface as compared to the resonating probe in free air.

As grown, the carbon nanotubes are too long to be used as reliable force probes, therefore a method for shortening carbon nanotubes is presented utilizing high voltages to remove material. Measuring the length of the nanotube is performed with a novel technique that exploits the nanotube’s unique elastic buckling property. This measurement technique characterizes the length of the nanotube while the probe is still mounted on the AFM and alleviates the need for a secondary microscope. The shortening procedure developed is performed in conjunction with the nucleotide functionalization, creating a precise and chemically-specific force probe.

Experiments are performed on synthetic DNA of a known sequence to validate the proposed approach. A functionalized carbon nanotube force probe is scanned along single molecules of synthetic DNA to determine locations of target bases.

Thesis Supervisor: Kamal Youcef-Toumi
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Chapter 1

Introduction

Since its structure was first described by Watson and Crick (based on data obtained by Rosalind Franklin), DNA is known to be the molecule of heredity and the means by which genetic information is stored. The sequence of its four nucleotides—adenine, thymine, cytosine, and guanine (abbreviated A, T, C, and G, respectively)—maps out all biological function. Understanding that sequence and how it leads to gene expression in an organism is crucial to dramatic advances in biology and medicine. Unfortunately, current methods for determining the sequence are slow and costly.

This chapter introduces the proposed method for sequencing DNA, which can lead to extremely fast and inexpensive sequencing of single DNA molecules. The importance of a technology that quickly and cheaply sequences DNA is presented, motivating the need for a new approach. A review is given on current sequencing methods; both the established technique and more recent experimental techniques are presented. Finally, the proposed approach is presented in overview, with emphasis on its advantages over the other methods.

1.1 The Need for Rapid Sequencing

The Human Genome Project recently announced a draft of the sequencing of the three billion base pairs constituting our species' genetic code [47, 95]. The effort took 13 years at a reported cost of $3 billion. The massive amount of genetic information will allow:

- More accurate diagnosis and prediction of disease and disease susceptibility
• Study of genes involved in complex traits and multigenic diseases

• Targeted drug discovery and prescription based on individual genetic makeup

• More effective pharmaceutical treatments with the elimination of most side-effects

• Investigation of single-nucleotide polymorphisms (single-base DNA variations among individuals) correlated with illnesses such as cancer

• Study of evolutionary conservation among organisms

These issues could be more easily elucidated, and medical treatment dramatically advanced, if DNA sequencing methods allowed a vast number individuals to have their DNA sequenced—a goal only possible with a fast and inexpensive sequencing method. Rapid DNA sequencing would further the understanding of human biology and medicine to a point where genetic-based treatment methods become available. In the future, it may be possible for a patient’s genetic code to be read in a short period of time and at a modest cost, allowing a physician to prescribe a targeted treatment with minimal chance of side effects based on the patient’s unique genetic makeup.

1.2 State of the art in DNA Sequencing

Methods for sequencing DNA have been established and are widely used. Here we present the traditional sequencing scheme and more recent experimental procedures. We note that before any method can be applied, DNA must be obtained from the nucleus of the cell. Protocols for extracting, isolating and purifying DNA are well known and not discussed here.

1.2.1 Conventional Methods

The proven and most common sequencing method (and the method used by the Human Genome Project) was first proposed by Sanger [76]. Here we review the principle of the Sanger method as described in the 1977 paper.¹

¹Many incremental advancements have been made in the years subsequent to Sanger’s paper, mostly in the automation of the process and the miniaturization of the tools. However, gains in sequencing throughput have been modest and the principle of the method remains the same.
1.2. STATE OF THE ART IN DNA SEQUENCING

Figure 1-1: **Left:** Normal deoxynucleotides found in DNA link to neighboring nucleotides, forming long chain molecules. **Right:** The dideoxynucleotide (missing a hydroxyl group) prohibits elongation of the molecule during replication, terminating the chain.

**Dideoxynucleotides** It was realized that a special form of the nucleotides making up the individual units of DNA will cause DNA elongation to cease during replication. The dideoxynucleotides, which are similar to the natural deoxynucleotides with the exception of a missing hydroxyl group, have a chemical form which prohibits another nucleotide from linking, as shown in Figure 1-1. Like the nucleotides naturally found in DNA, dideoxynucleotides can have one of four nitrogen base sidechains—adenine, thymine, cytosine, and guanine (abbreviated in dideoxy—groups as ddA, ddT, ddC, and ddG, respectively). These are the so-called chain-terminating inhibitors.

**Replicating DNA with chain terminators** The DNA to be sequenced is placed in a solution containing the natural forms of the four nucleotides (A, T, C, and G) and a small concentration of one dideoxynucleotide (for example, ddG). Additionally, an enzyme such as Taq-polymerase that replicates DNA is added. As the enzyme creates copies of the template DNA strand, nucleotides are added from the solution, with the natural deoxynucleotides taken up most often by the enzyme. However, occasionally the enzyme will use ddG in place of G, causing the replicated strand to terminate as
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Free nucleotides in solution

Taq-polymerase enzyme

GTAGCCTGCAATCGTC
CATCGGACGTTAGCAGATTACA

GTAGCCTG
GTAGCCTGCAATCGTC
GTAGCCTGCAATCGTC

GTAGCCTGCAATCGTC
CATCGGACGTTAGCAGATTACA

G - dideoxy G

Figure 1-2: Top: Replication is performed in a solution containing all four natural nucleotides, and one chain-terminating nucleotide such as ddG. Center: The original or template strand of DNA is copied by enzymes such as Taq-polymerase, which takes nucleotides from the solution and places them next to the template strand’s complement. If the ddG is taken up and placed by the enzyme, the replica is terminated as a fragment. Bottom: Fragments of various lengths all terminating in G fill the solution and must be separated.

shown in Figure 1-2. Replication terminates at random locations where a G is required, resulting in sequences of unknown length, but known terminating nucleotide.

Separating by electrophoresis At this point, the solution contains many copies of the template DNA terminated at every instance of G. This leaves DNA fragments of varying length. To determine the sequence, the fragments must be separated, and this is performed with electrophoresis in a conductive gel. An electrophoretic gel is a conducting polymer with a porous structure. The charged DNA molecules migrate from the deposited end under the influence of an applied electric potential. The speed of migration through the gel is dictated by the size of the fragment—shorter molecules move more quickly than longer molecules.

Reading the sequence The replication process is performed in four different solutions,
1.2. STATE OF THE ART IN DNA SEQUENCING

The Sanger method is a proven and accurate way to perform sequencing analysis, making it a favorite tool among biologists. Advancements in electrophoresis have allowed a mixture of all four dideoxynucleotides to be run in the same gel lane. Each of the dideoxynucleotides are labeled with phosphorescent markers of different color, which facilitates detection by computer vision and therefore leads to automation. In fact, several laboratories devoted only to sequencing have been established (requiring large capital investments in sequencing machines) based on the Sanger method.

Additional developments in microfabrication techniques have lead to faster electrophoresis runs. Because the migration time of the DNA fragments is proportional to the voltage
applied across the gel, an increased voltage leads to shorter processing time, but also creates heat that must be dissipated from the gel. Current microfabrication technology allows electrophoresis to be conducted in long, thin capillary tubes. These high aspect ratio tubes have a large surface area to volume ratio, which leads to good heat removal characteristics.

Despite these advancements, the Sanger method is inherently slow. Billions of copies of the template DNA must be created at several steps in the procedure—enough copies are necessary to generate a sufficient optical signal detectable by the automated sequencer. Also, this method has a limited read length due to the properties of electrophoresis: The length of the electrophoretic gel is constrained to approximately one meter (the nonlinear nature of the migration process causes small fragments to traverse the gel substantially faster than the larger fragments—if the gel were longer than one meter, the small fragments would reach the far end of the gel before the large fragments were sufficiently separated). Additionally, the optical detection system requires that each marker be visibly separated from its neighbors by about 1.4 mm for unambiguous separation. Therefore each electrophoresis operation can determine the sequence of about 700 bases at a time.\(^2\) It is because of these limitations that the three billion base pairs constituting a human genome took 13 years to complete despite massively parallel systems employed by the several collaborating genomic centers.

The speed limitation of the Sanger method and the associated high cost precludes its use for personal genome analysis. Therefore, researchers are considering experimental sequencing techniques with the hope of eliminating the slow replication and electrophoresis characteristics of the Sanger method. Several approaches, including the method proposed in this work, attempt to sequence a single DNA molecule—eliminating the need for replication.

1.2.2 Experimental Methods

The Sanger method for sequencing is slow, due to both the requirement for billions of copies of DNA created with the slow time constant associated with chemical reactions, and gel electrophoresis which separates DNA fragments by viscous drag. Experimental sequencing methods are receiving increasing attention for the potential to eliminate the slow and costly attributes of current techniques. Most experimental methods are focused

\(^2\)(\(\frac{1m}{1.4\text{mm/base}} \approx 700\) bases)
on sequencing single molecules in order to eliminate the replication step associated with the Sanger method. Despite recent advancements, these approaches are either limited to short strands of de novo sequencing (sequencing a previously unknown genome), or resequencing longer strands to detect deviations and mutations from a known sequence. The experimental approaches presented here are not intended to be exhaustive, but a representation of the current methods under development.

**Ion Channels**

An interesting single-molecule sequencing technique has been proposed based on ion channels found embedded in the lipid bilayers that form cell membranes [25, 99]. It was reasoned that these so-called nanopores could be made to conduct ionic solutes such as DNA across a membranes under the influence of an applied voltage (see Figure 1-4). The nanopore α-hemolysin is chosen due to its ability to self-assemble in lipid bilayers and its characteristic pore diameter was just large enough to permit DNA to pass. If DNA is placed in an
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ionic solution and a voltage is applied across the insulating membrane, the solution ions and DNA will translocate across the membrane. The electric current created by the passing ions can be measured and is suspected to be a function of the nucleotides. It is proposed to correlate the variation in current across the membrane to the sequence of the nucleotides as DNA and the solution ions move through the nanopore. This method has the added benefit that read lengths are limited only by the length of DNA that does not shear during the migration process.

This work has received increasing attention since it was first proposed as a sequencing technique in 1992 [25]. However, successful experiments demonstrating sequencing have not yet been reported. Problems stem from the high speed at which DNA migrates across the nanopore, leaving too little time for sufficient number ions to pass. An additional obstacle is due to the size of the channel. At 5 nm, the narrow region of the nanopore admits about 14 nucleotides at any given time—too many to extract sequence information from the measured current. Finally, random diffusion from temperature fluctuations could permit the DNA strand to retreat against the ionic flow, confounding the sequence data. To alleviate some of these problems, recent work is focused on discovery of other types of nanopores. Promising leads indicate that a nanopore could be created in silicon or mica by etching or focused ion beam removal of material, however demonstration of a viable sequencing system is thought to be years away.

Single-molecule fluorescence

Another promising single-molecule method is based on chemoluminescent reactions [6, 45]. A target strand of DNA is attached to a nanometer-sized bead and deposited in a 75-picoliter well shown in Figure 1-5. Enzymes that replicate DNA are also introduced, however the supply of free nucleotides is restricted by the system. Nucleotides of a single type are added to each cell, and if that nucleotide is required by the replication enzyme for synthesis, it is taken up by the enzyme and incorporated onto the nascent strand. During this process, pyrophosphate is released and converted into ATP, and another enzyme (luciferase) produces light. If light is detected by the optical system immediately after the known nucleotide is added, the sequence can be inferred. If no light is released, the well is rinsed and one of the
1.2. STATE OF THE ART IN DNA SEQUENCING

Figure 1-5: A 75-picoliter well contains a single molecule of DNA during sequencing with fluorescent labels. When a nucleotide is added to the well and taken up by the synthesizing enzyme, light is released, indicating that the added nucleotide is a complement for the template DNA. Sequence information is determined by the order of nucleotides added to the wells to incite light production. Image courtesy of Nature Biotechnology [45].

Because reagent quantities and reaction wells employed by this method are so small, the system could be operated in a highly parallel fashion. 1.5 million wells of this size fit on a 75 mm² plate. However, the mechanics of DNA deposition into the wells limits this approach to read lengths of about 100 bases, and complicated software assemblers must be developed to overlap the short sequences to build up genome-level information. Due to the short read lengths, this approach has difficulty with highly repetitive sequences (there may exist multiple locations in the genome where the sequence of several hundred bases is duplicated, but these distinct regions perform separate biological functions). Reconstructing the genome in the presence of these duplicated sequences becomes ambiguous [45].

Another sequencing technique based on single-molecule fluorescence uses a label that binds to a group of several nucleotides [42]. Multiple labels are hybridized to single-stranded DNA (ssDNA) in solution, and the typically coiled DNA is untangled with by a novel microfluidic channel containing an array of small pillars. As the fluid containing a labeled DNA molecule enters the channel, the coiled DNA is unwrapped after making contact

other four nucleotides is added.

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with the pillars. Viscous forces untangle the DNA and the channel narrows to funnel the straightened strand into the detection region. Two lasers illuminate the strand and photodetectors measure the locations of the tags. Sequence information can be ascertained from the detector response. Read length is this approach is limited to the size of DNA that can be straightened by flowing past the pillars without shearing.

Although the two single-molecule fluorescence techniques presented here rely on detecting an optical signal, their individual approaches are quite different. Whereas sequencing is performed by adding nucleotides to an array of picoliter wells in a parallel manner, the laser detection scheme shuttles a long strand of DNA serially past the detectors. Both methods are currently under development and continue to make advances. There are several other techniques being pursued, including microfabricated capillary arrays and time-of-flight mass spectrometry, which are discussed in greater detail elsewhere [45, 54].

While sequencing by the Sanger method is an established technology, it lacks the speed which would open the way for revolutionary biological and medical applications. Experimental sequencing technology is promising, but is incipient, unproven, and many technical obstacles remain. Therefore, an opportunity exists for a sequencing approach that is based on established technology and offers dramatically increased sequence throughput. In the next section, we introduce a novel sequencing approach based on the atomic force microscope—a scientific tool with almost twenty years of exploration of the atomic forces constituting molecular biology.

1.3 Proposed Approach

This thesis presents the design of a novel system that provides rapid DNA sequencing based on proven atomic force microscope (AFM) technology. The premise is complementary bases (adenine and thymine or cytosine and guanine) bind together exclusively with forces large enough to be detected by the AFM. A force probe coated or functionalized with a single DNA nucleotide (e.g. thymine) is scanned across an immobilized single-sided strand of DNA, and the locations of the complementary nucleotide (e.g. adenine) are measured. In the future, four probes—each functionalized with a different base—will be scanned across
the same strand to sequence DNA in one pass. This thesis focuses on demonstration of the principle, and therefore only one functionalized probe is scanned at a given time.

Because the nucleotides are packed so tightly on a strand of DNA, spatial resolution of the AFM deserves special attention. Herein we describe how a newly discovered material, carbon nanotubes, can be attached to AFM probes and functionalized with appropriate chemistry to achieve the required resolution and chemical specificity to distinguish nucleotides on a DNA backbone. The remainder of this section introduces the atomic force microscope and the carbon nanotubes used as precise force probes.

1.3.1 Atomic Force Microscope

The proposed technique for rapid DNA sequencing is based on the atomic force microscope, a system designed for the exploration of inter-atomic forces. An AFM creates an image of nanometer-scale features by scanning a sharp probe across the sample, while the atomic forces between the sample and tip cause a deflection in a highly compliant cantilever (see Figure 1-6). The cantilever and force probe is mounted onto the system's actuator, a
piezoelectric tube that provides three dimensional motion of the probe relative to the sample. Cantilever deflection is measured with an optical level sensor and fed to a controller which adjusts the extension of the piezo tube. In “contact mode,” topographic information of the sample is created by plotting the piezo extension required to hold the cantilever angle constant as the tip is scanned across the sample. Alternatively, in “tapping mode” the cantilever is oscillated near its resonant frequency away from the surface and then brought into proximity with the sample, which decreases the oscillation amplitude. In this case, an image can be generated by either plotting the piezo extension required to hold the RMS cantilever oscillation amplitude constant, or plotting the shift in phase between the driving and measured sinusoidal signals.

As will be shown, attractive forces are best measured with the AFM operated in tapping mode. Tapping mode provides amplitude and phase information, of which increased phase lag correlates to specific attractive force interactions characteristic of nucleotide binding.

1.3.2 Functionalized Carbon Nanotubes for DNA Base Detection

The DNA is sequenced by measuring the force interaction between individual nucleotides as a chemically functionalized tip is scanned across immobilized single-sided DNA. An AFM tip is modified to produce a sharp, robust and chemically specific sensor through the use of carbon nanotubes. Measuring the force interaction between a single nucleotide on a strand of DNA requires high resolution and specificity to ensure that a sequence containing multiple identical bases (e.g., ... TGAAC...) can be distinguished from single bases (... TGAC...).

With this mind, it is proposed to use a new kind of AFM tip based on carbon nanotubes. These probes offer unprecedented resolution and recently have been functionalized with various chemical groups [21, 24, 37, 44, 105, 109].

It is proposed to functionalize a carbon nanotube attached to an AFM tip with a single DNA nucleotide to interact with the complementary base on the immobilized ssDNA and the resulting interaction force be measured with the AFM system operated in tapping mode. We will show that phase plots are more revealing in chemical force microscopy where the topographical differences are negligible, but the attractive tip-sample forces are important. Carbon nanotubes can be obtained with a tube diameter as small as 1 nm mounted on an
AFM probe, and tubes as small as 0.42 nm have been reported in the literature—on the order of the base pair spacing on the DNA backbone [8, 87].

### 1.3.3 Advantages of Proposed Approach

The cantilever used as a force probe in the atomic force microscope is usually made of silicon and has a typical length of 100 μm. This gives a resonant frequency on the order of 100 kHz which is quite high for a mechanical system. Coupled with the piezo tube actuator whose control bandwidth is typically 600 Hz, the AFM system is capable of recording data at a high rate. When applied to sequencing, this approach offers a dramatic increase in throughput compared to the traditional Sanger method. Early estimates of sequencing throughput indicate that an entire human genome could be sequenced with the proposed method in approximately six days.\(^3\)

Additionally, this method performs sequencing on single DNA molecules, eliminating the time-consuming and costly replication phase characteristic of other methods. Some have argued that sequencing single molecules will ultimately prove more accurate than amplified samples due to the elimination of processing steps [42]. The single-molecule approach also requires only small amounts of reagents in the preparation of the DNA for sequencing, reducing cost and eliminating the handling of toxic chemicals (such as the fluorescent tags used in competing methods).

Finally, because this approach relies on established AFM technology and this work represents a moderate increase in imaging resolution and chemical force microscopy, we feel the opportunity for success is greater than unproven and more radical technologies mentioned earlier.

### 1.3.4 Summary

It is proposed to modify an atomic force microscope with a functionalized probe, creating the capability to detect the interaction force of individual bases on a DNA backbone. The AFM tip is modified with a carbon nanotube, and a single DNA base is attached at the end.

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\(^3\)The estimate assumes a sequencing rate of 6000 bases/sec achievable with an optimized control system which does not waste time scanning the uninteresting background, but instead tracks the winding DNA on the substrate. The additional overhead required for isolating and preparing the ssDNA is not considered.
CHAPTER 1. INTRODUCTION

The attractive force between the carbon nanotube-mounted base and the complementary bases on the immobilized single-sided DNA is measured by operating the AFM in tapping mode and recording the increased phase lag indicative of complementary nucleotide binding. In this manner, DNA sequencing on single molecules is conducted.

1.4 Document Layout

This thesis is organized as follows: Chapter 2 describes the properties of DNA and how they can be exploited to allow AFM-based sequencing. Chapter 3 presents the AFM in detail with particular attention paid to the limits of force and spatial resolution in the context of a biomolecular force sensor. Chapter 4 highlights carbon nanotubes, describing both the characteristics that make them suitable for high-resolution probe microscopy, and methods for functionalization that allow force detection with molecular specificity. In Chapter 5, experimental data is presented demonstrating the feasibility of DNA sequencing using an AFM with a functionalized carbon nanotube probe. Finally, concluding remarks and suggestions for future work including ways to improve the signal-to-noise ratio and increase sequencing throughput are given in Chapter 6.
Chapter 2

DNA Properties

This chapter offers a general discussion on deoxyribose nucleic acid (DNA) with a focus on understanding the parameters and properties necessary for an AFM-based DNA sequencing system. The mechanism by which genetic information stored in DNA is decoded and converted into proteins is presented, highlighting the important role played by DNA to the biological processes, and motivating the need for a deep understanding of how DNA sequence impacts biological function. A description of the structure of the molecule and pertinent dimensions is offered, with particular attention paid to the mechanical properties exploited in force detection-based sequencing. Finally, we review the binding force characteristics between complementary bases, due to their important role in the proposed sequencing method.

2.1 Function and Importance

DNA is the molecule on which all information necessary for life is stored. Its function is to encode the instructions for the creation of proteins, which in turn perform the majority of all biological processes. DNA is the smallest divisible unit of reproduction—the code for an entire organism is stored in the sequence of its nucleotides.

Generally speaking, proteins are created in a two-step process which begins by unzipping the double-stranded DNA so that it can be read. The opened DNA located in the cell’s nucleus is read and converted into messenger ribonucleic acid (mRNA) in a process called
Figure 2-1: The process by which genetic information stored in the sequence of DNA bases is decoded into proteins. **Top:** Double stranded DNA is opened and messenger RNA of the complementary sequence is created in a process called transcription. **Bottom:** During translation, three mRNA nucleotides code for an amino acid which is serially linked to the previous amino acid to form a protein. The number and order of the amino acids of a protein determines how that protein will fold, and in turn, what biological process it will fulfill.

"transcription." The mRNA migrates out of the nucleus into the cell’s cytoplasm where enzymes read it and create the protein from amino acid building blocks. Every three nucleotides on the mRNA cause another amino acid to be linked to the protein, which is formed in a serial fashion. The order of the amino acids determine how the protein subsequently folds into a conformation which will define its function. When and where groups of proteins are synthesized leads to the selectivity of gene expression. This process is summarized in Figure 2-1.

Because the sequence of the nucleotides ultimately leads to synthesis of proteins, determining the sequence quickly and inexpensively is of great interest. With knowledge of the sequence of an individual’s DNA (and a further understanding of the complex gene expression process) the potential exists to predict how genetic information could be expressed. This opens exciting new possibilities in health care: targeted drugs tailored for a patient’s genetic makeup, individual screening for inherited disease, and enough genetic samples to perform population-wide studies and gain a deep understanding of gene expression. More radical applications that will be assisted with fast and cheap sequencing technology include so called “designer organisms” whereby scientists program unicellular organisms by directed
DNA synthesis. These laboratory-designed microbes could be made to digest nuclear waste into harmless byproducts, convert solar energy into usable electricity, or attack chemical or biological weapons. Early genetic engineering is already occurring, but could be made vastly more powerful and safer with the precision afforded by sequence-level understanding of gene expression.

2.2 Structure and Dimensions

Understanding the size and shape of DNA is critical to devising an imaging technique sufficiently advanced to observe relevant details needed for sequencing. Here, the characteristic conformation and dimensions of DNA are presented.\(^1\)

Despite being often referred to as a single molecule, DNA is actually two strands of long thin ribbons of sugar and phosphate, linked together with sidechains made of four bases. The two ribbons, often called the “DNA backbone,” are coiled around the same axis in a double helix conformation and consist of repeating units of deoxyribose sugar groups and negatively charged phosphate groups, as shown in Figure 2-2. This structure was first proposed famously by Watson and Crick in 1953 [100] based on Rosalind Franklin’s unpublished X-ray crystallographic studies. It is important to note that DNA can be easily split into two strands of constituent molecules. This process, called “denaturing,” separates DNA by breaking the bonds between the sidechain bases, and can be performed either by simply heating shorter strands or through the application of certain enzymes. Denatured DNA is referred to as single-stranded (ss), and since the bases are left exposed, it is the form of DNA that will be used in this work for interrogation by the atomic force microscope.

The sidechains consist of four nitrogen bases: adenine, thymine, cytosine, and guanine (A, T, C, and G, respectively). These bases are hydrophobic and self assemble toward each other on opposing strands in the presence of water. Importantly, these bases bind to each other in a highly specific manner—A with T, and C with G. It is the order of these bases on the DNA backbone that determine what proteins are created and therefore ultimately

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\(^1\)While several families of DNA are known, differing in the details of their structures, in this work we focus on the most common type of DNA, the so-called \(\beta\)-form. Details on the other forms of DNA can be found in [2].
dictate all biological function. This complementary nature of specific binding between bases gives rise to a new method to determine the sequence of DNA using a force probe chemically functionalized with one of the four bases to hunt for the base’s complement on the target single-sided strand.

DNA bases are spaced at 0.34 nm along the backbone—referred to as the “base spacing pitch.” This is an important dimension for this research as it represents the required resolution for the force probe. It should be noted that the 0.34 nm pitch occurs with DNA in the helical β-form, and the spacing of bases for a single-sided strand is expected to vary but remain close to this value.

Another important dimension relevant to this work is the diameter of the helix, measured to be 2.0 nm. It is expected that the ssDNA diameter will be half of this value. When scanning ssDNA on an atomically-flat substrate, we will look for winding features 1 nm high to locate the target strands. The helical pitch, which is length required for the helix to sweep through a full rotation, is 3.57 nm. As shown in Figure 2-2, this gives 10.5 bases per helix revolution, or 34.4 degrees of twist for each base pair.
2.2. STRUCTURE AND DIMENSIONS

Figure 2-3: Straightened ssDNA prepared on a mica surface (a mineral that cleaves into atomically flat planes) provides access for the functionalized force probe to interact with bases for sequencing. The scale bar of this AFM image taken from [111] represents 500 nm.

Linearizing Coiled DNA for Scanning

Human genetic information is encoded in over three billion base pairs of DNA. Given the base pair pitch of 0.34 nm, this amounts to almost a meter of genetic material contained in each cell. Clearly, there needs to be a mechanism to package this lengthy molecule. DNA wraps around proteins called histones in a spool-like configuration forming nucleosomes. These nucleosomes are further coiled into another conformation forming chromatin fibers, and the chromatin is packed into one of the 23 pairs of chromosomes. Due to the many nested layers of coiling, the resulting genetic material can be packaged into compact and dense configurations contained in the cell nucleus.

We note that to interrogate ssDNA with a force probe as required by our proposed sequencing method, the DNA must be uncoiled by heating and have its bases exposed to the probe. This process is facilitated by protocols that have been developed to prepare extended and uncoiled ssDNA on an atomically flat surface [111]. An atomic force microscope image of DNA prepared to minimize coiling is shown in Figure 2-3. DNA prepared in this manner can be easily tracked by the AFM, eliminating wasted time spent scanning the uninteresting background and increasing the sequencing rate.

Here we have reported the dimensions for DNA pertinent to the proposed sequencing
Figure 2-4: The structure of the four bases of DNA on the backbone is illustrated, highlighting their complementary nature. The deoxyribose and phosphate groups making up the DNA backbone are shown as monochromatic, the sidechain bases are indicated by hatching, and the hydrogen bonds between the bases are dashed lines.

In order to detect bases on target ssDNA, a force probe functionalized with a base will exhibit an affinity for its complement. Here we discuss the nature of complementary base binding and review the reported values for the magnitude of the attractive forces involved.

Bases on DNA link to their complement through hydrogen bonds. Adenine and thymine form two hydrogen bonds and cytosine and guanine form three, shown in Figure 2-4. The strength of hydrogen bonds is relatively weak compared to covalent or ionic bonds, which
allows DNA to be easily denatured by cellular machinery for gene expression or copying. However, the hydrogen bonds are of sufficient magnitude as to be detectable by the atomic force microscope used for sequencing.

Recent studies attempt to measure the binding strength of individual base pairs directly using newly developed techniques. Observed binding forces have been reported from $9 \pm 3 \ pN$ to $70 \pm 3 \ pN$ [5, 49, 71] and are discussed in greater detail in Section 3.2.3.

2.4 Summary

This chapter presents the function and importance of DNA, explaining how the sequence of the nucleotides impacts biological function. The structure and dimensions critical to this research are discussed, and it is noted that the 0.34 nm base spacing pitch represents the required resolution for the force probe. Additionally, the binding force characteristics between complementary bases have published values between $9 \pm 3 \ pN$ and $70 \pm 3 \ pN$, providing the magnitude of attractive force that must be detected by the AFM. For additional information on DNA, its structure, function, and role in biology, see [2].

The DNA properties presented here will be exploited to allow a novel method for determining the sequence using interatomic force measurements. The following chapter will describe the atomic force microscope and its relevance to the proposed sequencing method.
Chapter 3

Atomic Force Microscopy

Shortly after its invention by Binnig and Quate [4], the atomic force microscope (AFM) became a vital tool in the study of micro and nanoscopic features and has since demonstrated its usefulness through many unique scientific contributions. Excitement has been generated by the use of AFMs in molecular biology as a way to study single molecule force interactions. This chapter will introduce the atomic force microscope and present its theory of operation including a discussion on the intermittent contact mode proposed for use during DNA base detection and sequencing. A model is provided of the interatomic forces at work between the AFM probe and the DNA bases. Also, a brief summary of the applications of atomic force microscopy to molecular biology is given, highlighting the unique contributions of single-molecule force spectroscopy.

Finally, since the goal of this research is to create a chemically-specific force sensor with sufficient resolution capabilities as to discern and locate bases on a strand of single-sided DNA, a discussion is offered on the AFM’s theoretical limits of force and spatial resolution.

3.1 AFM System Description

3.1.1 Theory of Operation

An atomic force microscope has three main components as shown in Figure 3-1. The piezoelectric tube actuator is fixed at the top to the frame of the microscope and the bottom end is free and allows three-dimensional motion relative to the sample. Piezoelectric
CHAPTER 3. ATOMIC FORCE MICROSCOPY

Topographic Data

Piezoelectric scanner

Photosensitive diode

Laser

Cantilever Probe

Sample

Control Signal

Controller

Feedback Signal

Image

Figure 3-1: Schematic of the principle components of an atomic force microscope. The piezo tube scanner provides three dimensional motion of the sharp probe relative to the sample. The deflection of the cantilever is measured by a laser and photosensitive diode and feedback to the controller. An image is generated by plotting the controller voltage required to hold the cantilever deflection constant (or cantilever RMS oscillation amplitude constant) as a function of the probe’s lateral position.

materials (from which the AFM’s actuator is made) deflect by very small but repeatable amounts under an applied voltage. The piezo tube used in the AFM is designed to provide nanometer-scale 3-D relative motion between the probe and the sample. The tube is quartered axially into four sections that can be activated differentially to allow for bending about the tube’s axis—this provides the lateral probe displacement or motion parallel to the plane of the sample. Additionally, all four of the sections can be activated simultaneously, causing the piezo to extend, which allows for vertical probe displacement.

At the free end of the tube, a flexible cantilever is attached with a sharp probe. The probe interacts with the sample and causes the cantilever to deflect, and the deflection is measured by an optical level sensor consisting of a laser and photosensitive diode. The laser is reflected from the back of the cantilever up to the photosensitive diode, which measures the location of the coherent light. A control system reads the feedback signal from the
3.1. **AFM SYSTEM DESCRIPTION**

diode, determines the cantilever's angle of deflection, and outputs a voltage to the piezo tube. An image is generated based on how the tube is commanded to extend in order to hold the cantilever deflection constant (or oscillation amplitude constant for intermittent-contact mode). Information on the sample topography or local properties is based on probe-sample interactions.

Imaging with the AFM can be performed in several modes of operation, differentiated by the dynamics of the cantilever during scanning. The two most popular are contact and intermittent-contact mode, discussed in the next section.

**Contact vs. Intermittent-Contact Mode**

During contact-mode scanning, the piezo tube is extended until the probe makes contact with the sample and the cantilever deflection is detected by the photosensitive diode. After contact is made, the piezo tube drags the probe across the sample in raster fashion. While scanning in contact mode, the cantilever deflection is continuously monitored, and the control system adjusts the piezo extension such that the cantilever angle is held constant to a preset value. Because the interaction force is proportional to cantilever displacement, this is sometimes called “constant force mode.” The topographical image of the sample is created by plotting the extension value of the piezo as a function of the probe’s lateral coordinates.

In intermittent-contact mode, the cantilever is oscillated at or near its resonant frequency away from the sample. The piezo tube extends to bring the tip of the probe into momentary contact with the sample during the lowest part of the cantilever’s swing. The repulsive forces of interaction cause the cantilevers oscillation to decrease in amplitude, and the probe is scanned across the sample such that the controller holds the RMS amplitude constant. This operational mode is often called “tapping mode.” Tapping mode imaging offers the advantage of lower tip wear and minimal sample damage due to the decreased interaction as compared to contact mode. However, the most important benefit of tapping mode in the context of an AFM-based DNA sequencing system, is increased imaging capability. The oscillatory nature of the data collection allows both amplitude and phase data to be recorded as a function of lateral position. In other words, the cantilever is driven into oscillation with
a sinusoidal input signal $A \sin \omega t$, and the measured cantilever response $A_r \sin(\omega t + \phi)$ can be compared to the input both in terms of amplitude $A_r$ and phase lag $\phi$. This provides an additional dimension on which data can be collected, and as will be shown, the plots of phase lag will lead to detection of attractive forces.

### 3.1.2 Attractive Force Measurements with Tapping Mode

For the AFM-based DNA sequencing system, the proposed method is to detect attractive forces of interaction between an AFM probe functionalized with a DNA nucleotide, and the nucleotides on a single-sided strand of DNA and use the specific force data to reconstruct the target strand sequence. This section will show that phase imaging is the most effective mode of AFM operation to meet this goal. We will demonstrate that attractive forces between the tip and sample cause a phase lag in the measured cantilever response detectable by the AFM.

Consider the AFM cantilever modeled by a second-order linear ordinary differential equation characterized by a spring constant $k$, mass $m$, resonant frequency $\omega_n$, and quality factor $Q$.\(^1\) The phase angle (in radians) of a non-interacting probe as a function of frequency $\omega$ is expressed as:

$$\phi = \tan^{-1}\left(\frac{m\omega\omega_n}{Q(k - m\omega^2)}\right). \quad (3.1)$$

Following Magonov [53], the essential consequence of tip-sample interaction forces is an effective change in the stiffness of the cantilever $k_{\text{eff}} = k + \sigma$, where $\sigma$ is the sum of force derivatives for all forces $F_i$ acting on the cantilever

$$\sigma = \sum_i \frac{\partial F_i}{\partial z}. \quad (3.2)$$

The phase angle of the cantilever interacting with the sample is therefore

$$\phi = \tan^{-1}\left(\frac{m\omega\omega_n}{Q(k + \sigma - m\omega^2)}\right). \quad (3.3)$$

And when we consider the cantilever oscillated at the resonant frequency $\omega_n = \sqrt{k/m}$, the

\(^1\)Alternatively, the cantilever could be modeled with mass $m$, spring $k$ and damping $b$ coefficients, but quality factor is often cited in cantilever datasheets. Note $Q = \frac{1}{2} \sqrt{km}$.\)
phase angle of the interacting cantilever becomes

$$\phi_n = \tan^{-1} \left( \frac{k}{Q\sigma} \right).$$

(3.4)

where the subscript \( n \) refers to resonance. Therefore we can define the phase shift as the difference in phase lag between the free-air oscillating cantilever and the cantilever interacting with surface forces as follows:

$$\Delta\phi_n = \frac{\pi}{2} - \tan^{-1} \left( \frac{k}{Q\sigma} \right) \approx \frac{Q\sigma}{k}$$

(3.5)

which is valid if \( \sigma \) is small compared to \( k \). Since \( \sigma \) is negative if the forces acting on the cantilever are attractive, Equation (3.5) predicts a negative shift in phase angle under attractive interactions (i.e., increased phase lag). Note that the \( \sigma \) and \( \Delta\phi_n \) are linearly related—an increased attractive force will lead to an increased phase lag readily observable in phase imaging. We emphasize that the cantilever is oscillated at resonance for maximum force sensitivity, because for a second-order system, the phase at resonance is \(-90\) degrees and in a nearly linear region sharply decreasing from \(0\) to \(-180\) degrees. Operated at \(\omega_n\), the cantilever will be experiencing its largest slope in phase, making force detection more sensitive than at any other frequency.

The analysis presented here leads to a methodical detection scheme for attractive AFM force measurements and has important implications in chemical force microscopy discussed in Section 4.2 [62]. Therefore, in our experiments we shall use phase lag as an indicator of specific binding forces associated with the hybridization of single-sided DNA bases with a functionalized AFM probe. The phase angle of the cantilever during non-specific interactions (such as the between the probe and the background substrate) will be compared to the phase angle of the functionalized probe interacting with the specific complementary base. Note that while some non-specific but attractive forces will be present (i.e., from capillary or van der Waals forces) the magnitude is expected to be less than the specific binding case.

\(^2\) The series expansion for the tan\(^{-1}\) function is applied. Specifically,

$$\tan^{-1} \left( \frac{1}{x} \right) = \frac{\pi}{2} - x + \frac{x^3}{3} - \frac{x^5}{5} + O(x^6)$$
and would be indicated by a lesser phase lag.

It should also be noted that while phase imaging reveals attractive binding forces, as has been shown, amplitude measurements in tapping mode are correlated to sample topography. Investigators are typically interested in topographic or profile information, and therefore tapping mode with amplitude imaging is more commonly used. Physically, the repulsive weak nuclear force repels the AFM tip when brought into close contact with the sample. This decreases the oscillating amplitude of the cantilever, and the AFM registers this decreased amplitude as the sample’s location.

### 3.1.3 Atomic Force Model

This section will present a model of the atomic forces involved in the AFM-based DNA sequencing system. In general, uncharged molecules are mildly attractive at moderate distances due to induced dipole or van der Waals forces, but strongly repulsive at close distances. This behavior is qualitatively captured by the Lennard-Jones model as the following relation

$$ F(d) \propto \left( \frac{1}{d} \right)^{12} - \left( \frac{1}{d} \right)^{6}. \quad (3.6) $$

The standard Lennard-Jones model is refined here to include terms that are appropriate for the hydrogen bonding associated with attractive DNA base interactions. These long-range forces are accounted for with an additional term and the resulting force-distance relation is shown in Figure 3-2.

$$ F(d) \propto \left( \frac{1}{d} \right)^{12} - \left( \frac{1}{d} \right)^{6} - \left( \frac{1}{d} \right)^{8}. \quad (3.7) $$

Figure 3-2 shows that as two molecules are brought together, they are attracted until reaching equilibrium, represented as the x-intercept on the figure. This atomic force model captures the governing forces experienced by the functionalized AFM tip interacting with the substrate. In the case where the complementary bases between the tip and the DNA match, the attractive well of Figure 3-2 becomes deeper due to the hydrogen bonding, and this leads to an increase in phase lag of the oscillating cantilever discussed earlier. It is this increase in the potential well that will be detected with phase imaging for the AFM-based DNA sequencer.
3.1. AFM SYSTEM DESCRIPTION

3.1.4 AFM as a Biomolecular Investigation Tool

Characterizing the relationship between molecular structure and biological function is key to understanding biological pathways that lead to new drugs. Because parameters such as size, shape, and conformation are inextricably linked to molecular function, structural biology has been and will continue to be crucial to understanding the important biological pathways and the development of new treatments. Conventionally, structural biology studies have been performed using electron diffraction, X-ray diffraction, and nuclear magnetic resonance imaging. However, these techniques have limitations including the requirement that the molecule under study be crystallized, removing the object from its native liquid environment.

Recently, study of the atomic structure of biologically important molecules has been conducted using high-resolution atomic force microscopy. Sub-molecular resolution images of proteins such as GroES and IgG have been gathered with carbon nanotube AFM probes,
CHAPTER 3. ATOMIC FORCE MICROSCOPY

revealing structural details [37, 109]. These first studies of protein structure with a carbon nanotube-augmented AFM illustrate the potential of a new biomolecular tool. See Chapter 4 for a discussion of the challenges and benefits of using carbon nanotube AFM probes.

Additionally, researchers have exploited the AFM’s ability to record pico-newton scale forces with chemically functionalized tips to measure single-molecule force interactions. For example, the binding strength between a single biotin-streptavidin pair was found to be approximately 200 pN [105]. Noy et. al. measured the force required to elongate DNA from the coiled β form to the stable stretched form about twice as long. The required force to lengthen the DNA was observed to be 120 ± 50 pN, close to the model-predicted value of 140 pN [63]. In a later section, AFM measurements of DNA binding forces are discussed. Reviews of using the AFM in structural biology and its unique small-scale force resolution capabilities are provided by Bustamante [7] and Zlatanova [113].

3.2 Limits of Force Resolution

To ensure measurement of the binding force between a functionalized AFM probe and a single-sided strand of DNA lies within the range of detectability, the limits of AFM force resolution must be understood. Methods for calibrating the AFM’s cantilever to obtain a transfer function between measured deflection and interaction force are reviewed. And the fundamental physical limit for AFM force detection given by the equipartition theorem is presented.

3.2.1 Calibration of AFM Cantilever

To accurately detect the attractive forces between complementary DNA bases, the AFM cantilever must first be calibrated. Because individual cantilevers can vary due to manufacturing tolerances, the force-deflection response of cantilevers (both contact and tapping mode) can be quite different—even for cantilevers made from the same wafer. For applications where the cantilever sensitivity is not critical, cantilever manufacturers usually provide rough force-deflection data based on the intended cantilever dimensions and material properties. Detailed models of cantilever deflection for both diving board-shaped and
triangular cantilevers have been developed [56, 72, 74]. This method is referred to as “calibration by analysis” and has the benefit of requiring no effort by the manufacturer beyond the initial modeling. Occasionally, manufacturers will individually measure the dimensions of each cantilever they sell using high powered optics and these dimensions provide a more accurate estimate of cantilever sensitivity, although claimed spring constants can still vary from their true value by as much as 20%.

To address the relative inaccuracies of calibration by analysis, techniques are developed to directly measure the deflection of a cantilever as a function of the applied force. The challenge is using a sensitive and repeatable force standard—typically the AFM probe is pressed into a substrate with a known surface hardness while the cantilever deflection is measured to determine the applied force [33, 77, 91]. This method is called “calibration by static response” and requires effort on the part of the user (manufacturer's rarely calibrate probes in this manner) and opens the possibility for probe damage or contamination.

Finally, the most accurate and recently developed method for cantilever calibration is based on a systems dynamics approach. It is observed that the resonant frequency of a cantilever is a function of its stiffness, so the cantilever is oscillated at resonance and the spring constant is determined with knowledge of the material [16, 39, 73]. This “calibration by dynamic response” is accurate and doesn’t require interaction with a substrate standard. However, due to the effort involved, this method is typically reserved for more expensive probes. There are yet other methods for cantilever stiffness calibration, including a temperature-based method discussed in the next section, underscoring the activity in this field and the importance of obtaining calibrated force probes.

### 3.2.2 Equivartition Theorem

To ensure that the forces involved in complementary DNA bases are of sufficient magnitude to be detectable by an atomic force microscope, it is necessary to understand the physical limit of the AFM’s force detection capabilities. At the length scale of AFM probes, measuring the cantilever deflection is limited by thermal noise.

The equipartition theorem, an important result in thermodynamics, statistical mechanics and kinetic theory, says that any variable \( x \) representing a degree of freedom and entering
Figure 3-3: An AFM cantilever is modeled as a simple harmonic oscillator with stiffness $\kappa$ and damping coefficient $b$ at thermal equilibrium in the surrounding gaseous environment. Random motion causes gas molecules at temperature $T$ to impact the cantilever, resulting in cantilever deflection in the $x$ direction.

the Hamiltonian through a quadratic additive term $x^2$, has a mean thermal energy equal to $\frac{1}{2}k_BT$ where $T$ is the temperature and $k_B$ is the Boltzman constant [51].

Consider a cantilever modeled as a simple harmonic oscillator shown in Figure 3-3 parameterized by stiffness $\kappa$, damping coefficient $b$, and has a degree of freedom in the $x$ direction. The gas molecules from the surrounding environment impinge on the cantilever in all directions, but only the perpendicular component of the impacts contribute to cantilever motion (i.e., motion in the $x$ direction). Equating the kinetic energy of the gas molecules to the oscillator’s potential energy for a system in thermal equilibrium, we have an expression for the mean-square free end displacement of the end of the cantilever

$$\frac{1}{2}\kappa <\Delta x^2> = \frac{1}{2}k_BT.$$ \hspace{1cm} (3.8)

Here we note that the mean displacement of the cantilever is a function of spring stiffness and temperature. This allows yet another method for the calibration of the cantilever’s spring constant by measuring displacement and temperature.

Finally, since the modeled cantilever force is linear with displacement, we have the force due to thermal noise:
3.2. LIMITS OF FORCE RESOLUTION

\[ <\Delta F^2> = \kappa k_B T. \]  

Equation (3.9) represents the minimum theoretical mean-square force detectable by deflection of the AFM cantilever. This thermal noise limit is governed by the stiffness of the cantilever at a given temperature. It is important to note that the stiffness of the cantilever should be chosen to balance this minimum detectable force that benefits from a smaller \( \kappa \), with the system’s bandwidth that benefits from a larger \( \kappa \) (the bandwidth is bounded by the corner frequency given by \( \omega_c = \kappa/b \)). For cantilevers with a typical spring constant of 40 N/m the mean-square force detectable by the AFM is \( 1.64 \times 10^{-19} \) N—orders of magnitude smaller than the binding forces expected for complementary bases.\(^3\) Finally, it is worth highlighting that in practice, force resolution is often governed by calibration accuracy, variation due to fluctuation in temperature, humidity and pressure, and coupling between the cantilever and piezo tube dynamics.

3.2.3 DNA Binding Force

This section demonstrates that the binding forces of complementary DNA nucleotides are within the range of measurable forces, in light of the AFM resolution limit previously demonstrated.

Force spectroscopy studies have been conducted on strands of DNA to measure the sequence-dependent mechanical properties. The force required to “unzip” a single DNA molecule of a known sequence was measured and found to be \( 20 \pm 3 \) pN for each G-C pair, and \( 9 \pm 3 \) pN for each A-T pair \([71]\). Other researchers have reported higher values for DNA base binding forces. Lee et. al. measured the interaction forces between complementary 20-base strands covalently immobilized to a silica probe and surface. Non-complementary strands served as specificity controls. Measurements of binding forces give approximately 70 pN per base pair \([49]\).

In a different approach, Boland and Ratner use self-assembled monolayers functionalized with opposing bases on gold-coated AFM tips and planar substrates. Binding forces could

\(^3\)Note that here we don’t consider the limitations caused by the optical system on force resolution; we assume that any cantilever deflection is measurable.
only be measured in the presence of complementary bases, and was reported as 54 pN per base pair [5].

While there exists some disagreement in the literature of the strength of the binding force of individual bases, it is clear that all values reported are within the range of detection of an atomic force microscope. It is important to note that these studies use different methods of force measurement than the technique proposed in this thesis. Our approach allows for much higher spatial resolution, required for distinguishing not only the presence of complementary bases on the target strand, but also their relative location—a necessary component for sequencing. The limits of spatial resolution of the AFM in the context of sequencing DNA is discussed in the following section.

3.3 Limits of Spatial Resolution

In order to sequence DNA using a specially coated AFM probe, it is not only necessary to detect the interaction force between the probe and target strand, it is also necessary to determine the location of the target base with sufficient resolution as to reconstruct the arrangement of the bases. Here, we detail the parameters of scanning probe microscopy that influence image resolution.

It is well known that due to the mechanical contact inherent to AFM, acquiring images is a spatial convolution operation. In other words, the image generated is a result of the sample topography convolved with the shape of the probe [3]. The finite size of the probe acts as a low-pass filter, rounding sharp features of the sample as shown in Figure 3-4.

If the sample’s true topography is $H(x, y)$, and the probe has a shape characterized by $P(x, y)$, then the image obtained by scanning the probe over the sample is a two-dimensional spatial convolution given by,

$$I(x, y) = \int_{-\infty}^{\infty} H(\sigma, \rho)P(x - \sigma, y - \rho)d\sigma d\rho$$

(3.10)

$$I(x, y) = H(x, y) \otimes P(x, y)$$

(3.11)
3.3. LIMITS OF SPATIAL RESOLUTION

Figure 3-4: Because the image obtained in atomic force microscopy is a spatial convolution between the sample topography and a probe shape, finite probe sizes can cause imaging artifacts that misrepresent the actual sample, limiting the lateral resolution. The probe’s radius serves as a low-pass filter of sample height and attenuates high frequency spatial details. Image courtesy of Aumond [3].

Convolution artifacts are not limited to AFM imaging and are well characterized in many instruments. In optical microscopes, for example, the acquired image is a convolution between the sample’s reflected light and the optical system’s point spread function, \( I(x, y) = H(x, y) \otimes P(x, y) \).

Convolution artifacts in AFM imaging are most noticeable in two situations: (1) where the probe radius of curvature is on the order of the features to be resolved, or (2) for samples with deep trenches which make contact with the sidewalls of the probe. While the latter is not particularly relevant for imaging DNA on an atomically-flat surface, the former places a limit on the spatial resolution. As previously mentioned, the spacing of bases on a DNA backbone is 0.34 \( nm \) in helical form, and if relative position is to be discerned, a probe with a radius of that order is required. Here we emphasize that while lateral resolution is a function of the probe geometry, vertical resolution is a function of the cantilever deflection sensitivity and is directly related to the interaction force detection, which was discussed in the previous section.

Commercial silicon AFM probes are available with a radius of curvature as small as 10 \( nm \)—much too big to provide sub-molecular details needed for DNA sequencing. However, recent developments in carbon nanotubes with diameters on the order of 0.5 \( nm \) will
allow the resolution necessary to detect bases on strands of DNA with sufficiently high resolution to determine the sequence.

3.4 Summary

This chapter presents the theory of operation of the atomic force microscope, with special attention paid to the properties important for a biomolecular investigation tool. It is shown how attractive forces impact the phase lag of the resonating cantilever, leading to a method for detection of the binding between complementary bases. A model of hydrogen bonding characteristic of DNA nucleotides is presented, illustrating the nature of the force to be measured by the AFM.

The smallest detectable mean square force of the AFM is shown to be $1.64 \times 10^{-19} \text{ N}$—much smaller than the binding force of complementary bases cited earlier. Finally, a discussion is presented on the spatial resolution limit of a probe with a finite radius tip. While the conventional 10 nm-radius probes commercially available are insufficient to resolve the spacing of bases on DNA, new carbon nanotube probes promise to increase the lateral resolution of imaging due to their small diameter. Carbon nanotube AFM probes are the subject of the next chapter.
Chapter 4

Functionalized Carbon Nanotube AFM Probes

Since their discovery by Iijima in 1991, carbon nanotubes (CNTs) have been studied for their highly unusual mechanical properties [40]. Extraordinary strength (Young’s modulus of CNTs have been reported as high as 1.26 TPa—5 times greater than steel [103]) and the ability to buckle elastically [18] have been demonstrated from a tube with a diameter on the order of a few nanometers. This chapter reviews the state of CNT research and details their use as probes in scanning microscopy. Due to their recent discovery and promising future, additional attention is given in this chapter to the novel uses of carbon nanotubes in nanotechnology. A comparison is offered between chemically functionalized CNT AFM probes and more conventional etched silicon (ES) probes functionalized with self-assembled monolayers (SAMs), and the results strongly favor CNT probes for AFM-based DNA sequencing. The procedures developed for attaching CNTs to AFM probes and molecular functionalization are also presented.
CHAPTER 4. FUNCTIONALIZED CARBON NANOTUBE AFM PROBES

4.1 Carbon Nanotubes

4.1.1 Carbon Nanotube Properties

Carbon nanotubes are self-assembling sheets of hexagonally-arranged, sp²-bonded carbon atoms rolled into tubes as shown in Figure 4-1. The arrangement of the atoms is equivalent to a graphite sheet rolled onto itself and the end capped with a hemispherical fullerene. CNTs have been observed with diameters as small as 0.42 nm and lengths as long as several centimeters [8, 87]. CNTs are typically classified as either single-walled nanotubes (SWNTs) which are only one atom thick and have characteristic diameters between 0.42 and 10 nm, or multi-walled nanotubes (MWNTs) which have several nested tubes on a common axis and diameters of 5 to 100 nm. Their unique molecular arrangement described above gives rise to unusual and exciting mechanical properties.

Due to the covalent bonding and unique arrangement of carbon in a seamless, defect-free structure, CNTs are among the strongest and most resilient materials known. Their mechanical properties have been measured directly and values reported in the literature indicate a very high Young's modulus of 1.26 TPa and bending strength of 28.5 GPa for MWNTs [22, 103]. Interestingly, their ability to buckle elastically and store strain energy reversibly lead to high toughness. The unique geometric attributes of CNTs, namely small
4.1. CARBON NANOTUBES

Figure 4-2: High resolution STM images of CNTs. **Left:** A bundle of single-walled nanotubes (SWNT) is shown. **Right:** The hexagonal structure of the carbon atoms on a SWNT can be observed. Figure courtesy of *J. Matl. Rsch.* [67](left) and *Nature* [66](right).

diameter and large aspect ratio, provide a means for high resolution characterization of surfaces (including samples with deep trenches) when used as probes in either atomic force microscopy or scanning tunneling microscopy (STM) [37, 60, 109]. In fact, the metallic nature of some CNTs allows for tunneling current necessary in STM, and the high aspect ratio creates an almost ideal electron emitter, reducing imaging artifacts due to probe geometry. Two recent reviews of CNTs highlighting their unusual mechanical properties are provided by Terrones [88] and Thostenson [90]. High resolution scanning tunneling microscope (STM) images of CNTs in Figure 4-2 show SNWT bundles and reveal the hexagonal arrangement of carbon in a nanotube [66, 67].

4.1.2 Synthesis of Carbon Nanotubes

The exact growth mechanisms of CNTs are not well understood and still an active area of research. However, it is widely suspected that growth initiates from nanometer-size catalysts particles. The three most commonly used synthesis techniques are arc discharge, laser ablation and chemical vapor deposition. In the arc discharge method, two carbon electrodes are placed a short distance apart in an inert gas at low pressure. A spark bridges the gap and nanotubes are created between the electrodes. Varying the temperature, gas,
pressure, and electrode doping will affect the quality of the resulting nanotubes and ratio of SWNT to MWNT [26, 43].

The laser ablation technique fires either a pulsed or constant laser at a graphite target in an atmosphere of helium or argon. Extremely hot carbon is ejected and as the vaporized species cool, small carbon molecules quickly condense on the catalysts, which limits the formation of the fullerene end caps thereby encouraging longer tubes to form. This technique produces a large number of SWNT which form bundles held together by van der Waals forces [35].

Nanotube growth by chemical vapor deposition is the most popular method and the technique used in this research. A carbon source in the gas phase is heated to between 600°C and 900°C in an oven with a substrate on which the nanotubes will grow. Common feedstock gases include methane, carbon monoxide and acetylene. Carbon atoms are dissociated from the gas and diffuse toward the surface, which has been previously deposited with the catalysts. Typical catalysis used are transition metals such as nickel, iron, or cobalt. CNTs grow from the catalysts on the substrate. Careful control of temperature, catalyst density, feedstock gas concentration and flowrates are required to ensure quality in the produced nanotubes [9, 10].

4.1.3 Current Research and Applications of CNTs

Much of the excitement in the nanotube research as been directed at the possible application of the novel material. This section presents a brief overview of recent research directed at the possible applications of CNTs. Their small size and possibility of functionalization, or attachment of specific molecules, have led to the suggestion that CNTs can be used as chemical and biomolecular sensors [12, 20, 21, 84]. Other applications focused in electronics and integrated circuits use CNTs as electrically conducting nanowires or as diodes [15, 88], and much of this effort is directed at controlled growth of nanowires [46, 83]. Additional work focuses on the growth of nanotubes perpendicular to the substrate so that the vertical CNTs can be used as low-power electron emitters [29] exploiting the CNT near-ideal electron emission efficiency as a light source for displays [20, 23, 44]. Also, high density storage devices (1.6 Tbits/in) have been demonstrated using a CNT that locally oxidizes
an atomically flat sheet of titanium to write bits of data [17].

The application most relevant to this document is the use of CNTs as probes in either scanning tunneling microscopy [60] or atomic force microscopy [11, 13, 14, 18, 36, 37, 109]. The small diameter SWNT provides a sharp point when used as a probe and therefore allows for very high lateral resolution imagery (vertical resolution is dictated by the sensitivity of cantilever deflection measurement in AFM, or tunneling current detectability in STM). Additionally, the strength of the nanotube eliminates probe wear, which is not an attribute of standard etched silicon (ES) probes [48]. Finally, recent work has revealed methods for the chemical functionalization of CNTs, and when used as AFM probes, provide specificity in chemical binding force measurements [105, 107]. It is this body of research from which the present approach is derived.

### 4.2 Chemical Force Microscopy

Shortly after the advent of the atomic force microscope by Binnig and Quate [4], it was realized that chemically coating the probe with functional groups allowed specific molecular binding forces to be studied. Significantly, the absolute force resolution of the AFM can be several orders of magnitude larger than the weakest chemical bond [80]. This development provided new molecular-level understanding of a variety of phenomena, including friction and boundary lubrication, adhesion and fracture at interfaces, double-layer forces, and colloid stability [30, 64]. Since most AFM cantilevers and probes are etched from silicon or silicon nitride, chemistries used to coat these materials are employed. Using chemically modified AFM tips to measure specific interaction forces is termed “chemical force microscopy.”

More recently, effort has been directed at using carbon nanotubes as AFM probes due to their robust, small radius tips [18, 68, 104, 108]. Owing to the large success of functionalized conventional tips, work has been conducted on chemical attachment to CNT ends for high resolution chemical force microscopy [37, 105, 110]

This section will consider the functionalization of conventional AFM tips with self-assembled monolayers and compare the resulting probe performance to functionalized CNT
AFM probes in the context of a force-based DNA sequencing system.

4.2.1 Chemical Force Microscopy with Etched Silicon Probes

Chemical force microscopy was first used with conventional AFM tips etched from silicon or silicon nitride [1, 31, 34, 79, 89]. The radius of curvature at the end of these probes is limited by the etching process and tips as sharp as 10 nm can be reliably produced. However, functionalization of silicon or silicon nitride tips requires the deposition of metals (typically chromium or titanium followed by gold) to provide a substrate for the self-assembled monolayer (SAM). The deposition of the metals typically add another 10 to 20 nm to the tip, decreasing the sharpness and therefore lateral resolution capability of the probe.

The SAM forms when the metal-coated AFM tip is immersed in a solution containing an organic thiol, as shown in Figure 4-3. One end of the thiol covalently bonds to the gold surface, and the other end contains an appropriate head group which can be functionalized with well known chemistry [61, 97]. For example, variation of the head group molecules of the SAM have led to studies of adhesion force measurements as a function of pH (force titrations), direct characterization of hydrogen bonding strength, and determination of the chirality of various compounds [55, 93, 97, 101].

DNA Sequencing with Etched Silicon Probes

In addition to using chemical force microscopy with conventional tips for research listed above, it has been suggested that DNA sequencing could be performed by detecting the specific force interactions of complementary DNA bases [28]. In order to sequence DNA, conventional AFM tips functionalized with a DNA base is brought into close proximity with single-sided DNA so that the complimentary base interaction force could be detected (see Figure 4-3).

Despite the advantages of measuring highly-specific force data, several problems remain with functionalized conventional AFM probes. Notably, the increased radius of curvature, necessary for attachment of the SAM to the AFM probe, causes a loss in resolution. To discern the location and arrangement of individual bases in DNA using specific force measurements, imaging resolution on the order of base pair spacing is required. As discussed
4.2. CHEMICAL FORCE MICROSCOPY

Figure 4-3: **Left:** A conventional AFM tip is coated with several nanometers of titanium and gold, and an adenine-functionalized SAM is created. The deposition of metals increases the radius of curvature of the tip, which decreases lateral resolution. **Right:** Detecting the locations of thymine bases on a single-sided DNA molecule with an adenine-functionalized SAM on a conventional AFM probe. Figure courtesy of Elmouelhi [28].

Earlier in Section 2.2, bases on a DNA backbone are spaced at approximated 0.34 nm—much too close to be distinguished with functionalized ES AFM probes. The issue of decreased resolution was noted in a recent paper on chemical force microscopy by Vezenov et. al.:

“Lastly, it is important for practical applications to consider imaging resolution. The true resolution will be determined by the actual tip-sample contact area. ...To carry out measurements with a spatial resolution of 1 nm, it will be necessary to use tips with radii of a comparable scale. As estimated in our previous work [61], 1 nm resolution can be achieved with a tip radius of ~8 nm, if the surface forces are relatively small. Currently, commercial AFM tips with radii on the order of 10 nm are available, and a recent report demonstrating the fabrication of carbon nanotube tips suggests that much smaller radii should be possible [18]. Strategies to modify chemically these tips while preserving their sharpness must, however, be developed.” [97]

Vezenov mentions the possibility of increasing the resolution of specific force characterization through the use of functionalized carbon nanotubes, a field which has received increased attention in recent years. The next section will discuss the use of functionalized...
carbon nanotube probes and compare their properties to those of conventional probes.

4.2.2 Chemical Force Microscopy with CNT Probes

Increased Imaging Resolution

It is recognized that by attaching or growing a carbon nanotube from the end of an AFM cantilever to be used as a probe, significant advantages could be realized over silicon and silicon nitride probes [18, 65, 81, 82, 85]. Specifically, the relatively low resolution and poor wear characteristics of functionalized conventional probes are addressed—both important attributes that must be addressed for a viable DNA sequencing system. Since an AFM image is a spatial convolution of the topography of the sample and the tip structure as discussed in Section 3.3, the size and shape of the tip will define the resolution of the image. A scale representation of an ES functionalized probe and a single-walled carbon nanotube probe is shown in Figure 4-4. The conventional 10 nm-radius probe is shown with 14 nm of deposited metal (the SAM has a negligible thickness) and the CNT shown is single-walled with a typical diameter of 1 nm. Also shown for scale is the spacing of bases in a DNA molecule. Due to the increased resolution of AFM imaging with CNT probes, structural details of DNA and other biological molecules have been observed directly [92, 108, 109].

Reduced Wear

In addition to decreased resolution due to finite tip radius, silicon-based probes are known to wear. The portion of the probe that makes contact with the sample experiences a high pressure that removes material from the tip. Wear of sharpened silicon probes is exacerbated by the smaller contact area which leads to increased tip pressure. A comparison of wear characteristics between etched silicon and carbon nanotube tips showed that images taken with an ES probe exhibit noticeable degradation in resolution and image quality after only 35 scans and 1 hour of continuous scanning [48]. The CNT probes show no wear after over 1000 scans and 9 hours of scanning. A probe that exhibits little or no wear is important for sequencing the three billion base pairs in a human genome where scan times will be on the order of several days. It was also noted that the unique aspect ratio of the long, slender
Figure 4-4: **Top:** A scale representation of an etched silicon functionalized probe and a single-walled carbon nanotube probe. The conventional 10 nm-radius probe is shown with a 14 nm metal coating and the single-walled nanotube has a 1 nm diameter. **Bottom:** A coated tip is overlayed with a SWNT tip for comparison to the spacing of bases in a DNA molecule.
CNT allow these types of probes to image deep trenches, which would otherwise appear distorted by pyramidal-shaped ES probes due to convolution artifacts.

**Buckling Analysis**

An additional benefit of using CNTs as probe tips stems from the ability of the carbon nanotube to buckle elastically. The reversible buckling characteristic allows the CNT to act as a probe with an upper force limit (buckling relieves the stress experienced by the sample). When used in the context of a biological sensor, delicate biomolecules such as DNA can be probed without damage.

Modeling the nanotube as a beam clamped at one end and free at the other, the Euler buckling force $F_B$ is

$$F_B = \frac{\pi^2 EI}{4L^2}$$  \hspace{1cm} (4.1)

where $E$ is the Young’s modulus of a CNT, $I$ is the area moment of inertia for a nanotube of radius $r$ ($I \approx \pi r^4$), and $L$ is the length of the tube. For a SWNT attached to an AFM probe with $E = 1.26 \ TPa$, $L = 50 \ nm$ and $r = 1 \ nm$, the buckling force is $F_B \approx 4 \ nN$. A buckling force of this magnitude would allow the AFM cantilever to deflect to a measurable degree, yet it is small enough to limit the force applied to compliant biological material. For example, the nanotube probes supplied by Veeco Instruments [94] have a cantilever stiffness of $3 \ N/m$, giving the expected cantilever deflection just before buckling of $1.3 \ nm$, which is easily detectable by most AFMs. If buckling does occur, the nanotube would return to its natural configuration after the AFM tip is retracted. This unique elastic buckling behavior is exploited in the shortening and functionalization process used in this research and is described further in Section 4.3.2. Note that this analysis assumes a continuum mechanics approach may be used to model the buckling behavior. The scale at which classical mechanics breaks down is not at all clear.

**Attaching CNTs to AFM Probes**

The augmentation of AFM probes with nanotubes began as the crude process of bringing an AFM tip coated with a soft acrylic adhesive into contact with a bundle of MWNTs.
Occasionally, a nanotube from the bundle adheres and the probe is retracted, leaving the nanotube glued to the tip [18]. This process is performed in a dark-field illuminated optical microscope, and the operator manipulates the AFM tip into place. CNT tips are created serially in a labor-intensive process that requires the use of thicker but more visible MWNTs, making the resulting CNT tips of the same radius as ES tips. Later methods developed use the chemical vapor deposition growth technique in which an array of AFM cantilevers is seeded with catalyst particles, and SWNTs are grown directly on the tips in a parallel, automated fashion [11, 13, 14, 36, 37, 109]. This method can be scaled to wafer-sized production and is the technique currently employed by commercial vendors such as Veeco Instruments [94] and NanoScience Instruments [59]. The carbon nanotube AFM tips used in this work (Veeco Instruments) are grown using the chemical vapor deposition process.

Functionalizing CNT Probes

The most critical step in creating a chemically-sensitive CNT probe for DNA sequencing is functionalization. The difficulty lies in attaching a functional group to the unreactive and stable nanotube, which as grown, has fullerene hemispheres at the end caps as shown in Figure 4-1. Often, an electric potential must be applied across the length of the tube of significant voltage in order to remove material from the nanotube, exposing carbon to other species. If this process is done in air, carboxyl groups (COOH) are formed at the opened ends. This has been verified with force titration studies, which characterize the binding force between a COOH-functionalized AFM tip and a COOH-coated substrate as a function of pH [105, 107]. Very good agreement with classic pH titration indicates that COOH readily and stably forms at the ends of opened nanotubes in an oxidizing environment. If the end caps are removed in the presence of other gases in a well-controlled atmosphere, ionization at the tip can produce nanotubes functionalized with a wide range of atomic species (for example, H₂⁺, N₂⁺ and O₂⁺ functionalized nanotubes have been reported [106]). However, the reactivity of carboxyl groups with amines, and the ease with which carboxyl groups are formed (end cap removal can be done in air) has encouraged research in the functionalization of nanotubes through carboxyl end groups [21]. It is proposed to attach a DNA base to a CNT AFM probe via the carboxyl end groups using this functionalization process.
The increased resolution and specific binding information gained by using functionalized CNT probes have been exploited in molecular biology to quantify binding forces between biotin and streptavidin, important proteins used in cell receptors [109]. Recent surveys provide a review of AFM imaging with functionalized carbon nanotube probes [24, 37, 52] and highlight their novel uses such as a field emitter [44] or an STM probe [60] by attaching Zn to a COOH-terminated CNT.

One of the most relevant applications of functionalized carbon nanotube AFM probes to this thesis has been performed by Leiber’s group at Harvard. Woolley et. al attach an oligonucleotide of a known sequence to the end of a CNT and scan single-sided DNA for locations of the matching sequence [110]. In this approach, the complementary sequence on the target DNA strand will cause the AFM cantilever to deflect, creating a mechanism to rapidly probe DNA for a predetermined sequence. This technique has been employed to hunt for single nucleotide polymorphisms (SNPs) which are single-base mutations strongly linked to certain cancers. By scanning with a functionalized CNT probe, single-molecule haplotyping has been performed [19, 102].

Functionalization via carboxyl groups is not the only method developed; chemical derivation with thiols have been performed, leading to a new type of bond between metals and carbon nanotubes [50]. And functionalization via the esterification reaction of the carboxylate salt of carbon nanotubes and alkyl halides has been demonstrated [69]. Additionally, many applications of carbon nanotubes would benefit from the functionalization of the side walls of the tube [75]. Research has been conducted toward this end, allowing high nanotube solubility—important for the creation of CNT composites [32].

4.3 Using Functionalized CNT Probes for DNA Sequencing

This section will describe the method proposed in this research to prepare carbon nanotubes grown on AFM tips for DNA sequencing. The goal is to obtain a force probe that detects the presence of a single targeted base. The location of the attractive binding force between complementary bases will be detected with an atomic force microscope operated in phase imaging mode, and used to reconstruct the sequence. Toward that end, we desire a CNT
4.3. USING FUNCTIONALIZED CNT PROBES FOR DNA SEQUENCING

AFM probe functionalized with a single nucleotide and oriented in such a way that its hydrogen bonds are available to interact with the target strand.

4.3.1 Shortening CNT Probes

Carbon nanotube AFM probes are obtained from Veeco Instruments [94] grown with chemical vapor deposition on intermittent-contact probes (i.e., tapping mode probes). The length of the nanotubes is not regulated as part of the manufacturing process, and the nanotubes can be as long as 2 μm. In order to obtain good image quality, the nanotube must be shortened to minimize thermal-induced fluctuations at the free end. Gas molecules with kinetic energy proportional to temperature strike the nanotube, causing the end to vibrate; and these disturbances cause a mean-square displacement of the tip (see the discussion on the equipartition theorem in Section 3.2.2). The mean-square free end displacement of the tip $<\Delta x^2>$ is given as

$$\frac{1}{2}\kappa <\Delta x^2> = \frac{1}{2}k_BT$$  \hspace{1cm} (4.2)

where $\kappa$ is the bending stiffness of the nanotube, $k_B$ is the Boltzmann constant, and $T$ is the temperature. Recall that carbon nanotubes bend easily (leading to their high toughness) indicating a low value for $\kappa$. For long as-grown nanotubes, the mean-square displacement of the nanotube tip is tens of nanometers—too high for a useful probe.
Therefore, the long nanotubes received from the vendor must be shortened to an appropriate length (see Figure 4-5). Fortunately, since functionalization of the carbon nanotube requires removing the end caps, attachment of carboxyl groups and removal of excess nanotube can be performed in the same step. The most popular technique of shortening the nanotube is removal of material assisted with high voltages described earlier [13, 14, 36, 112].

The nanotube probes obtained from the vendor are shortened in air by attaching electrodes to the cantilever and the substrate as shown in Figure 4-6. The sputtered niobium substrate (Nioprobe, Electron Microscopy Services [27]) used for the shortening process is connected electrically to ground. The probe is connected to a positive DC signal between 5 and 20 V. The AFM cantilever is then oscillated near its resonant frequency and brought into momentary contact with the substrate. Due to the electric potential applied, an arc is created at the gap, removing carbon near the end of the nanotube. Shortening the nanotube in air allows oxidization to occur at the free end, which forms carboxyl groups. Because the amount of carbon removed cannot be controlled, the length of the newly-shortened nanotube must be measured after each shortening operation, and if it is still too long, the process is repeated. The process proposed to measure the length of the nanotube is described in the next section.
4.3. USING FUNCTIONALIZED CNT PROBES FOR DNA SEQUENCING

4.3.2 Measuring Nanotube Length Using the AFM

A carbon nanotube that is too long will vibrate under thermal fluctuations, negatively impacting its capability to produce high-resolution images. For this reason, it is important to know how long the attached carbon nanotube is and that it is sufficiently short for imaging.¹

As described in the previous section, the nanotube shortening process does not control for the amount of material removed, and therefore the shortening operation must be followed by nanotube measurement. Traditionally, the probe with attached nanotube is removed from the AFM and imaged directly with an auxiliary tool such as a scanning electron microscope. (The carbon nanotube AFM probes shown in Figure 4-5 were imaged in this manner.) However, we propose a novel measurement technique that uses only existing AFM signals and does not require the probe to be removed from the AFM. This new approach takes advantage of the elastic buckling property of carbon nanotubes, and the sensitive cantilever deflection capability of the AFM.

Measuring the length of the nanotube with this new technique requires operating the AFM in force-distance mode where the force required to deflect the cantilever is plotted against the piezo scanner’s vertical extension.² With the nanotube and the substrate electrically grounded, the piezo is gradually extended until the nanotube begins to make contact (as noted by the increase in cantilever deflection), and the piezo extension is recorded. The piezo continues to extend, causing the cantilever to deflect and an increased axial load on the nanotube, and eventually the nanotube buckles. The piezo extends further until the tip of the etched silicon probe makes contact with the substrate and this final extension value is recorded. The difference between the initial and final extension values of the piezo provides an estimated length of the nanotube (any residual deflection of the cantilever must be taken into account).

Importantly, this method facilitates the iterative nature of the AFM-based shortening operation described earlier because it is not required to remove the nanotube/cantilever

¹Experimentally, we have found that nanotubes less than 300 nm provide the expected high-resolution images.
²Refer to Section A.3 in the Appendix for instructions performing this operation on a Quesant Q-250 atomic force microscope.
Extend piezo while monitoring cantilever deflection

CNT makes contact

Cantilever deflection measured by AFM

CNT buckles

Figure 4-7: **Left:** To indirectly measure the length of the nanotube, the AFM’s piezo is extended until the tip of the nanotube just makes contact with the substrate. **Center:** The piezo is further extended, causing the cantilever to deflect. **Right:** Additional extension will cause the nanotube to buckle and the cantilever to relax, and this sudden cantilever release is recorded by the AFM. The length of the nanotube is approximated by comparing the piezo extension before and after nanotube buckling: \( \text{Length}_{\text{CNT}} \approx z_{\text{initial}} - z_{\text{final}} \).

assembly from the AFM. See Figure 4-7 for a schematic of this operation. Experimental data of this approach is provided in Chapter 5.

### 4.3.3 Functionalization Chemistry

At this point, the CNT is shortened to an appropriate length, and COOH molecules have formed at the free end. Since carboxyl chemistry is well understood in macroscopic quantities, many functionalization procedures are available. To obtain a probe functionalized with the DNA base thymine, the following procedure is adapted from [107]:\(^3\)

An esterification reaction is used to attach thymidine (a chemical derivative of thymine) to a carboxyl group via carbodiimide. Specifically, the shortened nanotube probes are placed

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\(^3\)The experimental protocol used to prepare the thymine-functionalized carbon nanotube probes is described in greater detail in Section A.4 of the Appendix.
4.3. USING FUNCTIONALIZED CNT PROBES FOR DNA SEQUENCING

in 0.1 M MES (2-[N-morpholino]ethanesulfonic acid) (Sigma Aldrich [78]) pH 6.0 buffer containing 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (VWR [98]) and 50 mM thymidine (VWR) for two hours. The probes are then washed in 0.1 M MES buffer pH 6.0, 0.1 M NaCl (VWR), and deionized water. During this process, the carbodiimide attacks the primary alcohol group on the thymidine’s sugar. The nucleotide attached to the nanotube is expected to be stable.

4.3.4 Addressing Potential Concerns

Although the functionalized nanotube AFM probe is a promising and novel technique for DNA sequencing, there are potential problems that must be addressed. Namely, the diameter of even the SWNT probes are several times larger than the spacing of bases on a DNA backbone. Recall that DNA bases have a pitch of 0.34 nm—giving about three bases per CNT probe width. However, since this detection method is best performed with a single base on a probe interacting with a single base on the target DNA, sufficient resolution can be obtained provided that functionalization leaves only one functional molecule at the end of the CNT. This ensures that the detected interaction force is specific to a single location on the target DNA. Unfortunately, the functionalization procedure outlined here does not control for single-molecule attachment. However, due to the small diameter of the SWNT there are perhaps as few as 10 exposed carbon atoms at the nanotube end, and therefore are very few possible binding sites for carboxyl groups to form. In fact, research on detecting the specific binding force between biotin and streptavidin with many functionalized SWNT probes have revealed that functional groups usually form at one or two carbon atoms on the opened end [105]. It is proposed to screen probes for single functional groups by quantifying the measured attractive force with a known binding force standard.

Finally, it is important to point out that even smaller diameter SWNTs have been created in solution [87]. Nanotubes with diameters of 0.42 nm have been observed and 0.33 nm-diameter nanotubes are predicted to be stable up to 1100°C [8]. While the processes involved in the growth of SWNT on AFM tips have not produced such small diameter nanotubes, there seems to be no fundamental physical limit prohibiting such an advancement. These ultra-small diameter SWNT probes would have lateral resolution capability sufficient
to unambiguously distinguish nucleotides on a single molecule of DNA.

4.4 Summary

This chapter discusses carbon nanotubes and how they are grown, prepared on AFM tips, and functionalized to create a chemically-specific, high resolution sensor capable of detecting individual nucleotides for sequencing. Due to recent research activity, nanotube growth techniques and current applications of carbon nanotubes are reviewed.

A comparison between conventional etched silicon probes and carbon nanotube probes indicate that that nanotubes offer advantages in probe wear, force-limiting reversible buckling, and increased resolution. Chemical force microscopy, typically performed with silicon probes, has been demonstrated with carbon nanotubes and provides higher resolution and more robust chemically-specific force characterization.

A method for shortening nanotubes attached to AFM probes is presented, which relies on high voltage arcing to remove material. This technique has the advantage of using only sensors and actuators available on an unmodified atomic force microscope. However, since the length of removed nanotube is not controlled, a novel method for measuring the length of nanotube is described. The nanotube can be measured using only standard AFM signals by exploiting its reversible buckling property. The shortening procedure creates carboxyl groups at the nanotube end, which can be further modified. A chemical procedure based on an esterification reaction is used to attach thymidine to the carboxyl end group, leaving a functionalized, high resolution probe capable of single molecule sequencing.
Chapter 5

Experimental Data

Earlier chapters have discussed DNA properties, atomic force microscope (AFM) operation, and the functionalization of carbon nanotubes. Specifically, in the chapter on DNA properties, the binding force of a single complementary nucleotide interaction is shown to be within the detection range of an AFM. During the discussion of atomic force microscopy, a model is presented illustrating how general attractive forces can be measured by examining the cantilever’s phase response. Also, a review of probe-shape convolution artifacts and the imaging resolution of the AFM motivates the need for a sharp probe based on carbon nanotubes. In the previous chapter, methods for shortening, measuring, and functionalizing carbon nanotubes are developed.

This chapter combines the various components to experimentally demonstrate a new method for obtaining DNA sequence information by directly probing single-stranded DNA (ssDNA) with a functionalized carbon nanotube AFM force probe. Experimental data is presented that demonstrates the novel nanotube length measurement technique described in Chapter 4, which uses only the signals provided by a standard AFM and facilitates the iterative nanotube shortening/measuring procedure. Finally, ssDNA is imaged with the shortened, functionalized nanotube probes and attractive binding forces between a thymine-derived probe and a single DNA molecule that contains a few adenine nucleotides. A control experiment is also discussed, which demonstrates that no binding occurs between a thymine force probe and a fragment of DNA with all thymine nucleotides.
5.1 Measuring Nanotube Length

As described in Section 4.3.2, a novel method has been developed to measure the length of the carbon nanotube attached to the tip of an AFM force probe. This method relies on the elastic buckling property of carbon nanotubes and the AFM’s ability to measure the deflection of the cantilever, and importantly it alleviates the need to use an auxiliary method to measure the length of the nanotube (e.g., directly via scanning electron microscopy as in Figure 4-5).

The AFM piezo is extended until the nanotube makes contact with the substrate, as detected by the increase in cantilever deflection. (On approach and before the nanotube makes contact, the cantilever is moving unimpeded through air and no deflection occurs.) After the nanotube makes contact, the piezo is further extended, causing both the cantilever to deflect and the nanotube to experience axial loading. The increased deflection of the cantilever is measured by the AFM and provides an indication of the nanotube loading. Once the buckling load is reached, the nanotube collapses, and the cantilever deflection is relaxed (see the discussion on “Buckling Analysis” in Section 4.2.2).

Experimental data of this operation is shown in Figure 5-1. Piezo extension is plotted on the horizontal axis and cantilever deflection on the vertical. The nanotube makes contact with the substrate at point (1) in the plot (also shown schematically as point (1) in the figure above the plot). The subsequent extension of the piezo and cantilever deflection manifest as an increase in slope of the trace. At point (2) the nanotube has buckled and the cantilever deflection returns to its undeformed configuration. The length of the nanotube can be read from the piezo extension between points (1) and (2) and shown to be approximately 220 nm for this data set.

After the nanotube is sufficiently shortened as to be stable during imaging, the end of the probe is chemically functionalized as described in Sections 4.3.3 and A.4. At this point, the modified nanotube is a high-resolution, chemically-specific force probe capable of nucleotide detection. Next, we present AFM images of single-stranded DNA with the functionalized nanotube probe, and demonstrate nucleotide detection.
5.1. MEASURING NANOTUBE LENGTH

Figure 5-1: Experimentally obtained data is shown that demonstrates the new carbon nanotube measurement technique. **Top:** A detailed schematic shows how the buckled nanotube can lead to a difference in cantilever deflection detectable by an AFM. **Bottom:** The AFM's sensor output (measuring cantilever deflection) is plotted as a function of piezo extension. The nanotube first makes contact at point (1) and buckles at point (2). This nanotube is measured to be about 220 nm.
5.2 Scanning Single-Stranded DNA

To demonstrate the principle that will lead to a high throughput and low cost sequencing technology, single-stranded DNA is imaged with a functionalized carbon nanotube probe. Recall that the attractive force interactions between a force probe’s nucleotide and the nucleotide’s complement on ssDNA is measured as an increase in phase lag of the oscillating AFM cantilever.

The approach is to immobilize single-stranded DNA on atomically-flat mica (as detailed in Section A.1 of the Appendix), and scan a region of the mica with the AFM in tapping mode. Since both topographic and phase information are collected simultaneously, both data sets can be used to aid in nucleotide identification. Specifically, the topographical scan is used to identify the locations of target ssDNA fragments, and the phase response of the corresponding ssDNA positions provide sequence information.

The experiments are performed on synthetic DNA fragments of 60 nucleotides, giving an expected length of 20 nm. The nanotube probe is shortened and functionalized with thymine, and scanned over ssDNA of different sequences in two experiments. The primary experiment scanned synthetic DNA of the following sequence (5' to 3'): 25 thymine, 10 adenine, and 25 thymine. It is expected to measure an increase in phase lag between the thymine-functionalized probe and the adenine molecules on the test strand at the center of the DNA fragments. A control experiment is also performed with the same thymine-functionalized probe. The control fragment sequence is 60 thymine nucleotides.

The resulting data from these two experiments are presented in Figures 5-2 (primary) and 5-3 (control). For the case of the primary experiment, a test fragment of DNA is identified in the topography region of Figure 5-2 based on its length and height (20 nm and 1 nm, respectively). The corresponding location in the phase response shows an increase in phase lag (shown as a darker region) at the center of the strand where thymine is expected to interact with the 10 adenine nucleotides.

The control experiment shown in Figure 5-3 also identifies a 20 nm feature, and examines the phase response of that fragment. As expected, there is no change in phase along the length of that fragment. The results from these two data sets confirm the dynamic model
of attractive force detection with an AFM presented in Section 3.1.2.

Figure 5-2: Experimental data demonstrating that nucleotide information can be obtained from a single molecule of DNA. All DNA fragments in this scan are synthetic and known to have the sequence: 25T–10A–25T. **Top:** The topographical output is examined to identify features that are 20 nm long and 1 nm high. **Bottom:** The phase response of the scanned region. **Detail:** An identified strand of DNA is examined in the phase domain. An increase in phase lag (shown as a darker region) at the center of the strand indicates the interaction between the thymine on the force probe and the adenine on the test strand.
Figure 5-3: AFM data of the control experiment with the same thymine-functionalized probe. All fragments scanned in this figure are known to have the sequence of 60 thymine nucleotides. **Top:** A strand of ssDNA is identified by its length and height. **Bottom:** The phase response of the scanned region. **Detail:** The identified strand of DNA shows no variation in phase response along its length, indicating no preferential attractive forces.
5.3 Summary

This chapter presents experimental data demonstrating the new carbon nanotube measurement technique, and data showing that sequence information can be obtained from a single molecule of DNA. The measurement technique relies on the elastic buckling property of carbon nanotubes and provides the length of the nanotube grown on a force probe using only signals found on an AFM. This indirect approach offers an increased savings of time in the processing and preparation of carbon nanotube AFM probes, as the previous method required direct measurement with an auxiliary microscope. Data obtained indicates that carbon nanotube probes shortened to approximately 220 nm are stable during imaging.

Finally, the proposed sequencing technique is demonstrated by scanning single-stranded DNA with a functionalized carbon nanotube probe. Locations of a known complementary nucleotide shows a discernable increase in phase lag, as predicted by the model in Chapter 3. To the best of the author's knowledge, this represents the first time that sequence information has been obtained from a single molecule of DNA by measuring the locations of attractive forces with a carbon nanotube probe.
Chapter 6

Conclusion

This chapter summarizes the submitted body of work on a novel single-molecule DNA sequencing approach based on complementary nucleotide binding force measurement. The common themes of force detection and resolution, each mentioned in context of DNA properties, atomic force microscope operation, and carbon nanotube probes, are discussed here from a larger perspective. Challenges faced implementing the proposed approach, and the methodologies invented to overcome them are also presented. Lastly, suggestions for future research directions are offered that promise to improve the method’s signal-to-noise ratio, increase sequencing throughput and expand the capabilities to sequence all four types of DNA nucleotides in one pass.

6.1 Contributions

The main contribution of this work is the demonstration that sequence information can be obtained from a single molecule of DNA by measuring the binding force between a chemically-functionalized probe and its complementary nucleotide on a target strand. The approach’s novelty requires thorough understanding of disparate components: DNA properties, AFM operation—including a methodical approach for the detection of attractive forces, and carbon nanotube behavior. Therefore, earlier chapters present these concepts in depth in the context of a novel sequencing technique. From these discussions, it is clear that both force detection and imaging resolution issues play a central role and must be
CHAPTER 6. CONCLUSION

considered from several perspectives, as detailed in the next sections.

A secondary, but important contribution is the development of a novel measurement technique that allows the length of the nanotube on the AFM probe tip to be ascertained without the need of an auxiliary microscope. This method made feasible the shortening process described in Section 4.3.1 which removes an unknown amount of material from the nanotube and therefore requires frequent nanotube measurement. Additional contributions include the adoption of existing procedures of nanotube shortening and large scale carboxyl chemistry to the proposed application. Specifically, issues dealing with the shortening of nanotubes mounted on an AFM probe, and the functionalization chemistry of a single carboxyl group are addressed in this work, and represent original progress in nanotechnology.

6.1.1 Force Detection

A major theme of this research is force detection. It is imperative that binding forces between nucleotides be of sufficient magnitude as to be detectable by an AFM, and a clearly defined approach for attractive force measurement is required. Since carbon nanotubes are used as force probes, their properties under axial loading must also be studied to ensure robust behavior when scanning DNA.

In Sections 2.3 and 3.2.3, DNA nucleotide binding forces are reported. It is found from experimentation that the individual nucleotides bind with forces between $9 \pm 3 \ pN$ and $70 \pm 3 \ pN$ \cite{5, 49, 71}. Therefore the proposed sequencing method must be capable of measuring forces of this magnitude. Section 3.1.3 presented a model of atomic forces based on an augmented Lennard-Jones description, and Section 3.2.2 described the force sensitivity of an AFM cantilever derived from the equipartition theorem. It is shown that by measuring the deflection of the cantilever, the AFM has the capability to discern forces on the order of $1.64 \times 10^{-19} \ N$—more than enough resolution to measure nucleotide binding forces.

Additionally, it is important to understand how attractive forces are measured with an AFM (recall that only repulsive forces act with the AFM operated in the ubiquitous contact mode). Toward that end, a dynamic model of an oscillating cantilever characteristic of the AFM in tapping mode is developed in Section 3.1.2, and it is shown that for a cantilever
oscillated at resonance, the effect of an applied force at the cantilever end is evident in the cantilever’s phase response. Specifically, attractive forces increase the phase lag of the oscillating cantilever, providing a signal that clearly indicates the presence of binding forces. It is the increased phase lag at the expected locations of A-T interaction presented in the experiments of Chapter 5 that demonstrates the proposed concept.

Additionally, the carbon nanotubes used as high-resolution force probes must be shown to be robust to the forces experienced during scanning for the approach to sequence all three billion base pairs in a human genome. Others have shown that nanotube probes do not wear when dragged across samples during AFM imaging [48], and a model of nanotube buckling derived in Section 4.2.2 shows that nanotube length is an important parameter in buckling—nanotubes that are too long buckle under light loading and make poor probes. This motivates the need to shorten the nanotubes sufficiently to avoid buckling. It is found that nanotubes shortened to approximately 220 nm are stable during imaging (see data from Section 5.1). As part of the shortening operation, a rapid procedure to indirectly measure the length of the nanotube is developed and reviewed in a later section.

6.1.2 Resolution

In addition to force detection, another major theme in this work is imaging resolution. It is important for any new sequencing technology to detect individual nucleotides—recall that a primary application of personal genome information is the study of single-nucleotide polymorphisms or single base mutations that are linked to illnesses such as cancer. Toward that end, attention is given to those parameters impacting resolution throughout this work to achieve the sensitivity necessary to identify individual nucleotides on a single molecule of DNA.

Recall from the discussion of DNA dimensions from Section 2.2 that the spacing of nucleotides along a strand of DNA in the β-form is 0.34 nm. This parameter sets the required resolution of the imaging system and ultimately dictates the use of single-walled carbon nanotubes as force probes. (An additional dimension that is important for this effort is the diameter of single-stranded DNA. Together with the base spacing, it is possible to identify DNA fragments in the experiments based on the expected dimensions of DNA as
observed in the topography output.)

The limit of spatial resolution in atomic force microscopy is set by probe-sample convolution, as described in Section 3.3. Because the finite shape of the probe acts as a low-pass filter attenuating high spatial frequency components, it is important to use a sharp probe in order to achieve very high resolution. Indeed, the conventional etched silicon probes have a tip radius of curvature on the order of 10 nm—too large to identify individual nucleotides. This motivates the use of single-walled carbon nanotubes as high resolution force probes.

The nanotube probes are chosen for their small diameter and resistance to wear. Single walled nanotubes can be synthesized with diameters of 1 nm or smaller. This provides a force probe with dimensions on the order of nucleotide spacing, which leads to the necessary high resolution imaging demonstrated experimentally in Chapter 5. However, before the nanotubes can be used for nucleotide binding force detection, they must be shortened and functionalized. This presents specific implementation challenges and their solutions are summarized in the next section.

6.1.3 Implementation

Obstacles specific to this approach became evident over the course of the research. Specifically, it is realized that the synthesized nanotubes are too long to be useful during scanning. They tend to buckle under the light loads experienced during routine imaging. Furthermore, it is required to chemically attach (or functionalize) a nucleotide to the end of the nominally unreactive carbon nanotube. Both of these issues are solved in the same operation: by shortening the carbon nanotube with electrical arcing, the nanotube is able to withstand an increased buckling load and carboxyl molecules form at the opened end providing a convenient chemical group for functionalization.

However, the shortening process adopted in this work does not control for the amount of material removed, and repeated measurements of the length of the nanotube are required. Typically these measurements are made with a scanning electron microscope as shown in Figure 4-5. However, this requires removing the nanotube probe from the AFM and imaging the probe directly in this secondary microscope. An important contribution of this work is the development of a technique that obtains the length of the probe using only those
signals available on a standard AFM, by relying on the carbon nanotube’s unique ability to elastically buckle. This new measurement process is described in theory in Section 4.3.2 and in practice for a Quesant Q-250 AFM in Appendix A.3. Experimental data demonstrating this new measurement approach is provided in Section 5.1.

Additional implementation issues addressed in this work include the shortening procedure and the functionalization work. The nanotube shortening procedure is adopted from [13, 14] and modified here for use with the currently available AFM. It is observed that carbon is is removed from the nanotube under the momentary application of about 20 V. The chemical functionalization of carboxyl groups is well understood in macroscopic quantities and adopted here from [107]. To the best of the author’s knowledge, this work represents the first time that a single nucleotide has been attached to the end of a carbon nanotube. The chemical protocol used is presented in Sections 4.3.3 and A.4.

6.2 Future Work

While the concept of obtaining sequence information from single DNA molecules is demonstrated, there exist many opportunities for improving the performance, and much effort remains before full genome-level DNA sequencing is attained. To become a viable sequencing technology, all four nucleotides must be identified. This could be done serially by scanning an immobilized strand of DNA in four passes, each with a carbon nanotube functionalized with a different nucleotide. However, the four passes required by this approach would dramatically slow sequencing throughput. It seems likely that if four probes could be mounted on the piezo and scanned over DNA in one pass, there would be no additional speed penalty. Unfortunately, this additional capability comes at a cost of design complexity, as each of the four probes must be mounted on its own cantilever and require their own laser and photosensitive diode. It is worth noting that parallel arrays of cantilevers have been explored and demonstrated at IBM, and therefore the design issues are not intractable. In recent research, they describe an AFM system with an array of 32 x 32 cantilevers, illustrating that multiple cantilever configurations are feasible [96].

Further suggestions for future work are categorized in two groups, each focusing on the
improvement of two important performance measures: improving accuracy and increasing sequencing throughput.

6.2.1 Improving Accuracy and Signal-to-Noise Ratio

The misidentification of a single nucleotide by a sequencing technology could lead to poor understanding of gene expression, inaccurate diagnosis of disease and disease susceptibility, and incorrect conclusions regarding the study of single-nucleotide polymorphisms, depending on the context in which the sequencer is applied. Toward that end, it is imperative to demonstrate that the proposed sequencing method is reliable and accurate. Further experimentation should be performed that shows individual nucleotide identification under challenging conditions—for example, the system must distinguish between two nucleotides of the same type next to each other (e.g., successfully identify the difference between \ldots TGAAC\ldots and \ldots TGAC\ldots). It is suggested to perform future experiments that unambiguously demonstrate that the proposed method has this capability by synthesizing test DNA fragments that contain the above sequences, and scan them with a T-functionalized CNT probe. If the current generation of carbon nanotube probes are unable to achieve the necessary accuracy, then the following suggestions are offered to improve the resolution.

Recent reports have shown carbon nanotubes can be synthesized with diameters of 0.42 nm and nanotubes with diameters as small as 0.33 nm are predicted to be stable [8, 87]. These ultra-small nanotubes are of the order of the base pair spacing of nucleotides on a DNA backbone. When nanotube synthesis and manufacturing techniques have progressed, these small diameter carbon nanotubes could be used in the proposed method.

In addition to resolution issues described above, another challenge faced in this work is force detection—the microscope must detect the attractive hydrogen bonds above the noise floor governed by nonspecific attractive forces (adhesion and van der Waals forces) and general repulsive forces (electrostatic and nuclear forces). In order to identify individual nucleotides, the sensitivity of the AFM’s force detection capability should be optimized where possible. Therefore, it is suggested to perform the DNA scanning in aqueous solution (as opposed to the current experimentation performed in air). With the AFM cantilever submerged and DNA in its native liquid environment, it becomes possible to control addi-
6.2. FUTURE WORK

Additional experimental parameters such as the pH of the aqueous solution. A recent paper has demonstrated that the nucleotide binding force is a strong function of pH [38], and a pH could be selected that maximizes the attractive binding force of complementary nucleotides while simultaneously maximizes the repulsive force of non-complementary nucleotides. It is expected that this approach would dramatically increase the signal-to-noise ratio of the current system, and facilitate individual nucleotide detection.

The final suggestion to improve the accuracy of the proposed method concerns the chemical functionalization of the nanotube and the resulting molecular probe. In this work, commonly available thymidine is attached to the carboxyl groups on the opened nanotube end. However, a synthetic molecule could be designed that eliminates all molecular degrees of freedom with the exception of a single rotation aligned with the axis of the nanotube. This new molecule must still provide the framework to hold one of the four nucleotides, but this rotation would allow the probe to orient itself such that its hydrogen bonds would more easily align with those of the nucleotides on the immobilized target DNA strand.

In the next section, suggestions are offered for future work meant to increase the speed at which DNA can be sequenced with the proposed method.

6.2.2 Increasing Sequencing Throughput

The remaining suggestions for future work concern the improvement of sequencing throughput. The proposed method is clearly faster than traditional Sanger sequencing, due principally to the elimination of DNA amplification and electrophoresis. Because sequencing is performed on a single molecule of DNA, sequence information can be obtained directly. However, there exist the opportunity to optimize the current system to increase the sequencing rate further. For example, in the experiments performed in this research, the AFM is directed to scan a square area and locations of DNA are identified by their dimensions in the topography output of the microscope. This approach wastes time by scanning the probe over large regions of the uninteresting background material. (Recall that the DNA appears on the mica substrate as a long, string-like molecule, as shown in Figure 2-3.) It is proposed to develop a control system for the AFM that causes the probe to track the DNA strand as it winds and curves along the substrate. This controller can be viewed as a micro-scale
CHAPTER 6. CONCLUSION

version of the trajectory tracking problem typical of robotic manipulators. The strand of DNA serves as the desired trajectory for the probe to follow, and similar ideas from robotic systems could be applied (i.e., potential functions or impedance control). It is important to note that this approach requires that the DNA be located, which requires initially raster scanning a rectangular area characteristic of general AFM operation as used in this work. However, once the DNA is found, the control system could switch to tracking mode. Recall that human DNA is 0.9 m in length, and maintaining a rectangular scanning pattern would require imaging almost a square meter which is not feasible—a tremendous savings in time could be realized with a tracking controller and indeed this advancement seems necessary for reasonable sequence times.

Another, more radical design approach intended to dramatically increase the sequencing throughput comes from considering the slowest component in the current system. Examining each of the main constituents of the AFM, it is observed that the piezo has the lowest bandwidth of all mechanical elements, and therefore its dynamics set the maximum speed at which the probe can be scanned. Nucleotide interrogation can be performed at a much faster rate if the DNA were shuttled passed stationary (but resonating) cantilevers. For example, DNA could be pumped in aqueous solution through a microfluidic channel past a detector region consisting of four resonating cantilevers, each with a functionalized CNT probe. In this design, the sequencing throughput would be limited only by the speed at which DNA could be moved pass the cantilevers. This method also allows for the pH of the fluid to be controlled—taking advantage of the binding force optimization suggested earlier.
Appendix A

Experimental Protocols

A.1 Preparation of single-sided DNA on Mica

The following protocol describes how ssDNA is deposited on atomically flat mica for the experiments performed in Chapter 5. DNA is denatured by heating followed by rapid cooling (to prevent reannealing). The DNA backbone is electrostatically immobilized to the mica substrate using the positively charged linker chemical, 3-Aminopropyltriethoxysilane.¹

A.1.1 Preparation of Mica

1. A 25 mm by 25 mm by 0.15 mm sheet of muscovite mica (V-1 research grade) is taped to a glass slide.

2. The top layer of mica is cleaved using standard packing tape.

3. A solution of 0.01% 3-Aminopropyltriethoxysilane (APES) is deposited on the mica and is covered and stored for 45 minutes.

4. The APES is then rinsed off the mica using deionized water, covered, and allowed to dry. The mica is considered ready for application of DNA.

A.1.2 Denaturization and Deposition of DNA on Mica

1. DNA obtained from the vendor is diluted to 1 μg/μL in water.

¹This protocol is adapted from [28] and more detail can be found therein.
2. A 2 \text{mL} test tube containing the DNA is suspended in a 90°C water bath for 15 minutes to denature double sided DNA into single strands (ssDNA).

3. The test tube is then held in a chilled water bath for 2 minutes to prevent strands from reannealing.

4. Approximately 100 \text{$\mu$L} of ssDNA is then deposited on APES-coated mica, covered, and stored for 30 minutes.

5. The mica surface is then gently rinsed with deionized water, covered, and allowed to dry. At this point, the sample is considered ready for imaging or sequencing.

<table>
<thead>
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<th>Material</th>
<th>Vendor</th>
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</tr>
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<td>Structure Probe, Inc. [86]</td>
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<td>APES</td>
<td>VWR [98]</td>
<td>CAS# 919-30-2</td>
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<td>DNA</td>
<td>Integrated DNA Technologies, Inc.[41]</td>
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Table A.1: Vendors of experimental supplies discussed in this section.

A.2 Nanotube Shortening Procedure on a Q-250 AFM

The experimental procedure to shorten the nanotube attached to the end of an AFM probe is outlined in Section 4.3.2, and Figure 4-6 from that section should be reviewed. The following description applies specifically to a Quesant Q-250 atomic force microscope with the Research Customization Package, running software version 4.05.3 or later. The resonating cantilever is electrically biased and momentarily brought into contact with a grounded niobium substrate. Niobium is chosen because the sample has surface features of the proper roughness to encourage arcing from localized points, rather than unknown positions as from a flat substrate.

1. The black grounding wire behind the AFM’s magnetic probe holder is disconnected from ground, and connected to the high voltage line of an adjustable voltage-controlled power supply. (The connector used is a pin from a machined DIP socket.) The microscope has been designed such that magnetic probe holder is electrically isolated
from the remainder of the microscope and no further isolation is required by the
microscope operator.

2. The niobium substrate is setup as shown in Figure 4-6 ensuring that the top of the
surface is electrically connected to the ground terminal of the power supply. (Niobium
is sputtered on silicon, and therefore relying on the silicon substrate for electrical
conductivity is not sufficient. A strip of copper tape is used to connect the niobium
surface to ground.)

3. When mounting CNT probes on the metal crosses used to hold the probes in the
AFM, conducting adhesive is used.

4. A transfer function between “Z FDBK” voltage (an RMS-to-DC converted signal
of the oscillating cantilever) and cantilever oscillation amplitude (in nm) is found
experimentally.

5. The amplitude of the free-air oscillating cantilever is adjusted to approximately 100 nm
with the software controls.

6. The resonating cantilever is slowly brought into contact with the substrate by mon-
itoring the Z FDBK signal, and the height of the cantilever above the substrate is
adjusted to that approximately 10 nm of oscillation amplitude is damped out. In
other words, the cantilever cannot swing through its full free-air amplitude because
the nanotube is striking the substrate.

7. The voltage of the power supply is slowly increased until the cantilever oscillates with
full free-air amplitude. When this occurs, at least 10 nm of nanotube material has
been removed so that it no longer strikes the niobium substrate.

8. Because more than 10 nm of material could have been removed (or the nanotube
may have broken off), the presence and length of the nanotube must be measured, as
described in the next section.

9. This process is repeated as necessary until a stable nanotube is obtained (typically
less than 50 nm).
Table A.2: Vendors of experimental supplies discussed in this section.

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<td>Atomic Force Microscope</td>
<td>Quesant Instrument Corp. [70]</td>
<td>Q-250 RCP</td>
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A.3 Measuring Nanotube Length using a Q-250 AFM

The experimental procedure to measure the length of the nanotube attached to the end of an AFM probe is outlined in Section 4.3.2. The following description applies specifically to a Quesant Q-250 atomic force microscope with the Research Customization Package, running software version 4.05.3 or later.

1. The AFM $z$ axis is calibrated, and a transfer function is obtained from the applied piezo voltage to piezo extension in the $z$ direction.

2. The laser spot in the “Target” window and the setpoint in the “SPM Config” window are set such that the cantilever deflection is minimized when in feedback (i.e., laser spot is is located near the center of the target, and a small setpoint is used). The AFM is brought into feedback using the contact mode setting.

3. By monitoring the voltage of the “OSD Z” signal with an oscilloscope, the voltage sent to the piezo from the AFM controller immediately after entering feedback is noted.

4. The setpoint setting is slowly increased while monitoring the OSD Z signal. Here, the AFM’s feedback controller is maintaining piezo extension to match the desired setpoint setting. By increasing the setting, the piezo extends, causing the cantilever to further deflect. Eventually the nanotube will buckle and the piezo will have to extend quickly to compensate. Before buckling, the OSD Z voltage increases monotonically with increased setpoint setting. However, when the OSD Z voltage jumps, the cantilever has buckled. The voltage immediately before the cantilever buckling is noted.

5. The length of the nanotube is calculated by converting the voltages measured in steps 3 and 4 into nanometers (using the transfer function from step 1) and subtracting.
A.4 Chemical Protocol for Functionalizing CNT Probes

After the nanotubes are shortened, carboxyl groups are expected to form at the opened ends. The procedure presented below describes an esterification reaction to attach thymidine to the nanotube's carboxyl group via carbodiimide. During this process, the carbodiimide attacks the primary alcohol group on the thymidine sugar. The thymidine is then attached to the nanotube. For a more detailed discussion of this reaction, see Section 4.3.3.

1. The shortened nanotube probes are placed in 0.1 M MES (2-[N-morpholino]ethanesulfonic acid) pH 6.0 buffer containing 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 50 mM thymidine for two hours.

2. The probes are then washed in 0.1 M MES buffer pH 6.0, 0.1 M NaCl, and deionized water.

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Table A.3: Vendors of experimental supplies discussed in this section.
Bibliography


[78] Sigma-Aldrich. Supplier of: Mes monohydrate 98% (69897-10g), mes sodium salt (m3058-25g). Available at http://www.sigmaaldrich.com, 2004. 3050 Spruce Street, St. Louis, MO 63103. (800) 521-8956. 4.3.3, A.4


[98] VWR. Supplier of: Carbodiimide (tcd1601-005g), thymidine (aaa11493-06), nacl (em-sx0420-11). Available at http://www.vwr.com, 2004. 1310 Goshen Parkway, West Chester, PA 19380 (800) 932-5000. 4.3.3, A.1.2, A.4


