Abstract:
A single molecule of DNA is denatured and immobilized on an atomically flat substrate, and a carbon nanotube force probe functionalized with a nucleotide is scanned along the molecule to detect locations of the probe nucleotide’s complement. Discussion is offered regarding the preparation of single-walled carbon nanotube AFM probes and their chemical attachment of DNA nucleotides. Additionally, a model of the oscillating cantilever under the influence of an arbitrary tip force is reviewed and it is shown that attractive forces are measured as a decrease in the cantilever phase, relative to its driving signal. Finally, single-stranded DNA of a known sequence is scanned with a thymine-functionalized carbon nanotube force probe.

1. INTRODUCTION

Since the announcement of the complete human genome, scientists have begun sifting through the tremendous amount of genetic data (Lander et al., 2001; Venter et al., 2001). While much progress has been made toward the understanding of the genetic foundation of our species, it must be emphasized that any conclusions drawn from data obtained from the Human Genome Project comes from a very small sample of individuals. However, if a technology could be developed that facilitates inexpensive and rapid DNA sequencing such that personal genomic information becomes available, dramatic advancements would occur in medical diagnosis, drug development and prescription, and the study of genes associated with complex traits or multigenic diseases.

Modern genomic sequencing and analysis is performed via chain-terminating inhibitors known as the Sanger method (Sanger et al., 1977). Target DNA is chemically amplified with fragments terminating at known nucleotides, the resultants are separated by electrophoresis, and the sequence is deduced by computer-aided assembly of the overlapping fragments. The speed at which sequence information can be obtained is therefore limited by the time constants associated with the chemical reactions of amplification, and the viscous drag of electrophoresis (Marziali and Akeson, 2001). The throughput limitations inherent to the Sanger method and the associated high cost precludes its
use for personal genome analysis (Kling, 2003). Therefore, a need exists for a new technology that facilitates rapid and inexpensive DNA sequencing. Several approaches, including the method described in this work, attempt to sequence a single DNA molecule—eliminating the need for replication, electrophoresis and analysis.

Two experimental sequencing technologies receiving attention are Harvard’s nanopore (Deamer and Branton, 2002) and 454’s sequencing-by-synthesis (Braslavsky et al., 2003; Margulies et al., 2005). The nanopore approach attempts to measure individual nucleotides’ physical or electrical properties as a strand of DNA translocates across a small-volume opening in either lipid bilayer or silicon. However, problems stem from the high speed at which DNA migrates across the pore, leaving too little time to obtain an accurate measurement. Also, random diffusion from temperature fluctuations could permit the DNA to retreat, confounding sequence data.

In sequencing-by-synthesis, short fragments of DNA are attached to beads deposited in picoliter-sized wells, along with enzymes that release light when free nucleotides are added to the fragment. The system is designed to allow only one type of nucleotide into a well at a given time, and an optical system measures light released from each well. When light is emitted from a well after a solution of a known nucleotide is added, sequence information is inferred. Because the length of the DNA fragment attached to the bead is limited to around 100 nucleotides, repetitive sequences in the genome with periodicity greater than the fragment length become problematic.

The next section describes the method in detail and outlines our approach for single-molecule DNA sequencing based on the detection of the specific binding forces between complementary nucleotides, using a nucleotide-functionalized single-walled carbon nanotube as the force probe. In this approach, high throughput sequence data may be obtained from a single DNA molecule, with no anticipated limit to the molecule’s length, eliminating the need for analysis required to reconstitute a genome sequenced by millions of overlapping fragments.

This paper is organized as follows: Section 2 provides a description of the method, with special attention paid to the preparation of the single-walled carbon nanotubes used as force probes in Section 2.1. A mathematical model of AFM cantilever is reviewed in Section 2.2, illustrating that attractive forces are measured with an AFM by examining the phase response of the oscillating cantilever. Section 3 presents data of AFM scans of single-stranded DNA taken with a thymine-functionalized carbon nanotube. A general discussion is offered in Section 4. Finally, a summary and directions of future work are provided in Section 5.

2. METHOD AND DESIGN

This paper presents the design of a novel system that provides rapid DNA sequence information based on proven atomic force microscope (AFM) technology (Figure 1-A) (El Rifai and Youcef-Toumi, 2000). The premise is complementary nucleotides (adenine and thymine or cytosine and guanine) bind together with forces large enough to be detected by an AFM. A force probe coated or functionalized with a single DNA nucleotide (e.g. thymine) is scanned across single-stranded DNA immobilized on mica, and the locations of the complementary nucleotide (e.g. adenine) are measured (Figure 1-B). In the future, multiple functionalized probes will be scanned across the same strand to sequence DNA in one pass. This paper focuses on demonstration of the principle, and therefore only one probe is scanned at a given time.

Because the nucleotides are spaced so closely on the DNA backbone (0.34 nm for β-form), spatial resolution of the AFM requires special attention. Toward that end, a single-walled carbon nanotube is grown from the AFM probe, shortened and functionalized with a nucleotide to act as a high-resolution, chemically-specific force probe.
(see Section 2.1). Recent reports have shown that 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 (Cabria et al., 2003; Sun et al., 2000). These ultra-small nanotubes are of the order of the base pair spacing of nucleotides on a DNA backbone (Figure 1-C) and could potentially provide sufficient spatial resolution as to determine individual nucleotides on a target strand. Carbon nanotube AFM probes offer unprecedented resolution and could potentially provide sufficient spatial resolution as to determine individual nucleotides on a target strand. Carbon nanotube AFM probes offer unprecedented resolution and recently have been functionalized with various chemical groups (Davis et al., 2003; de la Torre et al., 2003; Wong et al., 1998b).

Finally, this approach requires sensitive attractive force measurements, and a mathematical model relating the cantilever dynamics to attractive forces acting at the probe is discussed in Section 2.2.

2.1 Carbon Nanotube Force Probes

In order to achieve the spatial resolution and chemical selectivity required to distinguish individual nucleotides, a single-walled carbon nanotube is used as a force probe due to its small diameter, robust mechanical properties, and ability to be functionalized. However, because the chemical vapor deposition process used to synthesize carbon nanotubes does not control for the nanotube's length, a shortening operation must be performed.

The purpose of the nanotube shortening operation is twofold. First, nanotubes that are too long tend to buckle under the axial loading experienced during imaging, producing poor or unrecognizable images. Second, removing carbon by electrical arcing in air has been shown to produce carboxyl molecules at the opened end of the nanotube and can be further modified with appropriate chemical protocols (Wong et al., 1998a). Carbon is removed from the free end of the nanotubes via arcing by applying a voltage to the probe/nanotube assembly relative to a grounded niobium substrate. The nanotube at the tip of the cantilever is then slowly advanced toward the substrate with the cantilever resonating while monitoring changes in oscillation amplitude.

Importantly, it has been demonstrated that typically one or two carboxyl groups are formed at the opened end of the nanotube, providing a single molecule onto which further functionalization can be performed (Wong et al., 1998b). In this work, the carboxyl molecule is used to attach a nucleotide via standard amide chemistry.

After the single-walled carbon nanotube has been shortened and carboxyl molecules are formed at the opened end of the nanotube, chemical processing is required to functionalize the force probe. Since carboxyl chemistry is well understood in macroscopic quantities, many procedures are available. Here, an esterification reaction is used to attach thymidine to carboxyl via carbodiimide. A detailed description of the chemical protocols used to prepare the functionalized carbon nanotube force probes are available elsewhere (Burns, 2004; Burns and Youcef-Toumi, 2006). This procedure provides a stable, high-resolution, and chemically-specific force probe based on a single-walled carbon nanotube that is used in the experiments in Section 3.

2.2 Attractive Force Measurements with Tapping Mode

Conventionally, the AFM is operated in contact mode, wherein the cantilever is brought into close proximity with the sample, and repulsive forces of interaction cause the cantilever to deflect away from the sample’s surface. However, because the interaction forces of interest are attractive, a different approach is required. In this section, we show that attractive forces are best measured with the microscope operated in tapping mode and by examining the phase of the oscillating cantilever relative to its driving signal.

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 $Q$ (Figure 2). The base of the cantilever is actuated with a sinusoidal forcing input $F_0$ in the vertical direction, and the cantilever deflection is measured at the tip and denoted by the coordinate $z$. The phase angle (in radians) between the input and the measured response of a non-interacting probe as a function of frequency $\omega$ is expressed as:

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

where $m$ is the mass of the cantilever, $k$ is the stiffness of the cantilever, $Q$ is the quality factor, $\omega_n$ is the resonant frequency, and $\omega$ is the frequency of the sinusoidal input.
Following Magonov (Magonov et al., 1997), the principal result 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 derivatives for all forces \( F_i \) acting on the cantilever

\[ \sigma = \sum_i \frac{\partial F_i}{\partial z}. \]  

(2)

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

\[ \phi = \tan^{-1} \left( \frac{m\omega_n}{Q(k + \sigma - m\omega_n^2)} \right). \]  

(3)

And when we consider the cantilever oscillating at the resonant frequency \( \omega_n = \sqrt{k/m} \), the phase angle of the interacting cantilever becomes

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

(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:

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

(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 (5) predicts a negative shift in phase angle under attractive interactions (i.e., increased phase lag). Therefore, in our experiments we use phase lag as an indicator of specific binding forces associated with the hybridization of single-sided DNA nucleotides with a functionalized AFM probe. While phase imaging reveals attractive binding forces, amplitude measurements in tapping mode are correlated to sample topography. Topographic information is used to identify the DNA fragments, and the phase response provides information on the presence of complementary nucleotides.

3. EXPERIMENTAL RESULTS

To demonstrate the method, single-stranded DNA is imaged with a functionalized carbon nanotube probe. The attractive force interactions between the force probe’s nucleotide and the nucleotide’s complement on single-stranded DNA (ssDNA) are measured as an increase in phase lag of the oscillating AFM cantilever. The approach is to immobilize ssDNA on atomically-flat mica, and scan a region of the mica with the AFM in tapping mode. A detailed explanation of the procedures used to prepare the synthetic DNA on mica for imaging are provided in (Burns, 2004; Burns and Youcef-Toumi, 2006).

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 sequence 5'-25T-10A-25T-3'. 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 on a synthetic strand of the sequence 5'-60T-3'.

The resulting data from these two experiments are presented in Figures 3 (primary) and 4 (control). For the case of the primary experiment, a test fragment of DNA is identified in the topography region of Figure 3 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 4 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 2.2.

4. DISCUSSION

A discussion on the relative strengths of expected interaction forces is presented, followed by advantages of the presented single-molecule sequencing method.

In Equation (5), note that \( \sigma \) and \( \Delta\phi_n \) are linearly related—an increased attractive force leads 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 \(-\pi/2\) and in a region sharply decreasing from 0 to \(-\pi\). 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 phase angle of the cantilever during non-specific interactions (such as that between the probe and the background substrate) is

\[ 1 \] More explicitly, the sequence of the synthetic DNA (listed from the 5' end of the deoxyribose molecule to the 3' end) is 25 thymine, 10 adenine and 25 thymine nucleotides.
Fig. 3. Experimental data demonstrating that nucleotide information can be obtained from a single molecule of DNA. All DNA fragments in this scan are synthetic and have the sequence 5′-25T-10A-25T-3′. 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.

compared to the phase angle of the functionalized probe interacting with the specific complementary nucleotide. Non-specific attractive forces may be present (i.e., van der Waals forces), but are expected to be approximately an order of magnitude weaker than the forces generated between multiple hydrogen bonds characteristic of nucleotide hybridization (Wade, 2003). Additionally, the effect of the non-specific forces are also minimized by the high aspect ratio of the carbon nanotube—the large surface area of the silicon probe, which is normally susceptible to these non-specific forces, is not allowed into close contact with the surface due to the long and thin nanotube. It is realized that non Watson-Crick hybridization may occur, and further refinement of the experimental protocols will focus on elimination of this effect.

The presented method could potentially provide sequence information at a very high throughput and accuracy. 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. Coupled with the piezo tube actuator whose control bandwidth is around 600 Hz, the AFM system is capable of recording data at a high rate (for example, topographical information can be collected at about 6 µm/sec with sufficient accuracy for sequencing). 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.  

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 (Jonietz, 2002). 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

2 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.
a moderate increase in imaging resolution and chemical force microscopy, we feel the opportunity for success is greater than unproven and more radical technologies.

5. SUMMARY

Early work is presented toward the demonstration of a novel sequencing technique by scanning single-stranded DNA with a functionalized carbon nanotube probe. Carbon nanotube AFM probes are grown from the tip, measured, shortened and functionalized with a thymine nucleotide. A model of the oscillating AFM cantilever is reviewed and it is noted that attractive forces at the tip cause a decrease in the measured phase response. Locations of a known complementary nucleotide shows a discernable increase in phase lag, as predicted by the model in Section 2.2.

Future progress will be directed at three major aspects of the described method: improving nucleotide detection capabilities, increasing force sensitivity, and speeding the sequencing throughput.

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