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# Atomic Force Microscopy Based DNA Analysis

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# ATOMIC FORCE MICROSCOPY BASED DNA ANALYSIS

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## ABSTRACT

This report explores dry and wet scanning of a surface and DNA pickup using an AFM, as well as fluorescent staining of DNA. Dry and wet scans of DNA were obtained using a cantilever AFM tip in tapping mode. Dry scans were found to be clearer than wet scans; however, the drying process was found to decrease the thickness of DNA 2–4 times less than its original thickness. Alternately, wet scans were found to be less clear than dry scans and introduced more noise into the images obtained. Additionally, DNA kept its initial thickness during wet scanning. DNA was capable of being picked up using an AFM tip in contact mode, and force curves were produced that signified pickup, as supported by theoretical predictions. Fluorescent DNA staining and visualization were attempted using DAPI nucleic acid stain and a fluorescent microscope. No significant results were found using restricted protocols as a result of an unfortunate time constraint. The DAPI stain was, however, confirmed to react to fluorescent exposure. Fluorescent imaging was attempted for stained DNA both as a solution between glass slides and as a dried sample bound to mica. Through the combination of the processes explored in this experiment (i.e. AFM scanning, DNA pickup, and fluorescent microscopy), future research may be able to explicitly prove that DNA can be picked up and transported via AFM.

## INTRODUCTION

Atomic force microscopy (AFM) is a technique that has been widely used throughout fields of research such as medicine, physics, and biology due to its ability to scan nanoscale surfaces [2]. The AFM does this by using a cantilever with a sharp, downward pointing tip at its end. To properly image a sample, a laser is reflected off the end of the cantilever as it moves across the sample. Deflections in the cantilever are then picked up by a photodiode and mapped using AFM imaging software. These high-resolution images can even be sensitive enough to pick up individual atoms on crystal surfaces [4].

By oscillating above the sample, the tip is able to make only very small contact, allowing the tip to inflict minimal damage to the sample; this setting is called “non-contact” or “tapping” mode. Alternately, “contact” mode drags the tip across the surface. These imaging modes can be seen in Figure 1. Due to direct contact, contact mode is capable of inflicting more damage to the sample than tapping mode might. As such, tapping and contact modes are generally used for fragile and rough surfaces, respectively. Tips are typically manufactured to be specific to one particular mode.

The cantilever tip may also be used to measure certain material properties of a sample, such as Young’s modulus, stiffness, or strain rates [5]. This is accomplished by pressing the tip to a surface with some force. During tip retraction, adhesion forces of a sample may also be measured, and molecules sticking to the tip may be stretched or moved; this process may be used for protein unfolding or DNA transportation [5]. It should be noted that adhesion of DNA to an AFM cantilever tip is caused by a non-specific force between the DNA and the tip. Force curves may be generated during this process and can be represented using height and deflection (in volts) axes; these are known as “deflection curves” (see Figure 2). Operations such as these are done in contact mode.

Adhesion forces of water on mica can be observed in Figure 2. The blue retracting curve shows where water molecules cling to the tip as it retracts, causing adhesion. The flat blue line after adhesion represents the moment at which the force of the cantilever was strong enough to overcome the adhesion. A similar curve may be observed when samples such as DNA are picked up – an elongated retraction line with many ridges will be observed as the tip picks up the DNA; the retraction line will then return to a flat line once the DNA has been completely lifted from the surface.

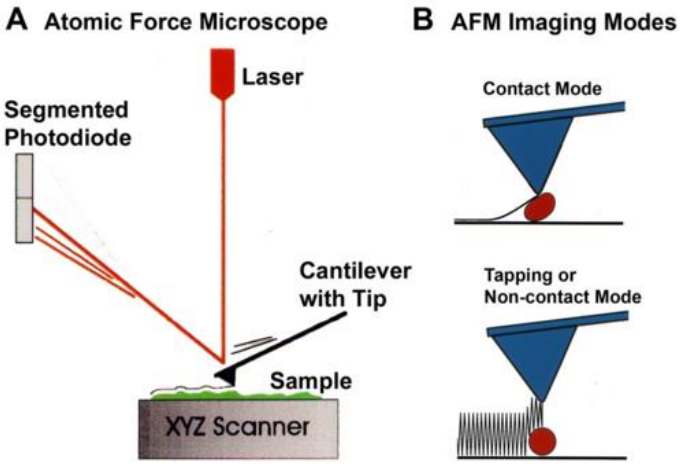


**Figure 1. AFM Mechanics [4]**

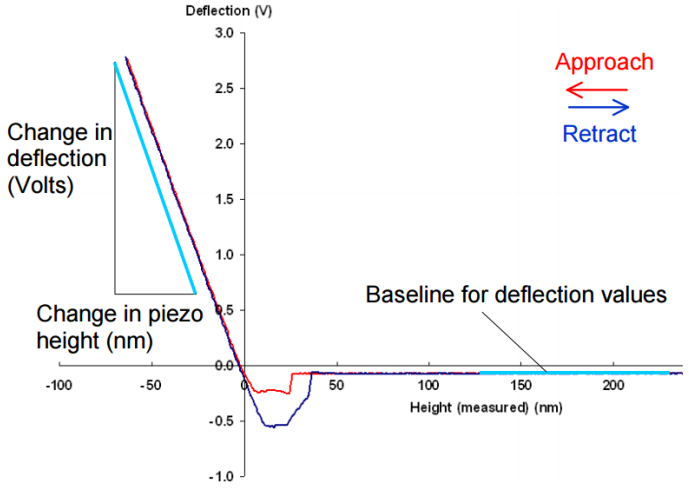


**Figure 2. Deflection Curve in Water [5]**

Fluorescent microscopy, a popular technique within biology and medicine, is a visualization technique that increases visibility of specimens such as microbes or DNA by staining them with a fluorescent dye and observing them under a fluorescent microscope. Fluorescent microscopes work much in the same way as standard microscopes, but instead of using visible light (400–700 nm), a fluorescent microscope uses long wavelength, low-energy light to visualize samples [6]. To do this, samples must first be fluorescently stained by "attaching a fluorescent tag to antibodies that in turn attach to targeted features, or by staining in a less specific manner" [6]. With this method, specific components of a sample may be visualized. In addition to visualization of a specimen, fluorescent staining may also be used in viability studies on cell populations to determine whether the cells are alive or dead [6]. Such stains include propidium iodide, which stains dead cells, and various cyanine dyes, which stain only living cells.

Many different dyes exist that stain different components. More specifically, there also exists many varying DNA dyes. Each dye has its own excitation and emission wavelengths and binds to different components. For example, DAPI and Hoechst 33258 are both A-T selective and bind to the adenine (A) and thymine (T) bases [1]. Dyes may also be selective of certain types of DNA; examples include PicoGreen and SybrGreen I which bind to double-stranded DNA (dsDNA) [1]. Additionally, certain factors may affect the performance of DNA stains (e.g. DAPI shows a 20-fold increase in fluorescence when bound to dsDNA) [1]. Though DNA staining is more expensive than other methods of quantification, it has been shown to be approximately 100–1000 times more sensitive than methods such as absorbance reading [1].

By picking up DNA with an AFM tip and dragging it across a nanowire connected to nano-electrodes, the base sequence of a DNA strand may be mapped by detecting the electrical signals produced by each DNA base. This experiment explores dry and wet scanning of a surface and DNA pickup using an AFM, as well as DNA visualization through fluorescent microscopy.

## MATERIALS & METHODS

AFM scans and images were collected using an Agilent 5500 Scanning Probe Microscope (SPM) (Agilent Technologies, Santa Clara, CA) operating in tapping and contact mode in air and water in conjunction with PicoView AFM imaging software (Agilent Technologies, Santa Clara, CA). Cantilever tapping mode tips had a spring constant of 3 N/m and a resonant frequency of 75 kHz (BudgetSensors, Multi75). Cantilever contact mode tips had a spring constant of 0.02–0.77 N/m and a resonant frequency of 6–21 kHz (NanoSensors, PPP-CONT-10).

This experiment utilized three procedures for analyzing lambda DNA (λ-DNA): dry scanning, wet scanning, and fluorescent staining. A TE buffer solution was prepared and was comprised of 10 mM Tris and 1 mM Ethylenediaminetetraacetic acid (EDTA). This TE buffer was then added 10:1 to a 10 ng/μL DNA solution to obtain a final DNA concentration of 1 ng/μL. This sample solution was used for both dry and wet scanning.

### AFM Sample Preparation

Samples were scanned and imaged on mica. Since mica exhibits a negative charge in the presence of water, and DNA exhibits a -1 charge, the sample DNA solution was combined with a 10 mM $Mg^{2+}$ ion for a net charge of +1. This positive charge effectively neutralizes the mica surface and allows the DNA in the solution to bind to the mica surface. This neutralized mica is known as a "treated mica surface."

The mica surface was prepared by using a piece of tape to peel off a single layer of mica. The peeled mica was then applied to a piece of double-sided tape that was stuck to a magnet; this magnet was attached to the AFM sample base. Once the mica had been attached to the magnet, the initial tape used to peel the mica was removed.

After treating the mica surface, 20 μL of the sample DNA solution was deposited onto the mica and allowed to incubate in a petri dish at room temperature for approximately five minutes. By allowing a sample to incubate for longer periods, the DNA in the sample is allotted more time to bind to the surface and better binding can be observed; however, longer periods of incubation also allow more particles in the air to fall into the solution, resulting in dirtier images.

Once incubation is concluded and DNA has been allowed to bind to the surface, the sample is ready for a wet scan. Alternately, for dry scanning, the sample is then rinsed with distilled deionized water ($ddH_2O$) and dried with a soft nitrogen flow. After the sample has been dried, it is ready for a dry scan.

### AFM Scanning Procedure

The following procedure was given by research mentor Bo Ma, and was used for all AFM scans.

1. Turn on computer
2. Turn on MAC Mode III (Agilent Technologies, N9621B)
    a. Let it zero out

3. Turn on the controller (Agilent Technologies, model number: N9610A)
    a. Check that numbers are visible
4. Open PicoView software
5. Choose scanner
    a. Large scanner (model number: N9524A) (closed loop)
        i. Used for dry scan
        ii. Allows for larger scanning area
            1. 100 μm x 100 μm
    b. Small scanner (model number: N9520A) (multipurpose)
        i. Used for wet scan
        ii. 10 μm x 10 μm scanning area
        iii. High sensitivity desired, since wet scans introduce a lot of noise
6. Choose and attach cantilever tip
7. Adjust laser
    a. Focus microscope on cantilever
    b. Move cantilever under laser
        i. Watch cantilever shadow – darkest shadow confirms correct placement
    c. Adjust photodiode to receive laser
        i. Tune knobs until deflection and friction ≅ 0.0X (where X is a positive integer)
8. Auto-tune frequency
    a. For dry scan only
    b. Wet scan uses a manual scan (choose ~16 kHz)
9. Attach scanning base and sample
    a. Confirm that the sample will not collide with the tip
    b. "Withdraw" to ensure this does not happen
10. Move cantilever over desired scanning area
11. Calibrate scanning size and speed
    a. Generally 1 line per second
12. Approach
    a. By clicking "approach" on PicoView
13. Scan
14. Withdraw and repeat Steps 10–13 until desired scanning image is obtained

To shut down the machine, stop the scan, withdraw, and turn off PicoView software. Once software is closed, all other machines may be turned off at the operator's discretion.

**DNA Staining**

This experiment utilized DAPI nucleic acid stain (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) to fluorescently stain λ-DNA. DAPI, a blue-fluorescent dye, stains preferentially to dsDNA and is A-T selective. The DAPI stain used in this experiment was purchased from Thermo Fisher Scientific (10 mg, catalog number: D1306). Excitation and emission maximums bound to dsDNA are 358 nm and 461 nm for DAPI, respectively [3].

Antifade reagents such as SlowFade® Gold or ProLong® Gold may be used to preserve fluorescence of a sample, however, none were used in this experiment.

Fluorescent microscopy was done using a Leica DM IL fluorescent microscope (Leica Microsystems, Wetzlar GmbH) with a DAPI filter.

Samples were prepared similarly to the supplier's instruction [3]. The dry DAPI dye purchased was combined with $ddH_2O$ to a concentration of 5 mg/mL. This stock solution was then diluted to a final molarity of 300 nM; this was done by first adding 2.1 μL of DAPI stock solution to 100 μL of 10 mM Tris (resulting in a 300 μM DAPI solution) followed by a 1:1000 dilution in 10 mM Tris.

A DNA solution was prepared similarly to AFM samples. A 10 ng/μL DNA solution was combined 1:10 with 10 mM Tris for a final DNA concentration of 1 ng/μL.

DNA staining was completed using two procedures. First, 100 μL of DNA solution was combined with 4 μL of DAPI solution; concentration of the DAPI solution varied between stock, 300 μM, and 300 nM solution to observe differences. These DNA-DAPI solutions were allowed to incubate at room temperature for at least five minutes. After incubation, the solution was deposited in 4 μL droplets between glass slides to view under a fluorescent microscope.

Second, similarly to dry AFM scanning, mica was peeled with tape and stuck to a glass slide. A 4 μL droplet of DNA solution was deposited on the mica followed by a 4 μL droplet of DAPI solution. Alternately, other iterations utilized a pre-mixed DNA-DAPI solution that was deposited on the mica. The sample was then covered and allowed to incubate at room temperature for approximately five minutes. After incubation, the slide was washed with Tris three times and dried with a gentle nitrogen flow. A second glass slide was then placed on top of the sample, and the sample was viewed under a fluorescent microscope.

DAPI was chosen for this experiment, because it possesses a +2 charge. This dye, in combination with the DNA solution described above, produces a +1 charge, which allows the DNA to bind to the surface of the mica, which exhibits a negative charge in the presence of water.

Samples were exposed to fluorescent light at varying intervals between 250 ms and two minutes; more exposure time was allotted if no fluorescence of any kind was observed. After each time interval, the fluorescent light was shut off, and the sample was viewed.

It should be noted that numerous volumes of DAPI solution (ranging from 4 μL to 10 μL) were planned to be tested during the first DNA staining procedure listed above. Unfortunately, due to time constraints, this and several other procedures were not able to be carried out or trialed; these processes are discussed in the "Results" and "Conclusions" sections.

## RESULTS

Similar to the mention of force curves in the "Introduction" section, cantilever deflection due to adhesion from mica can be observed in Figure 3. The retraction line (blue) is seen to have a sharp jump around 0.52 µm. This jump signifies the point at which the force of retraction exceeds the mica's force of adhesion on the tip, and the tip breaks free.

Alternately, Figure 4 shows the deflection curve as the tip picks up DNA. As previously mentioned, the DNA clings to the AFM tip due to a non-specific force. As opposed to Figure 3, Figure 4 shows no sharp peak due to adhesion; instead, there exist several smaller ridges as the DNA lifts off of the mica. The DNA can be seen to fully lift off of the mica surface around 0.78 µm (see Figure 4).

By measuring the deflection in volts in Figures 3 and 4 (approximately 20 mV and 40 mV, respectively), the force of adhesion can be calculated using

$$F = k\alpha V \qquad (1)$$

where $F$ is the adhesion force (N), $k$ is the spring constant of the AFM cantilever tip (N/m), $V$ is the cantilever deflection (mV), and $\alpha$ is the deflection sensitivity coefficient (m/mV) [13]. The deflection sensitivity coefficient, $\alpha$, is determined using the slopes of the linear curves in Figures 3 and 4; it can also be determined using AFM software.

Using Equation (1), adhesion forces on the tip are approximately 40 nN for the mica (Figure 3) and 2 nN for the DNA (Figure 4). Adhesion forces between mica and a tip are measured to be approximately 30 nN on average [12]. This value supports that Figure 3 shows adhesion from mica. Additionally, adhesion forces between DNA in water and a tip are measured to be approximately 3 nN on average [11]. This value supports that Figure 4 shows adhesion from DNA and signifies DNA pickup.

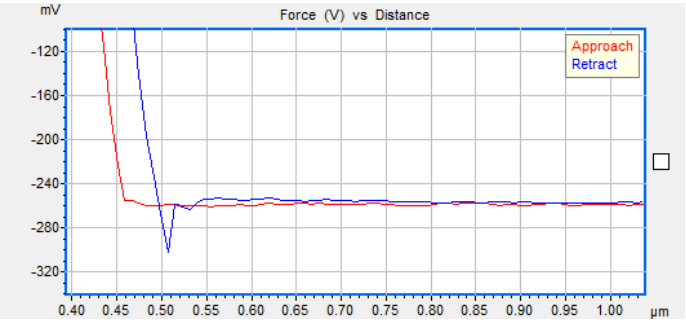


**Figure 3. Tip on Mica Deflection Curve**

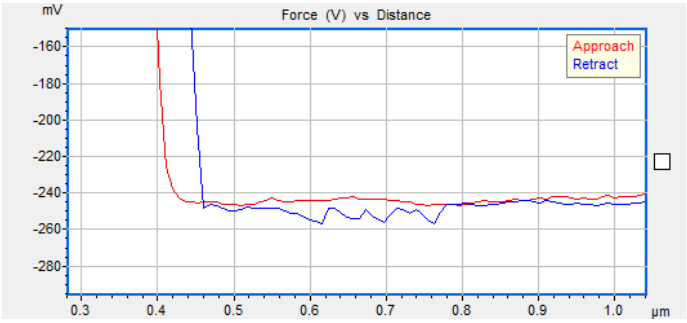


**Figure 4. DNA Pickup Deflection Curve**

Images were edited using Gwyddion SPM data analysis software (version 2.44) [7].

### Dry Scans

Dry scan images were taken as practice in preparation for wet scanning and can be viewed in Figures 5 and 6. Additionally, scans of DNA in Figures 5 and 6 verify that the buffer does not kill DNA.

In Figures 5 and 6, DNA was observed to form in some sort of branching formation with all the DNA seeming to flow in one direction. The uniform direction is likely due to the rinsing phase of dry scan sample preparation. As the water runs along the mica surface during a rinse, DNA flows with it, resulting in the DNA's uniform direction (see Figures 5 and 6). Additionally, particles can be seen at the ends of DNA as well as where DNA strands seem to connect (marked by red circles). It may be that the DNA is binding to these particles during incubation, ultimately settling into this peculiar branched fashion.

Due to the dry nature of the scan, DNA tends to become compressed. This compression is due to the strong interaction between DNA and the mica surface as the solution is washed away. The drying of the sample prior to scanning also affects the diameter of the DNA. These two things together tend to compress the thickness of DNA 2–4 times less than its initial diameter (2 nm) [9]. When the mica surface is treated and the solution is present, DNA tends to keep its original diameter [9].

By measuring the height of the strands in Figures 5 and 6 using Gwyddion, the average height is found to be approximately 1 nm. This measurement, supported by Reference [9], serves as a basis to conclude that the branches in Figures 5 and 6 are, in fact, DNA.

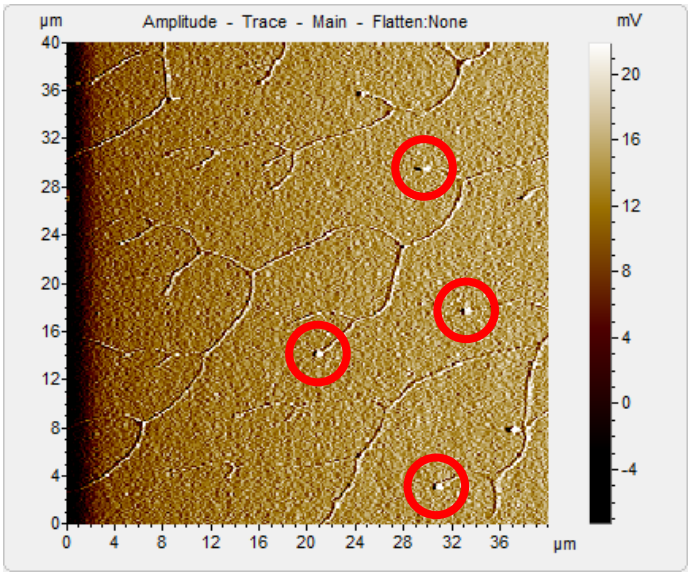


**Figure 5. Dry Scan (40 µm)**

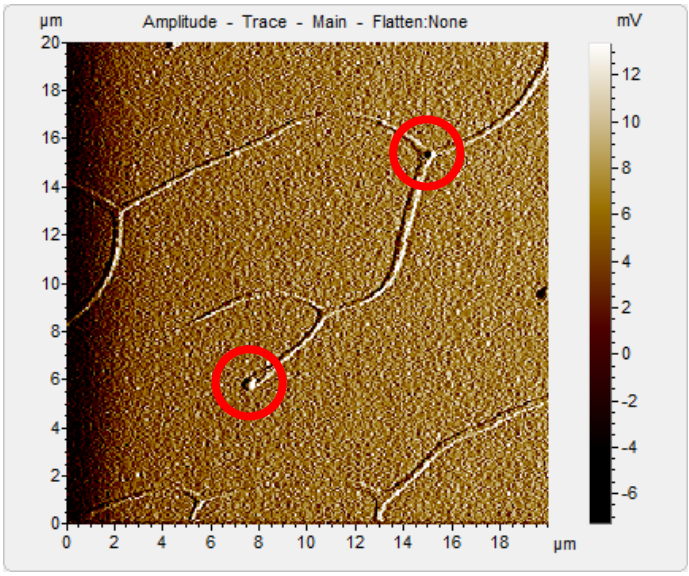


**Figure 6. Dry Scan (20 µm)**

## Wet Scans

Clear images of DNA in a solution are much more difficult to obtain than they are for dry scans. The presence of a liquid introduces large amounts of noise, distorting the image, and making it difficult to visualize. Some of this interference may be observed in Figure 9 (marked by a blue square).

Wet scans also show high concentrations of DNA at seemingly randomized positions across the surface. While DNA can be seen in these dense regions, it can be difficult to make out individual strands of DNA. Figures 7 and 8 show two dense regions in different locations on mica. It is currently unknown why DNA tends to concentrate in areas such as these.

Figure 9 shows a close-up of two DNA strands in a less dense region. Despite being noisier, these strands are much more distinguishable than the large quantity of DNA in Figures 7 and 8.

Similarly to the dry scans, the height of the DNA in Figures 7–9 were measured using Gwyddion. An average height was calculated to be approximately 2 nm; this measurement matches the average thickness for DNA as stated in Reference [9]. This thickness confirms that the wet scans in Figures 7–9 display DNA.

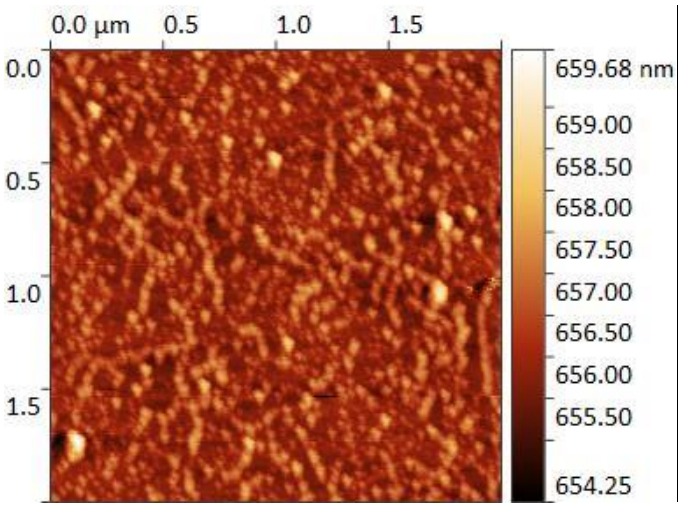


**Figure 7. Wet Scan 1**

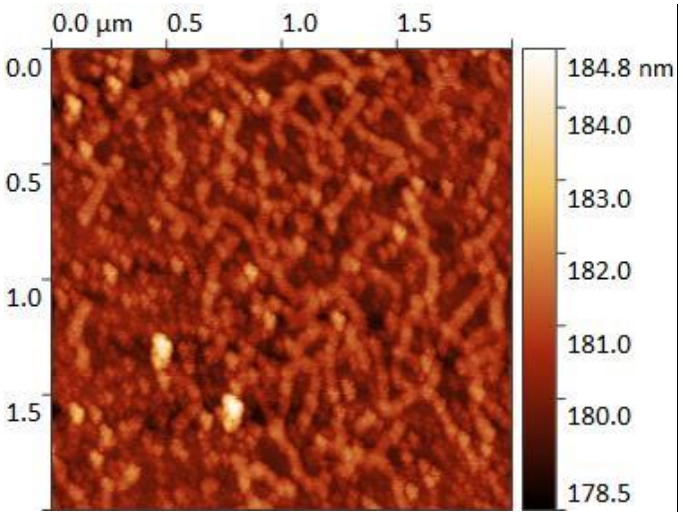


**Figure 8. Wet Scan 2**

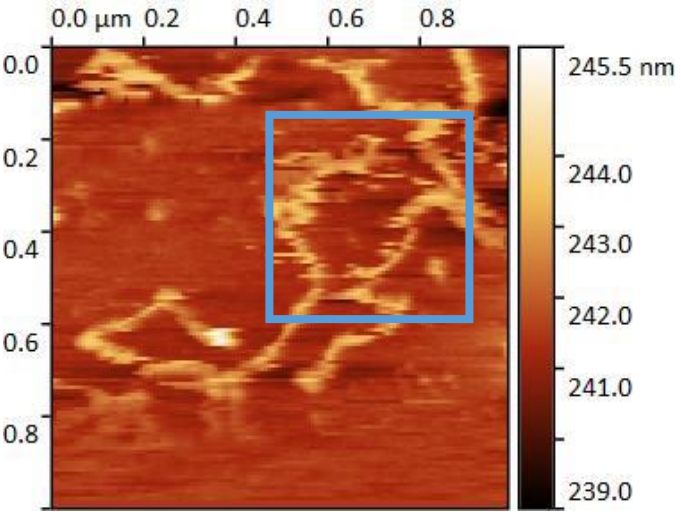


**Figure 9. Wet Scan 3**

**Fluorescent Microscopy**

Due to time constraints, no notable results were obtained for DNA staining and fluorescent microscopy.

Fluorescence was first tested using plain DAPI solutions in an attempt to see how the stain reacted to fluorescent light exposure. It was confirmed that the dye reacted, producing a pale blue-gray light, but the reaction was not particularly bright. When DNA stained samples were exposed and viewed, there was a similar result; the DNA could not be seen clearly nor was the image particularly bright. This may have been due to the dye being spread throughout the solution, causing an overall fluorescence; however, the same result occurred in the second DNA staining procedure (DNA bound to mica) mentioned in the "DNA Staining" subsection.

The second DNA staining procedure produced "better" results than previous procedures, and very faint molecules were believed to be seen. It's possible that this was an effect of observer eye strain during sample viewing, since the room was not completely dark and extra measures had to be taken to clearly view samples under the microscope. To determine whether these faint samples were actually DNA or not, a long-exposure picture was suggested to see what the eye could not. To accomplish this, a Nikon camera in conjunction with a microscope-to-camera adapter would be used to snap a picture. Unfortunately, a Nikon camera could not be obtained (the adapter was Nikon specific).

It was found that longer fluorescent exposure times (approximately one minute or so) were found to be more effective as they produced brighter fluorescence. Extra-long exposures were avoided as to not cause photo-bleaching or DNA sample damage [10].

## CONCLUSIONS

AFM scans of DNA varied in quality due to the nature of the scans and were obtained using a cantilever AFM tip in tapping mode. Dry scans produced very clear results, but the drying process compresses the DNA to 2–4 times less than its original diameter (0.5–1 nm as opposed to 2 nm). Alternately, wet scans produced less clear results than the dry scans. DNA strands often concentrated on particular spots on the mica, and much more noise was introduced into the scans as a result of the DNA solution. Images were confirmed to be DNA using Gwyddion software.

Picking up DNA with an AFM tip produces a force curve which signifies forces on the tip and can be used to determine various properties of a sample or surface. By observing the peaks in the retraction line of these curves, it can be deduced whether DNA or any other sort of molecule has been picked up. Additional research should be done to explicitly confirm that the molecules are being picked up off of the surface. DNA was picked up using a cantilever AFM tip in contact mode.

Due to time constraints, fluorescent images of stained DNA could not be obtained or successfully viewed. Fluorescent staining and sample viewing was not completed in a dark room, and excess light exposure may have been a factor toward the poor results obtained. Future experiments should be done to understand the optimal settings and constraints for viewing fluorescently stained DNA; constraints should include: varying DNA and fluorescent dye concentrations, several DNA-dye ratios, numerous exposure times, and experimentation in a dark room. Other methods should be explored to bind DNA to the surface on which it's viewed to ensure it does not move. Two such methods include binding DNA to mica by combining the DNA solution with a +2 charged dye or coating the glass slides used for fluorescent imaging such that the DNA binds to them.

By combining the various methods discussed in this report, a procedure could be put together that would prove the existence of DNA on an AFM tip after DNA pickup. By fluorescently dying DNA, binding it to mica, and then using an AFM to scan and pick up the stained DNA, one could detach the AFM tip and view it under a fluorescent microscope in an attempt to visualize the DNA strand bound to the tip. This process, coupled with force curves that signify the retrieval of DNA, would prove as sufficient evidence that AFM force curves are accurate, and that DNA retrieval and transportation via AFM is possible.

## ACKNOWLEDGMENT(S)

The author would like to thank Dr. Steve Tung and Bo Ma for their integral guidance, instruction, and assistance when needed. Additional thanks goes to Dr. Tung for access to his research lab and facilities.

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