# Imaging DNA with the Agilent 6000 ILM AFM

## Application Note

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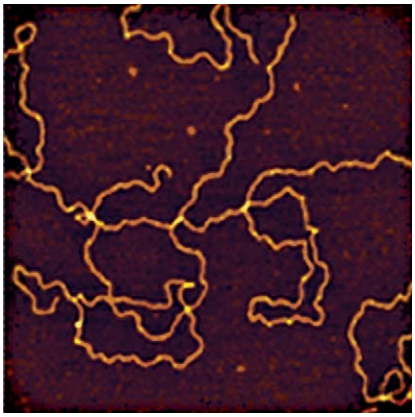

Figure 1. AAC mode AFM image of lambda phage DNA in ambient conditions. The image was acquired using the Agilent 6000ILM AFM. The DNA was immobilized on the mica in HEPES buffer containing the divalent cations $Ni^{++}$ and $Mg^{++}$ then was rinsed with double distilled water and dried under a stream of dry nitrogen.

### Introduction

In the field of biology, the atomic force microscope (AFM) can be used to examine, probe and measure biological molecules in their native environments. This application note presents examples of imaging DNA molecules in ambient and physiological conditions using the Agilent 6000 ILM AFM operating intermittent contact (AC) mode. The 6000 is an ideal platform for studying live cells, individual protein molecules, DNA, RNA, and a multitude of other biological molecules. It integrates the capabilities of an atomic force microscope with those of an inverted light microscope permitting nanoscale resolution of biological molecules.

**AFM**

AFM is a nondestructive scanning probe technique in which the tip of an AFM probe is used to scan the surface of a sample. AFM probe tips are extremely sharp (typically on the order of tens of nanometers), so AFMs can routinely image extremely small features on sample surfaces. Resolutions of just a few nanometers, even on soft biological molecules, are not uncommon.

As the tip of the AFM probe interacts with a sample surface, numerous short range and long range forces are encountered between the probe and surface. This causes the AFM probe cantilever to bend and twist. The bending changes the angle of an incident laser, which is reflected off the back side of the cantilever, to change position. A quad cell photo-diode detector measures the probes position, which is recorded and processed by the AFM software to generate a topographic map of the sample surface.

Dry DNA can be imaged by either AFM or electron microscopy; however, a significant advantage of AFM over SEM is that DNA samples can be prepared without staining or other, more arduous forms of sample preparation. Since most biological molecules are in liquid environments in their native states, there are significant advantages to studying them in liquid. Using AFM, DNA can be imaging in buffers and in the presence of ions, permitting the DNA to be imaged in a state which closely mimics physiological conditions.

**DNA**

In 1953 the three dimensional structure of the DNA double-helix was published by Watson and Crick. This was a monumental achievement, especially considering that all life as we know it is dependent on the information that is stored within the genetic code. As a result, deoxyribonucleic acid and ribonucleic acid may be the most important of all biomacromolecular structures.

The human genome is a complex storage medium within which there are nearly $10e^5$ genes. Of which approximately 10% code for proteins. Besides coding for other biological polymers, nucleic acids can perform a multitude of diverse functions so new applications are also constantly being sought for DNA and RNA.

For example, both DNA and RNA molecules can bind to a diverse set of molecular targets in a manner that mimics the specificity of monoclonal antibodies. In the fledgling field of nanobiotechnology, nucleic acids are being used as components of bioelectronic devices, computers, and as scaffolds in nanostructural engineering projects. An increasingly invaluable tool in these endeavors is the AFM.

The typical DNA duplex is composed of two polymeric chains that run in opposite directions to form a helical structure. These chains are each composed of the nucleotide residues dA, dC, dG and T. In the interior of the helix, purine and pyrimidine nucleobases exchange chemical information through specific hydrogen bonding interactions. This allows complementary DNA molecules to form extremely specific, high affinity complexes. Running along the DNA backbone are deoxyribose sugar moieties which are connected by negatively charged phosphate groups. As discussed below, the negatively charged phosphate groups can be exploited to immobilize DNA on smooth substrates of defined chemistry, which allows DNA to be imaged by AFM.

**AFM Imaging of DNA**

AFM has proven to be a useful tool to observe DNA conformations, substructures and protein-DNA complexes. From a biological perspective, one of the most appealing features of AFM is that it can operate in aqueous environments, making it a useful tool to image DNA under physiological conditions. None-the-less, careful sample preparation is critical to successful AFM imaging, and this is especially true for the case of DNA. Covalent and noncovalent immobilization techniques can permit the imaging of biological samples with AFM. With respect to DNA imaging, the noncovalent techniques are the simplest and most straightforward.

In order to image biological polymers, such as DNA, proteins and RNA, the biopolymers are generally dissolved in physiological buffers, then deposited and immobilized onto a substrate.

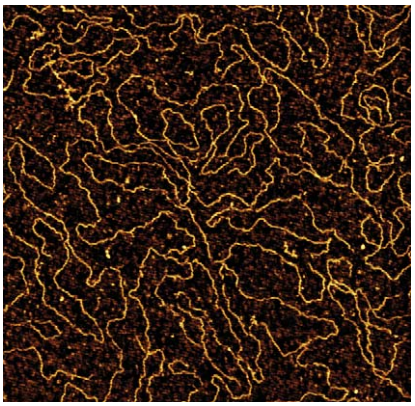

Figure 2. AAC mode AFM image of lambda phage DNA in liquid (aqueous buffer). The image was acquired using the Agilent 6000 ILM AFM. The DNA was immobilized on the mica in HEPES buffer containing the divalent cations $Ni^{++}$ and $Mg^{++}$.

Immobilization is a critical point regarding imaging samples in liquid using AFM- the samples must be firmly affixed to an immobile substrate. In the case of DNA, which has a diameter of only 2-3 nm, the substrate must approach an atomic level of smoothness in order to resolve the DNA from the underlying substrate. Substrates with well known, well characterized surface chemistries that have been successfully utilized to image DNA include bare mica, silanized mica, calcite, barite, flurite, carbon, carbon-coated mica, and thiolated gold-coated substrates.

Mica is an atomically smooth, well defined surface. It provides a convenient and versitile substrate to which the DNA can be immobilized from a variety of aqueous solutions. Freshly cleaved mica has a net negative charge on its surface due to the presence of siloxy (deprotonated silanol) groups. The siloxy groups are spaced 0.5 nm apart with potassium ions bridging the negative charges in adjacent layers.

Mica can be treated with molecules that contain easily protonated functional groups. For example, poly-L-lysine, poly-L-ornitihine, or APTES (aminopropyltriethoxy silane) can be used to to induce a positive charge on the surface of mica; permitting the immobilization of DNA by its negatively charged phosphate backbone. DNA adsorbs to amine-treated mica much more tightly than to bare mica in the absence of a divalent cations; even in the presence of a metal ion chelating agent such as EDTA.

A number of multivalent ions mediate attraction between like-charged molecules and functional groups in solution. This has proven useful in imaging DNA by AFM, particularly in cases in which the DNA is to be imaged in liquid on atomically smooth substrates such as muscovite mica. When mica is exposed to an aqueous solution containing divalent cations, such as $Ni^{++}$ or $Mg^{++}$, it undergoes an ion exchange process in which the potassium ions on the surface of the mica are replaced by the divalent cations in the solution.

When DNA in a buffer solution that contains $Ni^{++}$ or $Mg^{++}$ is added to the mica, these divalent cations can form a charge bridge between the negatively charged phosphate groups on the DNA backbone and the negatively charged siloxy groups on the surface of the mica. The amount of DNA that can be adsorbed to bare mica through the use of divalent cations depends on the buffer composition, buffer pH, as well as the species and concentration of divalent cations.

DNA in HEPES buffer with divalent nickel and magnesium ions absorbs relatively tightly to mica; for example, on the order of 100 times more tightly than the same buffer containing only monovalent cations such as sodium or potassium. Compared to HEPES buffer, less DNA adsorbs to mica from a Tris buffer solution that contains the same divalent cations. This is probably due to the fact that Tris is positively charged in solution at neutral pH. Much less DNA adsorbs to mica from water or Tris buffer that contains only monovalent cations, and practically no measurable DNA adsorbs to bare mica in Tris buffer containing the metal ion chelating agent EDTA.

DNA concentration, buffer composition and buffer concentration effect the conformation of DNA before, and probably after it is absorbed onto mica. For example, DNA tends to

aggregate on mica in water at low DNA concentrations. Therefore, when imaging under ambient conditions, the sample must be blown dry with a clean compressed gas immediately after rinsing with water in order to prevent aggregation. In contrast, even at high DNA concentrations, DNA tends to spread much more evenly on mica in HEPES buffer that contains $Mg^{++}$ compared to water. DNA solutions that contain even small quantities of the divalent cation $Zn^{++}$, which interacts strongly with the DNA nucleobases, exhibits significant DNA kinking.

Electrostatic models can explain the results obtained for the binding of DNA to silanized mica and to other mineralized substrates. It has been suggested that DNA immobilization on mica occurs by 'salt bridging' or by fluctuations in the positions of counter ions between the negatively mica surface and negative charges on the DNA backbone.

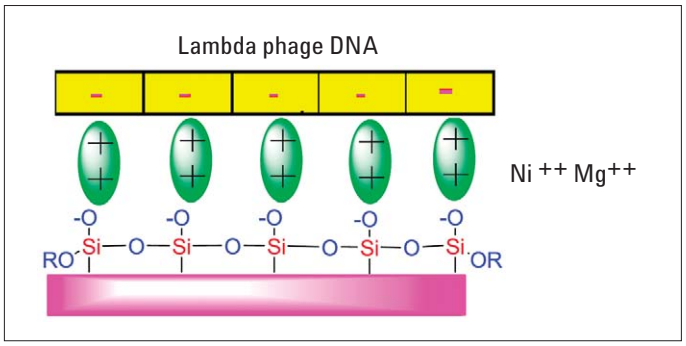


Figure 3. Immobilizing negatively charged DNA on mica using divalent cations $Mg^{++}$ and $Ni^{++}$ in HEPES buffer.

## Materials and Methods

### Instrumentation

AFM images were acquired using an Agilent ILM 6000 mounted on a Zeiss AxioObserver D1 with a coverslip sample cell. The AFM was controlled by PicoView. Images were acquired under ambient conditions or under DNA imaging buffer as described above. Applied Nanostructures HYDRA6V-100WG AFM probes (nominal k = 0.402 N/m) were driven to resonance in AAC (acoustic AC) mode at frequencies of approximately 66 khz in ambient conditions and 20 kHz in liquid.

Amplitude-distance cycles were used to adjust the free cantilever oscillation amplitude to 3 nm and in order to determine the optimal amplitude set point, which was approximately 20% less than the free amplitude.

### AFM Probe Spring Constant Calibration

In order to accurately determine the spring constants of each AFM probe, the Thermal K feature of PicoView, which uses equipartition theorem to deduce the spring constant from the thermal vibration of the cantilever, was used. The spring constants of the probes were found to be within +/-10% of the reported nominal specification (0.405 N/m). The resonance frequencies of the the AFM probes were also within +/- 10% of their reported nominal values in air (66 kHz).

### Image Processing

Images were acquired at scan rates of 1 Hz and a resolution of 512 x 512 pixels. Post image processing was performed with PicoImage.

### Sample Preparation

Lambda phage DNA was imaged under ambient conditions or in an aqueous buffer using AAC Mode AFM. Lambda phage DNA, HEPES DNA imaging buffer concentrate (pH 7.6) and concentrated solutions of divalent cations were utilized to immobilize the DNA on mica. 50 uL of a 2.1 µg/ml solution of lambda phage DNA (EcoR1 digest; SigmaAldrich) in 10 mM HEPES pH 7.6, 4 mM $MgCl_2$ was spotted onto the center of a freshly cleaved 25 x 25mm muscovite mica substrate. 50 uL of DNA imaging buffer (10 mM HEPES pH 7.6, 4 mM $MgCl_2$, 2 mM $NiCl_2$) was immediately added and the sample was allowed to stand for 5 mins. After 5 mins the sample was washed ten times in DNA imaging buffer. For imaging in buffer, the sample was immediately loaded on a 6000 ILM cover slip sample holder, which was filled with 500 uL of imaging buffer and loaded onto the 6000 ILM. For imaging in air, the sample was washed ten times in double distilled water, blown dry with compressed research grade nitrogen and allowed to stand overnight in a covered dish before being loaded on the AFM.

### Discussion

As discussed, mica is an atomically flat substrate that has a negatively charged surface, so it is a useful substrate to immobilize DNA in buffers that contain divalent ions. The divalent cations mediate the interaction between the negatively charged phosphate groups of the DNA backbone and negatively charged groups on the surface of the mica.

The DNA images presented here were obtained using dilute solutions of DNA in HEPES buffer (pH 7.6), and freshly cleaved mica that was treated with $Ni^{++}$ and $Mg^{++}$. The samples were allowed to stand for several minutes, then washed to remove excess and loosely bound DNA. The samples were either imaged directly on the Agilent 6000 ILM AFM in a liquid cell or rinsed in water and quickly dried for imaging under ambient conditions.

## Conclusion

The structure of DNA is known to play an important role in many physiological processes, including but not limited to the control of gene expression, replication, recombination, and packaging. AFM is a powerful analytical tool that can be used to image many biological molecules, including DNA, RNA, protein and DNA–protein complexes. Bare mica is a well defined and characterized substrate that can be used to provide clear, crisp images of DNA.

DNA can be imaged with the AFM on dry mica under ambient conditions or in physiological buffers. AFM images

of DNA absorbed onto mica, whether it be under ambient conditions or in conditions that closely simulate physiological conditions, has provided a greater understanding of DNA's behavior. DNA imaging under ambient conditions has been proposed as a convenient method to assay DNA samples for certain important structural characteristics such as DNA bending. Mica and HEPES buffer that contains divalent cations, such as $Ni^{++}$ and $Mg^{++}$ is a relatively fast and reliable immobilization system. It closely mimics physiological conditions and firmly secures DNA for high resolution topographic imaging in liquid.

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