Probing DNA-Protein Interactions with Atomic Force Microscopy

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INTRODUCTION

Large, multi-component protein assemblies are involved in many DNA transactions such as recombination, replication, transcription, and repair. In order to progress in the understanding of different key steps of these mechanisms, it is imperative to analyze the structure of the DNA-protein complexes involved and the dynamic interactions that govern their assembly, function and disassembly. These complexes are usually studied *in vitro* using a combination of biochemical and biophysical methods. A direct visualization technique such as AFM imaging is unique in that it allows one to characterize mechanisms involved in DNA-protein recognition and DNA-protein complex formation in different conditions (air and liquids of varying composition) with nanometer resolution. By comparison, other single molecule techniques such as optical or magnetic tweezers give indirect measurements based on the mechanical properties of single DNA molecules, and place severe restrictions on DNA size. Fluorescence microscopy can be used in conjunction with such approaches, but requires staining of either the DNA or the proteins and has relatively low resolution (about 200 nanometers). Owing to successive improvements to sample preparation and experimental methodology – along with instrumentation progress, most notably the invention of TappingMode and the ability to operate in fluid – AFM has indeed become a powerful and complementary tool to probe DNA-protein interactions at the single molecule level. The purpose of this application note is mainly to illustrate the potentialities of AFM imaging in the analysis of the architecture and dynamics of DNA-protein complexes, while introducing readers to the issue of DNA-protein complex adsorption, key for rationalizing sample preparation.

MAPPING DNA-PROTEIN COMPLEXES IN AIR

Nucleic acids were among the first biological samples imaged with the scanning tunneling microscope[1], the precursor to AFM. It was recognized that by raster scanning a very sharp tip across the sample surface, a topographical map of DNA could be made based on the tip-sample interactions. Nanometer resolution imaging of DNA with the AFM has nowadays become routine, and has been extensively applied to the mapping of DNA-protein complexes. Sample preparation for AFM only uses very small quantities of DNA and proteins, in the range of a few nanograms or a few femtomoles. The most common way to image nucleoproteic complexes with the AFM is to deposit the molecules onto

Figure 1: Mix of relaxed and supercoiled pTZ18R DNA plasmids adsorbed onto mica and imaged in air.

DNA length: 2861 base pairs
1.5µm x 1.5µm scan
Z scale: 3nm

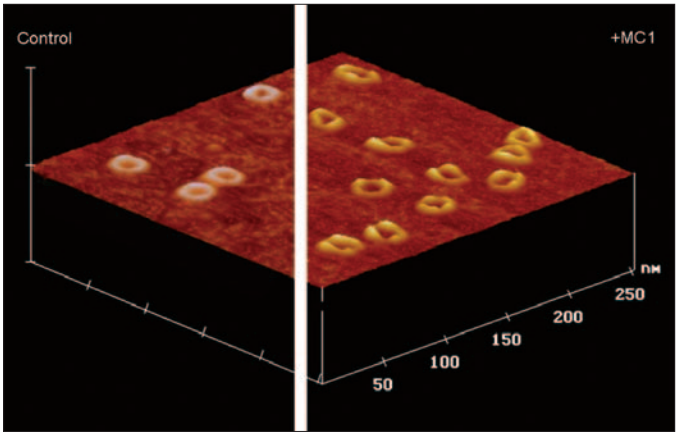


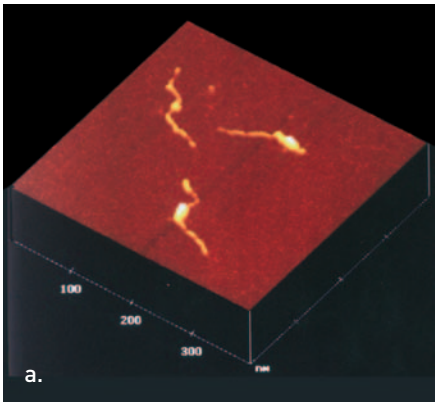
Figure 2: DNA minicircles naked (left) or complexed to the MC1 protein (right). The height of naked minicircles is very regular whereas minicircles bound to MC1 show important height variations, due to the torsion induced by the binding of one or two MC1 per minicircle.

DNA length: 207 base pairs
250nm x 250nm scans
Z scale: 15nm

an atomically flat substrate such as mica. Adsorption of DNA on the substrate is usually promoted by using a buffer containing Ni^{2+}, Mg^{2+}, or other divalent cations that favor the interaction between the negatively charged DNA and the negatively charged mica. Under such conditions of adsorption, the molecules are able to move and equilibrate thermodynamically on the surface[2] and good spreading of DNA on the surface can be obtained. The sample is then dried, after which the DNA or DNA-protein complexes are easily imaged in air.

As with transmission electron microscopy (TEM), it is possible to analyze different kinds of DNA fragments – linear DNA, supercoiled or relaxed closed circular DNA such as plasmids (Figure 1) or minicircles (Figure 2) - and to characterize different types of DNA-protein complexes. Unlike TEM, however, AFM does not require the use of a staining agent to visualize the biomolecules. Moreover it provides topographical information, in contrast to the purely 2D images produced by TEM, which can help to distinguish fine structural details. For example, AFM allowed the unambiguous identification of binding of Fur protein to two distinct adjacent promoters as shown in Figure 3[3]. In addition to mapping of protein position along DNA molecules, local DNA curvature, flexibility[4], or deformation upon protein binding, such as bending[5,6] or wrapping[7-9] of DNA, can be quantitatively analyzed by AFM.

DNA bending induced by proteins on linear DNA fragments can be determined by direct angle measurements. Such deformations have been illustrated with architectural proteins such as MC1, which induces a kink angle of 116°[10] and spatial deformation within DNA minicircles (Figure 2)[5,11]. Recognition of damage sites by proteins involved in DNA repair is also frequently related to DNA bending. DNA glycosylases such as hOGG1 recognize lesion sites, as



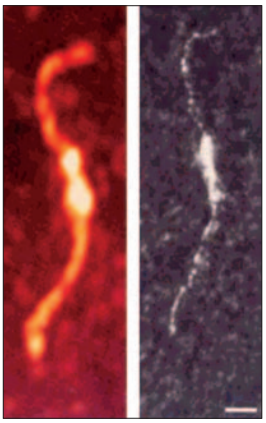


Figure 3: Visualization of Fur-DNA complexes by AFM.

(a.) 3D representation.

400nm x 400nm scan
Z scale: 25nm

(b.) High magnification image compared to that of a similar molecule observed by dark field Transmission Electron Microscopy; the constriction observed in the Fur coat by AFM corresponds to the occupation of the two adjacent promoters of the aerobactin gene.

Scale bar: 20nm
DNA length: 645 base pairs

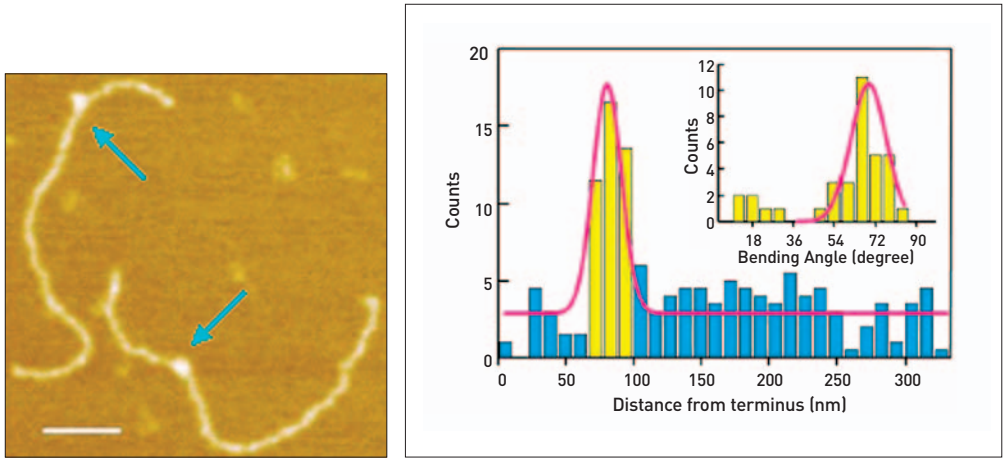


Figure 4: hOGG1 is one of the DNA glycosylases responsible for the recognition and removal of damaged bases from the genome. Protein position on damaged DNA molecules (as indicated by arrows) is well correlated with the position of the lesion site. Measurements of bending angles indicate a mean angle value of 70°.

DNA length: 1024 base pairs, with a lesion site 245 base pairs from one end
Scale bar: 50 nm

demonstrated by mapping of the complexes (Figure 4), and hOGG1 induces a mean bending angle of 70°[6]. Refinement of methods used to analyze bending angles is described in Cognet et al.[12].

The wrapping of DNA bound to proteins is well illustrated in the case of a chromatin fiber[13], where each nucleosomal core particle is able to wrap 146 base pairs of DNA. DNA wrapping associated with complex formation is characterized by a shortening of the apparent DNA length in AFM images. Interestingly, a few proteins involved in promoter regulation have been described to wrap DNA to form small loops. For instance, LrpC from *B. subtilis* wraps DNA in a right-handed superhelix to promote positive supercoiling. AFM has been used to demonstrate that the formation of these nucleosome-like structures results from a polymerisation of LrpC along DNA and an oligomerisation induced by curved DNA (Figure 5)[7]. The implication of wrapping in DNA regulation has also been illustrated for DNA gyrase, which promotes negative supercoiling by wrapping DNA in a left-handed helix. AFM has also been used to demonstrate a complete loss of wrap in the DNA-gyrase complexes (Figure 6)[9] in the presence of ADPNP cofactor.

Numerous other DNA-protein complexes have also been studied by AFM in air, and we invite readers to refer to more complete reviews [14-16]. These examples demonstrate that AFM imaging in air, combined with careful choice and control of biomolecules and immobilization conditions, can provide useful qualitative and quantitative characterization of nucleoproteic complexes. Although inferred from DNA molecules and complexes immobilized and dried on the surface, such data already offer a good basis for understanding the behavior of individual proteins along DNA molecules. However in order to probe dynamic aspects directly and to better approximate physiological

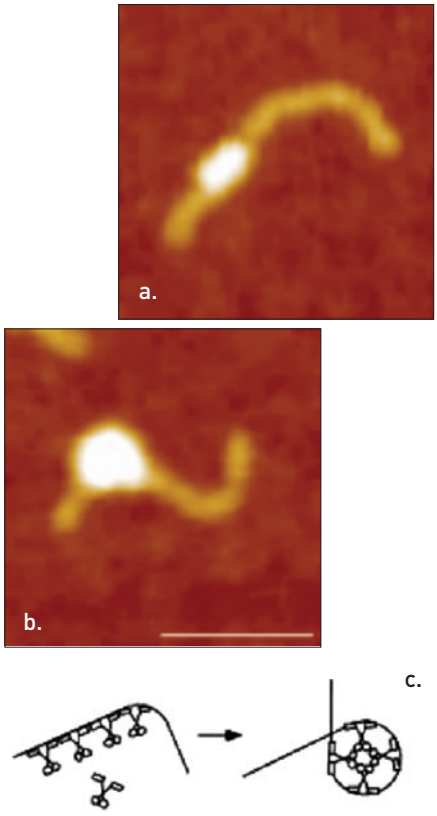


Figure 5: AFM visualization of LrpC-DNA complexes; (a) without wrapping, (b) with wrapping of DNA. Interaction of LrpC with flexible/curved DNA is able to induce a complete wrapping of the DNA around the protein to form a nucleosome-like structure (radius of curvature: 4.5 nm), as schematized in (c).

DNA length: 331 base pairs
Scale bar: 50 nm
Z scale: 5 nm

conditions, it is necessary to study DNA-protein complexes still able to evolve during the AFM observation, and thus to work in liquid.

PROBING DNA-PROTEIN INTERACTIONS IN LIQUID

This is made possible by the use of a fluid cell, which forms a closed fluid volume around the cantilever and sample surface (Figure 7). Working in liquid maintains DNA and proteins in their native state and allows the possibility of varying external parameters such as buffer conditions in the course of an experiment. Indeed within the last decade the emergence of single molecule force measurement techniques and of single molecule imaging techniques, including AFM in liquid, has created new opportunities to analyze the dynamics of DNA-protein interactions. One can imagine that the greatest synergy will be achieved by using complementary methods, particularly those that allow direct visualization along with more traditional biochemical and biophysical approaches. AFM has a special preferred position concerning direct time-resolved visualization of biological systems in liquid.

SENSING DNA-PROTEIN INTERACTION FORCES

The same cantilevers that can be used to image with AFM can also be used to measure nanoscale forces. The softest cantilevers have spring constants on the order of 0.01 N/m (or 10 pN/nm), which allows the AFM to measure forces in the piconewton range. These forces can be measured as the tip approaches the sample, contacts the surface, and then retracts from the sample, producing a "force-distance" curve with piconewton range force resolution and sub-nanometer distance resolution. This force and distance regime matches well with both intermolecular interactions (such as between DNA and proteins) as well as intramolecular interactions (such as in protein folding). Readers can find a detailed overview of the use of AFM as a force measurement tool in another Veeco Application Note 17.

In the case of intermolecular DNA-protein interactions[18-20], the typical experiment involves covalently attaching the ligand to the cantilever tip and bringing it into contact with the surface, where it interacts with its target to form a complex. Upon retraction, this complex is increasingly loaded until it finally ruptures. Repeating this process in different conditions with the same tip and surface produce distributions of unbinding strength probability, which allows one to estimate thermodynamic parameters related to the energy barriers involved in the interactions. This methodology has been used to study the DNA-protein interaction between the transcriptional activator ExpG (*S. meliloti* bacterium) and three

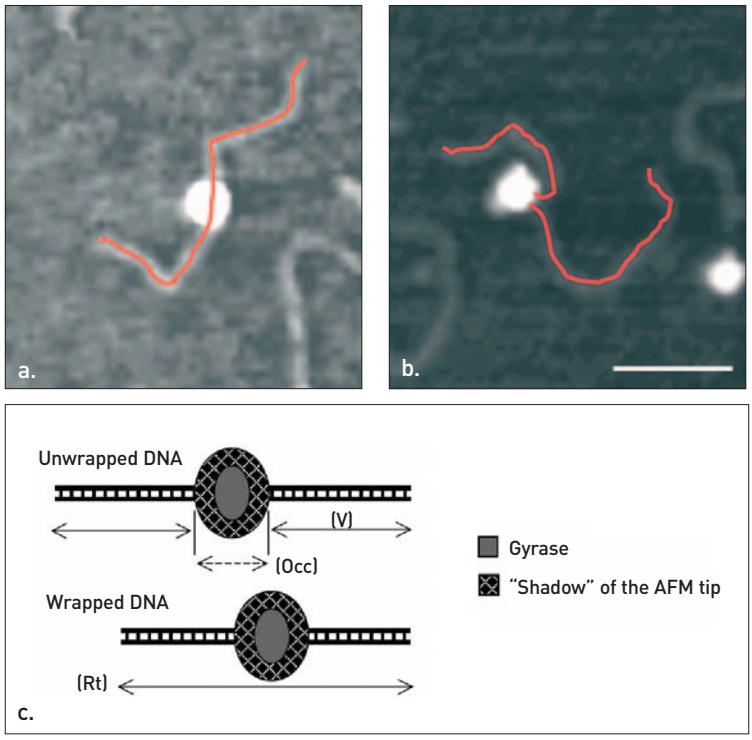


Figure 6: AFM measurements reveal whether DNA-gyrase complexes are (A): unwrapped (in the presence of ADPNP) or (B): wrapped (in the absence of ADPNP). The red lines show how the read-through DNA length (Rt) and visible DNA length (V) were respectively measured. Schematic in (C) illustrates the difference in contour length between wrapped and unwrapped DNA molecules.

DNA length: 1070 base pairs
Scale bar: 100 nm

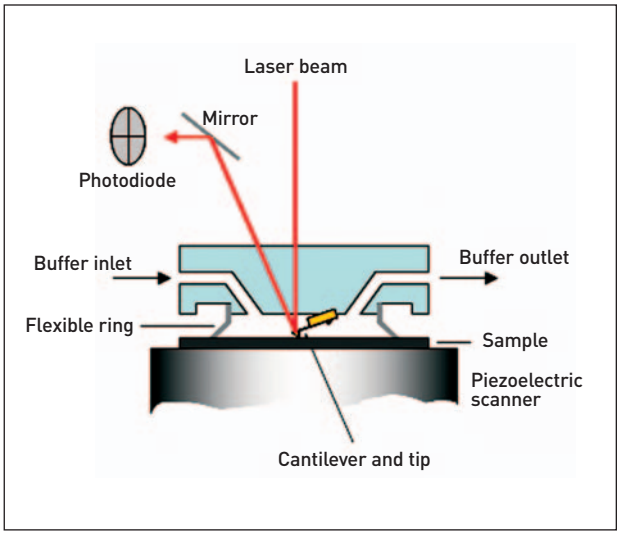


Figure 7: Cross section schematic of the AFM fluid cell setup.

domains of a promoter region[19]. In these studies ExpG proteins were attached to a mica surface treated with silanes and DNA wild type or mutated fragments were attached to the tip. Unbinding force measurements revealed that only one binding site within the promoter (core region) determines DNA recognition by ExpG (Figure 8), whereas two adjacent domains (box 1 and 2) affect the stability of the DNA-protein complex.

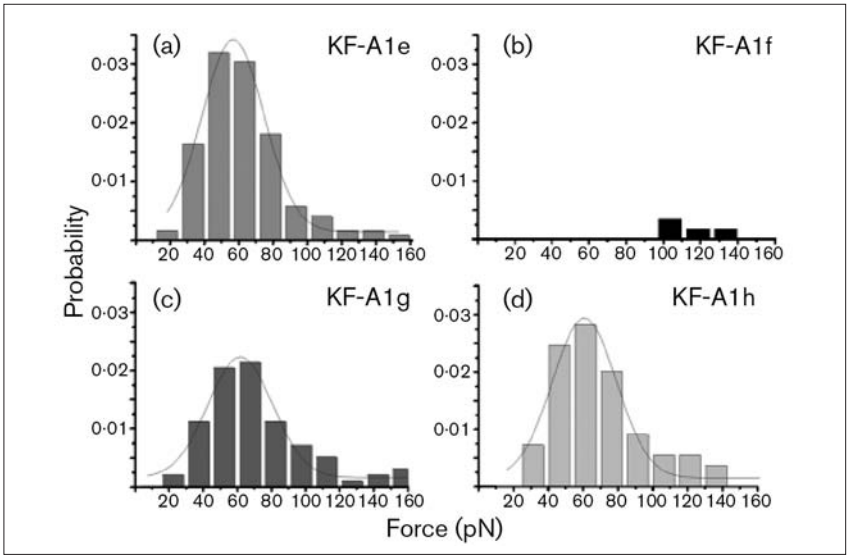


Figure 8: Unbinding force histogram between ExpG and (a) the wild-type DNA sequence, (b) the DNA sequence mutated in the core region, (c) and (d) DNA sequences mutated respectively in box 1 or box 2. Only the core region appears to be determinant for the DNA recognition by ExpG.

VISUALIZING DYNAMIC DNA-PROTEIN INTERACTIONS

Imaging dynamic events at the single molecule level is a fascinating and potentially powerful way to investigate DNA-protein interactions. The use of tapping mode in liquid allows one to observe DNA-protein complexes in a physiological environment, and to record their evolution over the course of time. A number of pioneering works have demonstrated the feasibility of these studies and their ability to measure dynamics parameters that describe the interactions. The dynamic behavior (over a timescale of minutes) of different types of complexes such as DNA with RNA polymerase [21, 22], photolyase [23], p53 [24] or DNase I [25, 26] have been investigated by AFM in liquid. For example, the RNA polymerase-DNA complex has been studied in different buffer conditions and in different situations, either where RNAP is stably bound to the mica surface and the sliding of DNA is characterized (Figure 9) [22] or, conversely, where the sliding of RNAP along immobilized DNA is analyzed [21].

Efforts are now being made to improve and rationalize further sample preparation methods for this kind of experiment. Indeed the complexes must be imaged with sufficient resolution but also with

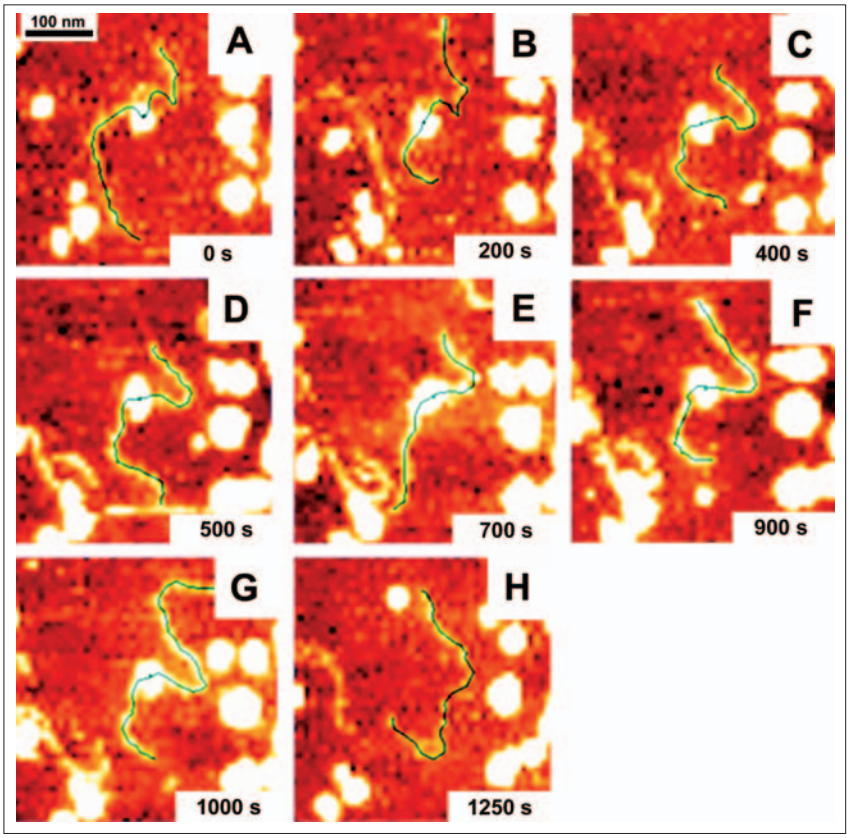


Figure 9: Characterization of the sliding of DNA along RNA polymerase. The DNA contour is traced with a thin line and the center of the RNAP is marked with a dot. The elapsed time after the initial observation of the complex is indicated above each image. Analysis of the sliding of the DNA molecule relatively to RNAP was shown to be consistent with a 1D random walk.

DNA length: 1001 base pairs
Scan size: 350 nm x 350 nm
Z scale: 5 nm

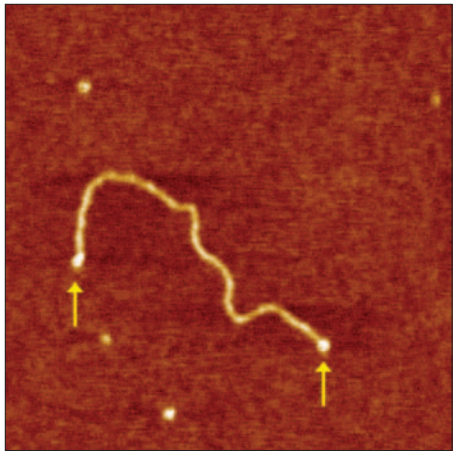


Figure 10: Streptavidin-biotinylated DNA complex; one streptavidin protein is bound to each extremity (arrows).

DNA length: 1444 base pairs
500 nm x 500 nm scan
Z scale: 3 nm

good control of interactions with the sample surface and good knowledge of their influence on DNA-protein interactions. Actually each kind of DNA-protein complex must be considered as an overall system, where specific versus non-specific interactions in the DNA-protein complex and interactions with the surface are both very dependent on the ionic conditions in the buffer.

The main actor of the binding to the surface is usually the adsorption of the DNA molecules. The presence of divalent cations (such as Mg^{2+} or Ni^{2+}) and a low concentration of monovalent cations (such as Na^{+} or K^{+}) in the buffer are known to enable reliable imaging of DNA molecules on mica. Recently, the forces acting between DNA and mica and their dependence on monovalent and divalent ion concentrations have been investigated and a rationalized framework describing the issue of DNA adsorption has been proposed [27].

Various attempts have also been made to design more deliberate and specific DNA attachment methods. One approach is to functionalize the mica surface with amino groups (APTES), which allows attachment of DNA molecules on the surface at random positions along the DNA chain, without the requirement of divalent cations in the buffer. This method has been used to image the dynamics of DNA structures such as supercoiled plasmids or Holliday junctions over the course of time [28]. Alternatively, the authors of this note are developing a strategy to tether the biotinylated ends of linear DNA molecules to mica using the biotin-streptavidin complex (Figure 10) and biotinylated tethers immobilized on the surface.

Sample preparation for imaging dynamic DNA-protein interactions in liquid essentially requires satisfying a compromise between the necessity to bring the DNA molecules onto the substrate surface and the necessity to conserve their mobility and their accessibility to the proteins. As a tool to modulate DNA accessibility and reactivity, we have designed a system in which one can alternately switch between various DNA adsorption conditions during a single AFM experiment. This is done by using mica pretreated with nickel ions to keep the molecules on the surface and varying the monovalent and divalent ion concentrations in the buffer to change the adsorption strength [27, 29]. Using this framework, we have started to examine the influence of adsorption strength on the binding of small ligands (ethidium bromide [30], bleomycin [31]) to DNA. Naturally we wish to extend this work to more challenging DNA-protein complexes and work toward this goal is currently under way.

CONCLUSION

The AFM instrument has become indispensable for probing DNA-protein interactions at the single molecule level. This application note only covers a few examples of the knowledge that AFM has brought forth. Imaging immobilized DNA-protein complexes in air has essentially shed light on their architecture. Now, by working in physiological buffer, it is possible to directly image dynamic interactions and to probe the actual interaction forces. We foresee a whole new range of fascinating DNA-protein interaction studies yet to come based on this solid foundation of successful investigations and increasingly rationalized approaches to sample preparation.

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