Atomic Force Microscopy Imaging and Probing of DNA, Proteins, and Protein DNA Complexes: Silatrane Surface Chemistry

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Chapter 21

Atomic Force Microscopy Imaging and Probing of DNA, Proteins, and Protein–DNA Complexes: Silatrane Surface Chemistry

Yuri L. Lyubchenko, Luda S. Shlyakhtenko, and Alexander A. Gall

Summary

Despite their rather recent invention, atomic force microscopes are widely available commercially. AFM and its special modifications (tapping mode and noncontact operation in solution) have been successfully used for topographic studies of a large number of biological objects including DNA, RNA, proteins, cell membranes, and even whole cells. AFM was also successfully applied to studies of nucleic acids and various protein–DNA complexes. Part of this success is due to the development of reliable sample preparation procedures. This chapter describes one of the approaches based on chemical functionalization of mica surface with 1-(3-aminopropyl) silatrane (APS). One of the most important properties of APS-mica approach is that the sample can be deposited on the surface in a wide range of ionic strengths, in the absence of divalent cations and a broad range of pH. In addition to imaging of dried sample, APS-mica allows reliable and reproducible time lapse imaging in aqueous solutions. Finally, APS mica is terminated with reactive amino groups that can be used for covalent and ionic attachment of molecules for AFM force spectroscopy studies. The protocols for the preparation of APS, functionalization with APS mica and AFM probes, preparation of samples for imaging in air and in aqueous solutions, and force spectroscopy studies are outlined. All these applications are illustrated with a few examples.

Key words: Atomic force microscopy (AFM), Force spectroscopy, Surface chemistry, Mica functionalization, Silanes, Silatranes, DNA local structures, Protein–DNA complexes.

1. Introduction

Atomic Force Microscopy (AFM; another name is Scanning Force Microscopy, SFM) and its predecessor, Scanning Tunneling Microscope (STM), are relatively new tools with enormous potential

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importance to structural biology. The prototype STM instrument was conceived by Binnig et al. *(1)*, an invention for which Binnig and Rohrer were awarded the 1986 Nobel Prize in Physics. AFM is a direct descendent of this early instrument *(2)*, but is capable of imaging nonconducting, as well as conducting surfaces. Unfortunately, early artifactual STM images of a graphite surface mimicking DNA molecules *(3, 4)* have seriously undermined hopes for immediate success for applications of both STM and AFM to structural biology. A serious practical limitation to the application of AFM to structural and conformational studies of DNA and its complexes with proteins and other biological macromolecules has been sample preparation. The macromolecules must be tethered to the substrate surface in order to avoid resolution-limiting motion caused by the sweeping tip during scanning. The breakthrough in reliable and reproducible imaging of DNA with AFM was made in early 1990s due to development of methods for sample preparation. Several of such methods were developed simultaneously in a number of laboratories *(5–14)*. A major feature of these methods is the use of a specially prepared surface that holds (usually electrostatically) the sample in place during scanning. We *(8, 14–18)* initially worked out a procedure for chemical modification of mica. A weak cationic surface is obtained if aminopropyltriethoxy silane (APTES) is used to functionalize the mica surface with amino groups (AP-mica). AP-mica allowed us to routinely perform visualization of DNA with AFM, achieving resolution as good as that of traditional electron microscopy (EM). We have also shown that AP-mica holds nucleic acids under physiological conditions, allowing for the ability to image *in situ*. Remarkably, we have achieved resolution for DNA in solution exceeding that of EM for dried DNA samples. Recently, we have developed an improved surface chemistry utilizing a more hydrolytically stable silatrane such as 3-aminopropylsilatrane (APS) instead of silanes. This method provides a more reproducible, robust surface for topographic, *(19–32)* as well as for force spectroscopy AFM applications *(33–37)*. The scheme for synthesis of APS is presented in **Fig. 1a**.

The method of functionalization of mica is based on covalent attachment of 3-aminopropyl silatrane (APS) to the surface of mica *(24)*. As illustrated by the scheme in **Fig. 1b**, the silicon residues of APS are bound covalently to exposed hydroxyl groups of the freshly cleaved mica resulting in a strong covalent attachment of alkylamino residues to the surface, giving it properties similar to an anion exchange resin used in affinity chromatography. The amino group after protonation in water solution becomes positively charged in a rather broad range of pH (aliphatic amines have a pK of around 10.5). Therefore, DNA, which is a negatively charged polymer, should adhere to this surface strongly. APS-mica preparation requires a very low concentration of APS

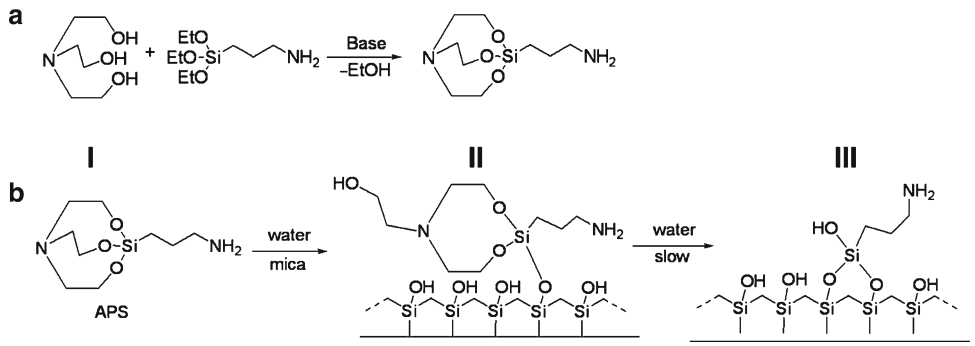

Fig. 1. (**a**) Scheme for the synthesis of APS. Chemical formula for APS is 1-(3-aminopropyl)silatrane (2,8,9-trioxa-5-aza-1-silabicyclo[3.3.3]undecane) (CAS [17869-27-1]). Molecular weight: 232.36. Molecular formula: $C_9H_{20}N_2O_3Si$. (**b**) Scheme for reaction of 3-aminopropylsilatrane (I, APS) with hydroxyl groups on a silicon surface. The initial adduct II may react with a second surface OH group forming III in a reversible equilibrium.

(~150 μM) allowing to prepare a smooth surface, so DNA and DNA–protein complexes can be visualized unambiguously (*see* **Figs. 2a, b** respectively).

The features of this procedure of sample preparation are as follows *(26)*:

1. Binding of DNA to APS-mica is insensitive to the type of buffer and presence of Mg^{2+} or other di- and miltivalent cations; hence, sample preparation can be done in a variety of conditions.
2. Deposition can be done in a wide variety of pH and over a wide range of temperatures.
3. Once prepared, samples are stable and do not absorb any contaminants for months with minimal precautions for storing.
4. As low as 2–10 ng of DNA is sufficient for preparation of one sample.
5. APS-mica is terminated with primary amines that can be used for covalent attachment of biomacromolecules.

These characteristics of APS-mica were crucial for routine imaging nucleic acids of various conformations (e.g., *(19, 20, 22, 24, 27, 38, 39)*); nucleoprotein complexes of different types (e.g., *(25, 26, 30–32)*), protein assemblies such as amyloid fibrils, spheres, and toroids *(33, 35, 37, 40–47)*; and for AFM force spectroscopy studies *(33–35, 47, 48)*.

We have shown that amino-terminated surface (APS-treated mica and AFM tips) can be functionalized further using molecules containing amine-reactive groups such as glutaraldehyde *(33, 34)* and hydroxysuccinamides *(49)*. This property of APS-functionalized surfaces was used for covalent attachment of biomolecules

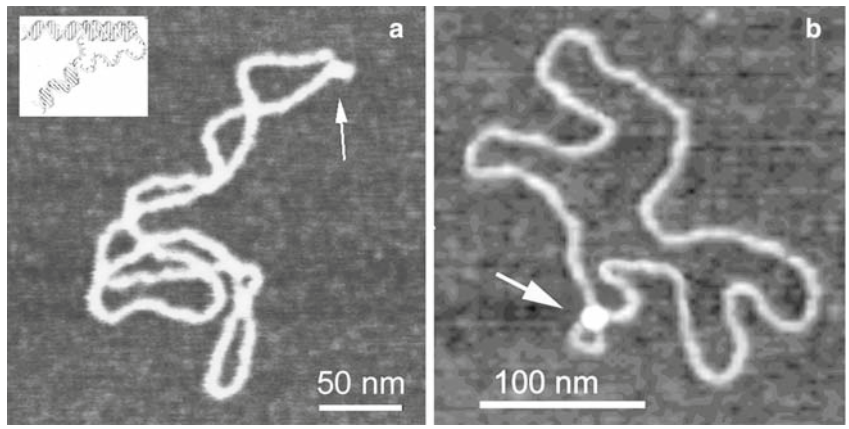

Fig. 2. AFM of images of supercoiled DNA with extruded H-DNA local structure indicated with an arrow (**a**) (*see* **ref.27** for details) and looped structure formed by the interaction of SfiI enzyme (indicated by arrow) with two recognition sites in the DNA template (**b**). Specifics for SfiI-DNA complexes are described in **ref.32.**

of various kinds for probing intermolecular interaction. Moreover, the use of relatively long flexible linkers made it possible to perform the force spectroscopy analysis at a single molecule level. **Subheading 3.3** outlined the specifics for the preparation of surfaces with covalently attached functionalized PEG linkers.

2. Materials

2.1. Mica Substrate

Any type of commercially available mica sheets (green or ruby mica) can be used. Asheville-Schoonmaker Mica Co (Newport News, VA) supplies thick and large (more than 5 × 7 cm) sheets suitable for making the substrates of different sizes.

2.2. Chemicals

1. 3-Aminopropyltriethoxy silane (Fluka, Chemika-BioChemika, Switzerland; Aldrich, USA; United Chemical Technology, USA) can be used without additional purification.
2. Sodium metal and triethanolamine were purchased from Sigma-Aldrich.
3. Xylene solvent was purchased from VWR.
4. Argon gas supplied in cylinders of reagent grade.
5. Bifunctional NHS-PEG-MAL (bf PEG, *N*-hydroxysuccinimide-polyethylene glycol)-maleimide, 3,400 MW (Laysan Bio, Inc., Arab, AL).
6. Dimethylsulfoxide (DMSO, Sigma-Aldrich Inc.)

2.3. Water

Double glass distilled or deionized water was filtered through 0.2-μm filter.

2.4. Minor Equipment

1. A vacuum cabinet or dessicator for storing the samples. A unit Gravity Convention Utility Oven (VWR) is recommended.
2. UV ozone tip cleaning unit for UV irradiation of the AFM tips before the functionalization. The ProCleaner™ system (BioForce Nanosciences, Inc., Ames, IA) or CL1000 UV Crosslinker (UVP, LLC, Upland, CA) is recommended.

3. Methods

3.1. Synthesis of APS

1-(3-Aminopropyl)silatrane (CAS [17869-27-1]) was prepared by a method briefly described previously *(24)*. (Silatrane refers to 2,8,9-trioxa-5-aza-1-silabicyclo[3.3.3]undecane. However, silatranes are generally accepted to contain an N->Si coordinate bond.)

A catalytic amount of sodium metal (5 mg) is added to 15.0 mL (16.8 g, 0.11 mol) of triethanolamine (Aldrich) in a 250 mL round-bottom flask under argon or nitrogen atmosphere and allowed to form a solution (sodium catalyst can be replaced with the equivalent amount of sodium ethoxide or sodium hydroxide in 1 mL of ethanol). A rubber balloon is then attached to the flask via a rubber septum and a needle to allow hydrogen to escape without building up pressure. Moderate heat (up to 100°C) can be applied to accelerate the process, but the mixture should be allowed to cool to room temperature before the next step. An equivalent amount of (3-aminopropyl)triethoxysilane (26.4 mL or 25.0 g, 0.11 mol) is added to the mixture; then the flask is placed into a 60°C water bath and connected to the vacuum line to absorb ethanol released in the reaction. This reaction can be simply performed on a rotary evaporator. At the end of the reaction, the mixture loses ~17 g of ethanol and is turned into a solid. This process can take more than 24 h, but mechanical or occasional manual stirring can reduce time to 1–2 h. Evaporation at the end with 150 mL of xylenes at 60°C can also accelerate the process and help crystallization. Vacuum-dried 1-(3-aminopropyl) silatrane obtained by this method can be used directly for AFM, or for better results and higher stability the product can be purified by crystallization. Minute amounts of sodium hydroxide in the product practically do not affect the performance of the reagent, or change the pH of stock solutions of APS used for AFM. Recrystallization from xylenes provides 20 g (80% yield) of colorless solid. The synthesized APS has the following

characteristics: m.p. 91–94°C (open capillary tube); lit. (US Patent 3,118,921) m.p. 87.2–87.9°C (sealed capillary tube). ^{1}H NMR (DMSO-d_6), ppm: 0.08–0.14 (2H, m, $SiCH_2$); 1.1 (2H, br. s, NH_2); 1.28–1.37 (2H, m, CH_2); 2.37 (2H, t J = 7.2 Hz, NCH_2); 2.77 (6H, t J = 5.9 Hz, NCH_2); 3.59 (6H, t J = 5.9 Hz, OCH_2) *(50)*.

3.2. Mica Functionalization with APS

1. Prepare 50 mM APS stock solution in water and store it in refrigerator. The shelf life of the stock solution is not less than 6 months.
2. Prepare working APS solution for mica modification dissolving the stock solution in 1:300 ratio in water; it can be stored at room temperature for several days.
3. Cleave mica sheets of needed sizes (typically 1 × 3 cm) to make them as thin as 0.05–0.1 mm, place them in appropriate plastic tubes, pour working APS solution to cover the mica sheet, and leave on the bench for 30 min. Depending on the size of the mica strip, the plastic disposable 3-mL cuvettes or plastic 15-mL tubes are suitable for these purposes.
4. After 30 min, discard the reagent and rinse the mica with water under the water stream or replacing the water in the tube 3–4 times.
5. Remove the mica sheets and dry them under Argon stream. The strips are ready for the sample preparation. Note, however, as prepared, the APS mica sheets can be stored under Argon for several days. This procedure allows one to obtain a weak cationic surface with rather uniform distribution of the charge. **Fig. 2a** shows uniform distribution of DNA fragments over the surface.

3.3. Covalent Attachment of Bifunctional PEG Linker to APS Surfaces

This procedure describes the surface functionalization steps of aminoterminated APS surfaces that are required for AFM force spectroscopy applications. References *(33, 34, 36, 37, 49)* are good examples for a few applications with the use of APS and other silatranes. The following procedure describes specifics for covalent attachment of bifunctional PEG (bf-PEG) terminated with maleimide and NHS groups. The latter provides covalent bond reacting with the surface-terminated amines allowing the use of maleimide termini for covalent attachment of molecules containing thiol groups *(34, 36)*. A typical force curve with clearly identified rupture event is shown in **Fig. 3**.

1. Prepare 1.67 mM solution of bf-PEG in DMSO, 10 mM of pH 3.7, TCEP hydrochloride (Hampton Research Inc, Lynchburg, VA), and 20 mM β-mercaptoethanol.

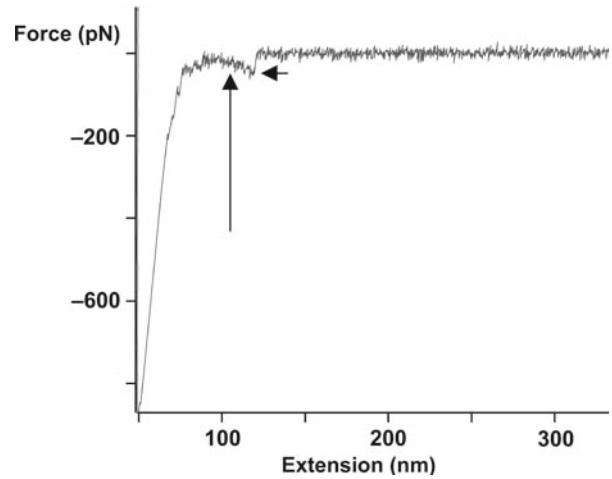

Fig. 3. Force curve illustrating the single molecule detection of interaction of two α-synuclein molecules at conditions facilitating the misfolding and aggregation of this protein *(35)*. The event for the rupture of the protein dimer spontaneously formed upon approaching tip to the surface is indicated with a short arrow. The long arrow points to the region of the extension curve corresponding to stretching of the PEG linker *(36.* The protein was immobilized on APS-functionalized mica and silicone nitride tips via PEG linker.

2. Apply 0.1 mL of the bf-PEG solution to APS-mica and allow the reaction to proceed for 3 h.
3. Rinse the mica three times with 1 mL of DMSO followed by a thorough rinse with deionized water and use for the covalent attachment of your sample. The following protocol for immobilization of α-synuclein via formation covalent bonds between maleimide moiety of immobilized PEG with cystein of the protein can be used as a template for development of the procedure for immobilizing the needed molecule.

3.3.1. Procedure for Immobilization of α-Synuclein on Mica

1. Prepare α-synuclein solution in the concentration 19 nM in water with 0.25 mM TCEP.
2. Place 10 μL of the protein solution on the PEG-activated mica sheets for 1 h.
3. Rinse the mica with DMSO for three times to remove NHS-PEG-MAL solution from the slide followed by a thorough rinse with water.
4. Rinse with HEPES buffer (pH 7.0) few times, deposit 50 μL of α-mercaptoethanol solution, and leave for 10 min.
5. Rinse with HEPES buffer (pH 7.0) and mount the mica sample on the microscope stage.

3.3.2. Procedure for Immobilization of α-Synuclein on the AFM Tip

1. Clean silicon nitride tips with 95% ethanol, immerse tips in 95% ethanol for 30 min, rinse in water and dry in argon flow, place tips under the UV lamp for 30 min, immerse tips in APS solution for 30 min.
2. *NHS-PEG-MAL treatment*: remove tips from APS solution, rinse with water, dry in argon flow, and immerse the tips in NHS-PEG-MAL DMSO solution (*see* **Subheading 3.3.1**.) for 3 h.
3. *Protein attachment*: mix 10μL of 10 mM TCEP stock solution with 37 μL of stock protein (500 nM solution) and allow to stay for ~15 min; rinse the tips with DMSO for three times to remove NHS-PEG-MAL solution from the tips, rinse the tips with water many times to remove DMSO and immerse the tips in TCEP/protein/buffer solution for 1 h, rinse tips in HEPES buffer (pH 7.0), immerse in α-mercaptoethanol solution for 10 min, rinse the tips in HEPES buffer (pH 7.0), and leave in this buffer.

3.4. Sample Preparation for Imaging in Air

1. Prepare the solution of the sample (DNA, RNA, protein-DNA complex) in appropriate buffer. DNA concentration should be between 0.1 and 1 μg/mL depending on the size of the molecules.
2. Place 5–10 μl of the solution in the middle of APS-mica substrate (usually 1 × 1 cm squares) for 2–3 min.
3. Rinse the surface thoroughly with water (2–3 mL per sample) to remove all buffer components. 5–10 mL plastic syringes are useful for rinsing. Attach an appropriate plastic tip instead of a metal needle.
4. Dry the sample by blowing with clean argon gas. The sample is ready for imaging. It is recommended 30 min drying of the sample in vacuum cabinet prior imaging especially if the humidity is high. Store the samples in vacuum cabinets or desiccators filled with argon. They remain unchanged for months.

3.5. Sample Preparation for Imaging in Liquid

1. Prepare the solution (DNA, RNA, nucleoprotein complexes) and preincubate it for 10–20 min to allow the temperature to equilibrate. Recommended concentration of DNA is 0.1–1 μg/mL depending on the size of the molecules.
2. Mount a piece of APS-mica on the microscope stage, and follow the steps under **Subheading 3.7**.

3.6. Procedure for Imaging in Air

The procedure for imaging in air is straightforward: mount the sample and do imaging. Although both contact and intermittent (tapping) modes can be used, the latter is preferable and allows one to get images of DNA and DNA–protein complexes routinely. Our experience is mostly limited to MultiMode system (Veeco, CA) running various NanoScope III, IIIa, and IV

controllers and MFP 3D microscope (Asylum Research, Santa Barbara, CA). However, the samples prepared on APS-mica were imaged on other commercially available instruments, for example, the microscopes manufactured by Molecular Imaging (Agilent, Tempe, AZ). With MultiMode system any types of probes designed for intermittent imaging modes (Tapping mode for the Veeco systems) can be used. Typical tapping frequency of 240–380 kHz and scanning rate of 2–3 Hz allow one to obtain stable images.

3.7. Procedure for Imaging in Aqueous Solutions

The capability of AFM to perform scanning in liquid is its most attractive feature for numerous biological applications allowing imaging at conditions closed to physiological ones. In addition, this mode of imaging permits one to eliminate undesirable resolution-limiting capillary effect typical for imaging in air (e.g., *(12, 16, 51)*). As a result, images of DNA filaments as thin as ~3 nm were obtained in water solutions *(18)*, and helical periodicity was observed when dried DNA samples were imaged in propanol *(52)*. In addition to APS-mica, our previously developed procedure with the use of AP-mica can be used as a substrate for imaging in liquid *(14)*; note that the first images of DNA in fully hydrated state were obtained by the use of AP-mica *(15)*. This type of imaging is recommended in cases when dynamics is studied. The following procedure is described for the use of MultiMode AFM (Veeco, Santa Barbara, CA) and can be easily adapted for other systems.

1. Install an appropriate tip in the holder designed for imaging in liquid (fluid cell). Use Si_3N_4 big triangle with thick legs or small triangle with thin legs cantilevers *(18)*.
2. Mount the APS mica sheet on the stage of the microscope. Mica pieces of 1 × 1 cm are sufficient for NanoScope design of fluid cell. Wrap the stage of the scanner for MM AFM with Parafilm to prevent potential shortage due to accidental leak of the fluid underneath of the mica sheet.
3. Attach the head of the microscope with installed fluid cell and make appropriate adjustments of the microscope.
4. Approach the sample to the tip manually, leaving ~50–100 μm gap between the tip and the surface.
5. Inject the sample solution or appropriate solvent with 1-mL plastic syringe. Take ~50 μl of the sample with 1-mL syringe. Use 200-μl plastic tips with capillary ends instead of metal needle. Cut both ends of the tip to fit to the syringe and the diameter of inlet hole of the fluid cell. The use of the second syringe attached to the outlet of fluid cell allowing for pulling out the solution is recommended for manipulating with small volume of solution.

6. Change the position of the mirror to maximize the signal on the photodetector.
7. Find a resonance peak. Typically it is a quite broad peak in the range of 7–12 kHz for the MultiMode system. Follow the recommendations given in the manual for the fluid cell operation how to find the peak.
8. Minimize the drive amplitude. The values vary from tip to tip, but amplitude as low as 20 nm or less provides good quality pictures (*see* **Notes 1–5**).
9. Allow the microscope to approach the sample and engage the tip.
10. Adjust the values for the set point voltage and drive amplitude to improve the quality of images and start the data acquisition using the continuous mode scanning.

3.8. AFM Force Spectroscopy

These specifics are made for MFP 3D system (Asylum Research, Santa Barbara, CA). Modify the protocol for your system.

3.8.1. Spring Constant Measurements

1. Install the tip into the AFM head and put a piece of freshly cleaved mica on the stage.
2. Based on thermal profile estimate the resonance frequency of the tip, type in start and end frequency. Do auto tune.
3. Approach to the surface.
4. When on the surface, click stop and change from AC mode to Contact mode.
5. Go to force menu. Click continue force and slowly move the red bar down (or arrow) until you see characteristic force curve. Click stop.
6. Find the linear slope on the force curve, put marks on the linear part of the force curve as far away from each other as possible. Press the arrow button in either direction to make sure that both marks are moving in the same direction (they should be on the same curve: approach or retraction, and when they move in the same direction, it indicates that they are).
7. Click the deflection button to see deflection value in air.
8. Withdraw and go to thermal and do thermal. Wait for several seconds to collect more curves then stop.
9. Do the fit to get the spring constant value. Use this value for the next step.

3.8.2. Force Measurements

Before you start force measurements you have to do tune and approach as it is described earlier. When you are on the surface do the following:

1. Type in the designated box the measured spring constant and measure the deflection of the tip in liquid- the same way as it

is described in the sections earlier. The deflection will be different from the value measured in air.

2. Set up the parameters for scan rate, trigger, dwell time (if any) and start collecting force curves.

4. Notes

1. *Silatrane synthesis.* In **Subheading 3.1**, sodium catalyst can be replaced with the equivalent amount of sodium ethoxide or sodium hydroxide in 1 mL of ethanol.
2. *DNA concentration.* This parameter depends on the length of molecules. If the molecules are as small as several hundred base pairs, the concentration ca. up to 1 μg/mL is recommended to avoid intermolecular crossing. To the contrary, low DNA concentration is recommended for large DNA molecules. For example, concentration of lambda DNA (~48 kb) ~0.01 μg/mL allows one to get images of individual DNA molecules *(15, 17, 52)*.
3. *DNA preparation.* Very small amount of DNA is needed. Typically 1–10 ng of DNA is sufficient for the AFM preparation of plasmid DNA (~3 kb long). Because one band of DNA in agarose gel usually contains 100 ng of DNA, DNA extracted from the gel can be sufficient for preparation of a set of samples. The following procedures can be used for purification of DNA extracted from the gel.
   - *Electrophoretical deposition of DNA bands onto DEAE paper.* The strips of the paper are placed into the slot 3–5 mm below the band and the DNA is electrophoresed onto the paper for 5–10 min (the time can be determined by a direct examination the gel under a UV transilluminator). The DNA is extracted from the paper by elution in 2 M NaCl solution for 30 min at 37°C followed by ethanol precipitation.
   - *Extraction from the gel.* The procedure is based on the use of the extraction kit UltraClean15 and the protocol provided (MoBio Laboratories, Inc., Solana Beach, CA). Generally, the purification consists of melting of the slice of agarose gel, immobilizing DNA on the sorbent, washing off all contaminants, and eluting DNA from the matrix with a low-salt buffer. At least one step of the ethanol precipitation is needed to remove UV-absorbing low molecular weight materials. A similar procedure can be applied to purification of the sample extracted from polyacrylamide gel *(53)*.

4. *Imaging conditions.* It was recommended to operate the instrument at the lowest possible drive amplitude. This recommendation is based on the following considerations. An oscillating tip provides rather large energy to the sample. A total energy deposited into the sample by oscillating tip can be as high as 10^{16}–10^{17} J at amplitude oscillations of 30 nm *(18)*. However, this value is almost three orders of magnitude lower if the microscope is operated at amplitudes as low as ~3 nm. Such imaging conditions allow one to minimize effect of the tip on the sample, to prevent damaging the tip, and get images with high contrast. In addition, such conditions considerably simplify the study with AFM dynamic processes such as segmental DNA mobility, local structural transitions in supercoiled DNA *(18, 20)* and conformational transitions of DNA Holliday junctions including branch migration *(26, 38, 39, 54)*. This capability is illustrated in **Fig. 4**, in which a set of time lapse images of a segment of supercoiled DNA containing a cruciform is shown.
5. *Alternative procedures for AFM sample preparation.* Among other techniques applied to AFM studies of DNA, the method based on using divalent cations *(7, 12, 55)* allowed to get images of a number of nucleoprotein complexes. In this approach, the mica surface is treated with multivalent ions (e.g., Mg^{2+}) to increase its affinity to DNA, which is held in place strongly enough to permit reliable imaging by AFM. An alternative is to deposit the sample in the buffer containing a multivalent ion. This cation-assisted procedure of sample preparation was used for studies of the process of DNA degradation with nuclease *(56)* and interaction of DNA with a site-bound enzyme. EcoP15I *(57)*. The mechanism of this technique remains unclear; detailed protocol depends on the

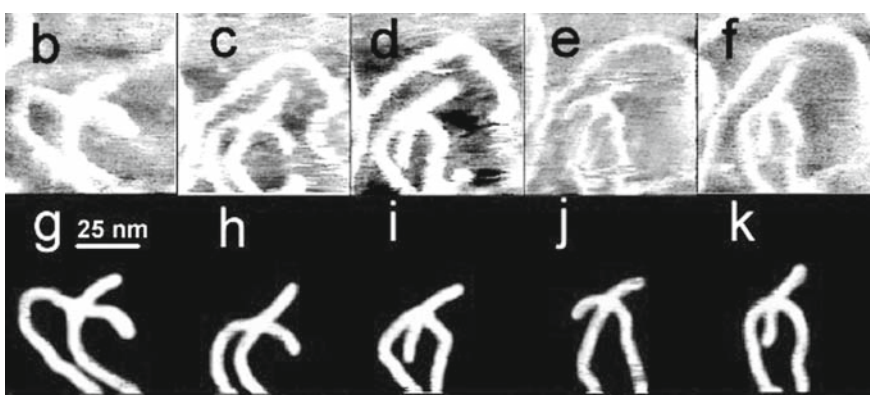

Fig. 4. Time lapse images illustrating dynamics of cruciforms formed within supercoiled DNA. Sections of the plasmid containing the cruciform are shown. Frames (**b–f**) correspond to times 11 min (**b**), 44 min (**c**), 60 min (**d**), 134 min (**e**), and 155 min (**f**). Traces of the same molecule on black background are shown in frames (**g–k**) (*see* **ref.** *39* for details).

system studied, the type of the cation used, and efficiency of DNA deposition is buffer sensitive *(55, 58)*. In some cases, a special class of tips (electron-beam-deposited tips) is required for reliable imaging *(59)*.

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