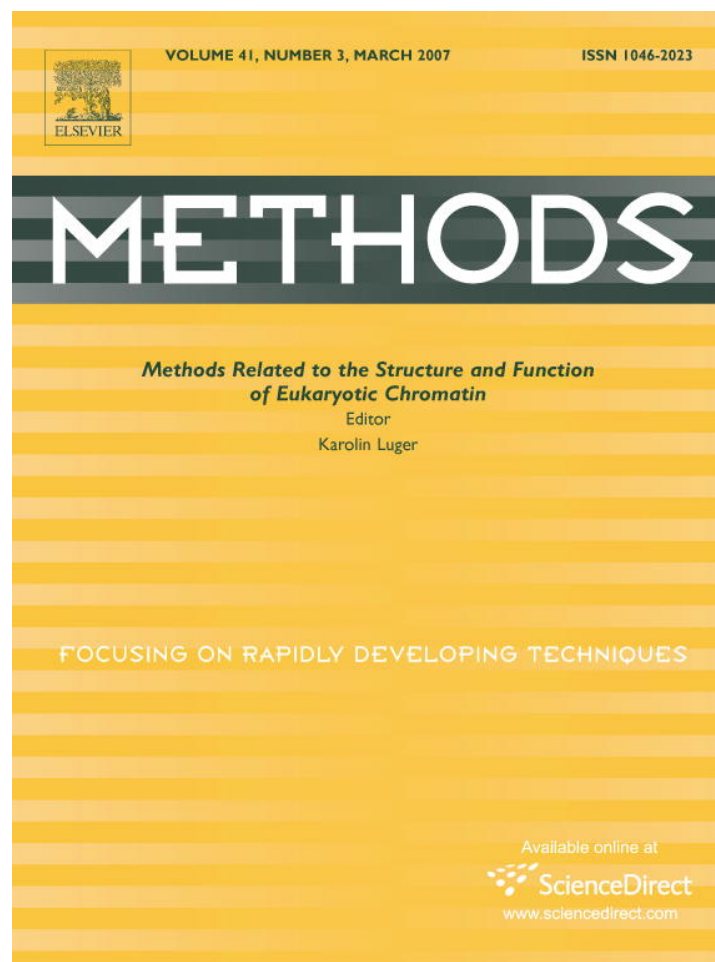


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Using atomic force microscopy to study chromatin structure and nucleosome remodeling

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Abstract

Atomic force microscopy (AFM) is a technique that can directly image single molecules in solution and it therefore provides a powerful tool for obtaining unique insights into the basic properties of biological materials and the functional processes in which they are involved. We have used AFM to analyze basic features of nucleosomes in arrays, such as DNA–histone binding strength, cooperativity in template occupation, nucleosome stabilities, nucleosome locations and the effects of acetylation, to compare these features in different types of arrays and to track the response of array nucleosomes to the action of the human Swi-Snf ATP-dependent nucleosome remodeling complex. These experiments required several specific adaptations of basic AFM methods, such as repetitive imaging of the same fields of molecules in liquid, the ability to change the environmental conditions of the sample being imaged and detection of specific types of molecules within compositionally complex samples. Here, we describe the techniques that allowed such analyses to be carried out.

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1. Introduction

The atomic force microscope “images” individual molecules by monitoring the flexing of a micromachined cantilever as it scans over a surface with a sharp tip of end radius 1–50 nm, contacting molecules deposited on the surface. The deflection of the AFM tip as it responds to sample topography is measured by reflected laser light and converted to a digital image map of molecule locations on the surface, plus their height profiles. This technique can be used in a number of ways (see Section 1) and various biological applications are rapidly developing [1–4].

A number of features of atomic force microscopy (AFM) are particularly suited to studies of chromatin.

AFM can provide information on fairly large biological complexes, a size range for which the number of useful techniques is limited. For example, nucleosomal arrays of tractable length (2–30 nucleosomes) fall in the size range most amenable to detailed AFM analyses and even larger arrays can be studied. The inherent single molecule nature of the technique can provide precise statistical distributions of many chromatin array structural features, for example, relative nucleosome numbers and locations on arrays (cf. [5]). That information reveals qualitative insights into DNA–histone binding strength, nucleosome occupational cooperativity and nucleosome positioning [6–10] which can be quantified via the application of a statistical thermodynamic model developed specifically to analyze AFM data ([9]; Solis et al., submitted). Detailed aspects of DNA association (wrapping) with the histone octamer [10–12] or actions of proteins on chromatin [13,14] can also be analyzed. The ability to image in solution and to alter

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the sample environment, while still monitoring the same individual molecules, can provide single molecule level information on nucleosome stability in arrays [8,15] and the actions of complexes that modify chromatin structure, such as ATP-dependent nucleosome remodeling complexes [16,17,43]. The recently developed ability to identify specific types of molecules in compositionally complex samples makes it possible to focus on changes involving specific proteins in multicomponent systems [18,43]. The strengths of interaction between molecules can be directly and quantitatively studied using force curves [18,19]. These features combine to make atomic force microscopy uniquely suited to the study of chromatin, as well as many other biological systems.

2. Description of methods

2.1. AFM Instrumentation

The atomic force microscope [1] is a fairly complex instrument, but the major US manufacturers (Agilent—<http://www.molec.com/>; Asylum Research—<http://www.asylumresearch.com/> and Veeco—<http://www.veeco.com/>) offer training in the use of their instruments. If at all possible, make a side-by-side comparison with a standard biomolecular sample before making a purchasing decision. Some workers choose to build home-made versions (cf. [11]).

There are some general points to bear in mind regarding instrument operation. These instruments use piezoelectric scanners, which have several important characteristics. They are hysteretic, i.e., they have a time dependent, non-reversible response. They show “creep”, i.e., distortion of an image due to scanning direction and tip placement changes, and their behavior changes over time. Therefore regular calibration of the instrument is essential. This must

be carried out for scanning in both the x - y plane and in the vertical axis. Standard reference calibration nanostructures are available from all the major manufacturers. When setting up the instrument, the manufacturer’s manuals will probably instruct you to set a deflection signal to some value on a readout. It is important to understand what these values mean in terms of real distances, forces, and deflection amplitudes. This is done by calibration.

It is also important to remember that the success of the technique and the results themselves depend on surface chemistry. Many kinds of surfaces are available [13,20–23]. Surface attachment inevitably results in some degree of constraint on the freedom of attached molecules and the local environment of attached molecules may differ from the bulk solution environment. These features are inherent to the technique. Also, it is important to remember that any contaminants present in the sample or in any of the solutions used for dilution, washing etc. will appear in the images. If the contamination binds the surface better than the target molecules, the contaminant may be all you see!

2.1.1. Operating modes of the AFM

In the text below, the word “substrate” is used to refer to both the surface and to molecules on the surface that are contacted by the AFM tip.

2.1.1.1. Contact mode. This was the original mode of operation for the AFM. It is the simplest mode but is not generally useful for “soft” samples like chromatin. In this mode, the tip is lowered onto the surface until a fixed (static) deflection of the cantilever is obtained. The force required to cause this deflection is given by the equation $F = kx$, where k is the spring constant of the cantilever and x is its deflection. The deflection may be calibrated using an “approach curve” (Fig. 1A). On approach, the

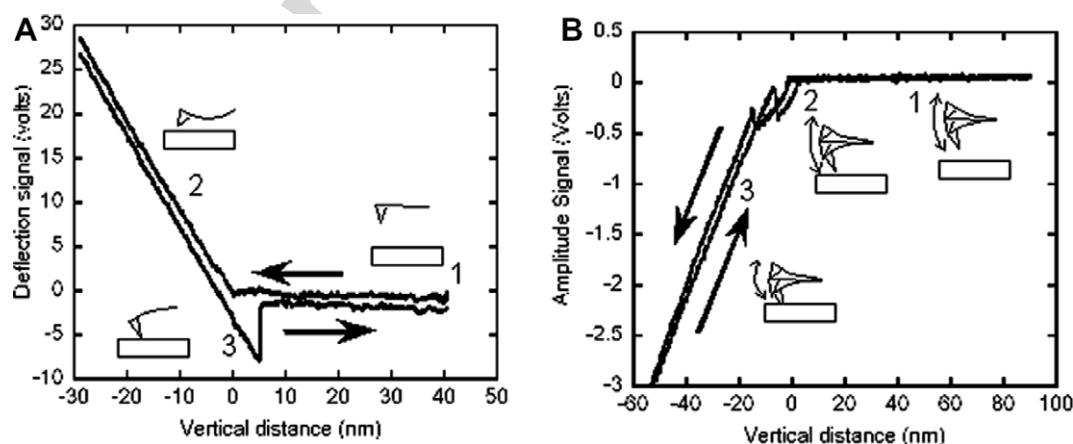


Fig. 1. Determining optimal instrument settings. Examples of curves used for determining optimal instrument settings are presented: (A) deflection vs. Distance (for contact mode microscopy); (B) amplitude vs. Distance (for dynamic force microscopy). The nominal contact point has been set to 0 nm on the horizontal axis in both figures. In (A), the approaching and retracting curves have been displaced vertically for clarity (arrows label approach and retraction on both panels). In (B) the vertical axis has been arbitrarily set to zero at the displacement where the probe is out of contact with the surface. The numbered regions on each plot correspond to probe displacements as shown (greatly exaggerated) in the adjacent illustrations.

cantilever is not deflected (region 1 in Fig. 1A) until contact with the substrate is established [24]. Once in contact with the substrate, the probe responds to changes in topography, being deflected in the z direction with changes in substrate height values (region 2 in Fig. 1A). In other words, the probe moves up and down in response to the substrate topography. The tip of the probe remains in contact with the substrate on retraction of the probe [25], owing to adhesion between the probe and surface (region 3 in Fig. 1A), finally losing contact when the force on the probe exceeds the adhesion force. The vertical axis in Fig. 1A (shown as raw voltage data) must first be converted into nanometers of displacement using the fact that the slope of the curve is unity when the probe is in direct (hard) contact with the surface. Once done, this calibration can be stored in software so that, for a given scanner, the software subsequently displays the vertical axis in nanometer (with the zero set to the constant level when the probe is far from the surface). This axis may be further translated into units of force using the above mentioned equation and the known stiffness of the cantilever (usually given in N/m or nN/nm). Soft cantilevers (<1 N/m) are generally best for contact mode imaging.

The contact mode of operation is established by choosing a set-point deflection corresponding to 1 nN or less. The lower limit on set-point is a function of the instrument (drift and noise) and the sample (adhesion between the probe and the substrate). A soft cantilever can “jump into contact” when the rate of increase of attractive forces exceeds the spring constant of the cantilever. Contact mode is generally disruptive of weakly surface-adsorbed samples, but imaging in a carefully chosen solution, as described by Muller and Engel [24] can minimize this problem, resulting in very high resolution on samples with a limited height range [25]. Samples with higher z values, i.e., large soft samples, yield lower resolution [26]. Thus, molecules like chromatin, with a significant height range, will produce higher ranges of probe topography and lower resolution.

2.1.1.2. Dynamic force microscopy (DFM). (Note: DFM is called “tapping mode” by VEECO and “acoustic mode” by Agilent). The signal-to-noise and the sensitivity of the AFM are generally improved by using the instrument in dynamic force mode [1]. In this mode, the probe is oscillated at or near its resonance frequency (determined by analyzing response versus tip drive frequency) and the amplitude of oscillation monitored as the sample surface is approached. An amplitude–distance curve for this mode of operation is shown in Fig. 1B. In this case, the microscope is set to image the substrate with some small reduction in amplitude (shown as a negative voltage on the figure because the amplitude out of contact with the substrate is arbitrarily set to 0 V). Once again, it is important to know the “operating point” (the relation of amplitude to displacement) of the microscope in absolute units. The amplitude signal can be converted from volts to nm displacement using the static calibration method described

above. The drive needed to achieve a specific amplitude of oscillation depends strongly on the response of the cantilever [27]. Thus, in this approach, setting a particular drive level has no meaning. It is the response that must be measured and controlled. That response will determine the best drive level.

Several distinct modes of DFM are commonly used. The amplitude–distance curves shown in Fig. 1B are taken in air with the cantilever driven slightly above its resonant frequency. The “jump” in amplitude just after nominal contact reflects a transition from a non-contact mode to an intermittent contact (or “tapping”) mode [28]. DFM in air must be carried out with stiff cantilevers ($k > 10$ N/m) to avoid the jump-to-contact problem and adhesion. These are common problems when imaging in air because the layer of adsorbed water and contamination that usually covers the substrate gives rise to a capillary adhesion effects [29].

DFM in solution generally yields the most reliable images because the problems owing to contamination layers are mitigated. Of course, this approach also has the significant advantage that the samples are in a state that more closely reflects the *in vivo* environment (compared to dehydrated samples). In solution imaging, the cantilever must displace a significant amount of solvent as it oscillates, and this gives rise to both viscous losses and an increase in the effective mass of the cantilever [27,30]. Resonant frequencies are lower and cantilevers can be harder to drive; these problems are mitigated by a direct magnetic drive [31].

2.1.1.3. Recognition imaging. This is a relatively new mode of DFM operation, requiring magnetic drive, in which the AFM tip is attached, via a flexible linker, to a substance like an antibody that is capable of specific interaction with a particular type of molecule in the sample being scanned [18]. The specific interactions of the tip-tethered antibody and its target antigen in the deposited sample are detected during scanning as small changes in the signal level (Fig. 2). In addition to antibodies [17–19], nucleic acid aptamers [32] have been successfully used as the specificity probe attached to the tip. Recognition imaging is a tremendously powerful technique with many potential applications because it allows a specific type of molecule to be identified in a compositionally complex sample, a common characteristic of biological materials.

At present the technique is still technically challenging. The AFM tips must be chemically modified in order to attach them to one end of the flexible, hetero-bifunctional PEG linker and the antibodies must be modified for attachment to the other end of the linker. These techniques are described in Section 2.4. We have also found it necessary to synthesize our own PEG linkers, because commercially available linkers are too long. The oscillation amplitude of the cantilever must be set to be approximately equal to the linker length, and excessive amplitudes are damaging to soft samples. Furthermore, the quality and consistency of commercially available antibodies are highly variable.

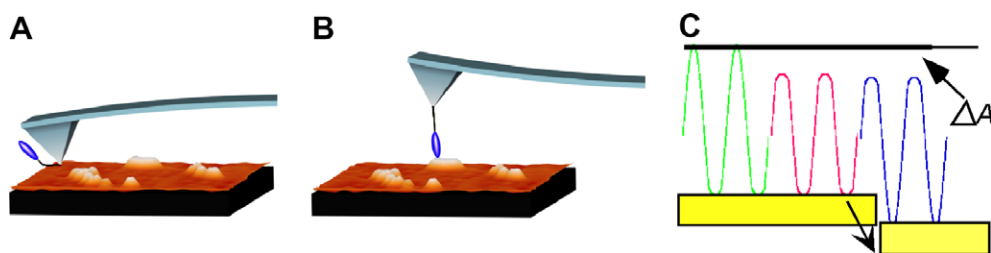


Fig. 2. How recognition imaging works. When an antibody attached to the cantilever via a flexible linker (blue blob) is not interacting with a target on the surface (A) the cantilever oscillates with its normal set-point amplitude as illustrated in the leftmost (green) curve in (C). On binding to a target antigen on the surface (B), cantilever amplitude is transiently reduced as shown in the middle (red) curve in (C). The microscope servo system restores the signal amplitude by pulling the sample away from the probe, as shown in the rightmost (blue) curve in (C). This results in the small shift downward in the position of a peak signal, shown as ΔA in (C). This peak shift provides the signal that is used to make the recognition image; it is thus acquired simultaneously with the topographic image information. (Reprinted from Proc. Natl. Acad. Sci. USA 101 (2004) 12504, with permission, copyright (2004) National Academy of Sciences, USA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

For example, commercial anti-H3 and anti-H2A antibodies are quite specific but anti-H4 and H2B from the same source are not [43]. In at least one case where commercial antibodies were not specific, we have turned to phage display to produce a more specific antibody [19]. The use of DNA aptamers [32] may provide a solution to the antibody specificity problem.

2.1.2. Using flow-through cells

Flow-cells can be used for both contact and DFM modes of operation, although they are far more common in DFM. The combination of a flow-cell and AFM imaging in solution offers the important possibility of carrying out in situ biochemical analyses of properties and processes, by changing the environment of a sample and monitoring the response of the same individual molecules to the change [15–17]. Cells that allow one to flow solutions into a sample are available for most instruments. We have experimented with many types of liquid feeding mechanisms, but prefer to use two micro-syringes connected to the flow-through cell by means of micro-PTFE tubing. Fluid is injected into the sample with one syringe at the same time and same rate as liquid is extracted from the cell with the second syringe (Fig. 3). It is extremely important that the injected fluid be at precisely the same temperature as the fluid in the liquid cell. Even minute temperature differences will cause significant drifts of the cantilever and sample cell, leading to an inability to repetitively reimage the same molecules, one of the most powerful advantages of the flow-cell approach. It is also extremely important that all components of the flow-through system are kept extremely clean. We clean our flow cells in a Soxhlet extractor (an apparatus for cleaning glassware by refluxing solvents like methanol:dichloromethane (80:20) through them) from time to time.

2.2. Preparing chromatin for AFM analysis

Sample purity is a critical issue in AFM studies since every molecule present in a sample shows up in the image. This reality is one of the biggest adjustments for those

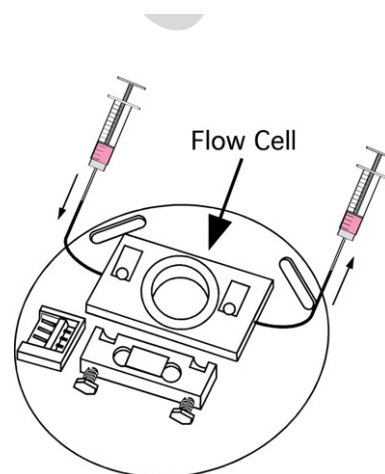


Fig. 3. Changing sample environment in the flow cell. The new solution (NaCl, ATP, peptides etc.) is carefully pushed into the flow cell by depressing one of a pair of syringes, cf. the left one, while solution in the flow cell is simultaneously withdrawn via the other syringe, cf. the right one.

beginning AFM work. The techniques below have proven satisfactory for producing clean DNA and histones for AFM studies.

2.2.1. DNA Purification

Our chromatin reconstitutions use specific DNA fragments cut out from plasmids with restriction enzymes. Rapid Alkaline Extraction [33] or Plasmid Mega and Giga Kits (Quiagen, Valencia, Calif.) have been used to isolate plasmid DNA.

2.2.1.1. Restriction digestion. Purified plasmid DNA (200–500 $\mu\text{g/ml}$) is cut with restriction enzymes in 1 mg batches, using a minimum of 250 U of restriction enzyme and digesting for at least 6 h (overnight preferred). Progress of the digest is monitored on analytical agarose gels. When completed, the DNA is alcohol precipitated and collected by centrifugation [34].

2.2.1.2. Isolation of DNA fragments. The DNA fragments we typically want to isolate are in the 1.5–3 kb size range. DNA from a restriction digest is dissolved in 1 \times loading

buffer (3% ficoll, 0.05% xylene cyanol, 0.05% bromophenol blue, 10 mM EDTA) at 5–10 $\mu\text{g}/\mu\text{l}$ (pellets may be incubated overnight at 4 °C to insure solution in the loading buffer). Approximately 50 μl is loaded into each well of a large (0.6–1 cm thick/at least 12 cm long) horizontal preparative agarose gel (1.2%) in TBE buffer (1.5 mM EDTA, 50 mM Tris–borate, pH 8.3) and run at 5–10 V/cm until the bromophenol blue dye has migrated $\sim 2/3$ of the way down the gel.

Electroelution seems routinely to produce the cleanest DNA for AFM work. The desired band is identified by “UV shadowing” [35] and cut from the gel using a sterilized razor blade. Commercial extraction apparatuses are available or the following can be done. Place the gel slice into dialysis tubing (Spectra/Por 23 \pm 2 mm Flat Width tubing, 6000–8000 MWCO p#132650, prepared as described in Maniatis et al. [34], clamp it at one end, submerge the slice in sterilized 0.5 \times TBE (2–4 ml) and clamp the bag shut. Place it in a chamber filled with 0.5 \times TBE, apply 150 V for 3 h, then reverse the current for 30 s. The buffer is removed and the bag is rinsed once with 0.5 \times TBE and the eluted DNA is ethanol-precipitated as above. The purified DNA is resuspended in sterile TE, extracted once with 50%/50% (v/v) phenol/chloroform:isoamyl alcohol, 24:1 (IAC), once with IAC alone, collected by ethanol precipitation, resuspended in sterile TE buffer (50–100 μl) and stored. The [DNA] can be quantified on agarose gels using DNA standards of known concentrations (small amounts) or by A_{260} measurements. We have also used kits to extract the DNA from ground-up gel slices but that technique can produce less clean DNA.

2.2.2. Histone preparation

2.2.2.1. Histone octamers. Non-acetylated or hyperacetylated HeLa histone octamers were prepared from nuclei (Cellec Biosciences, Minneapolis, MN) according to published methods [36]. To obtain hyperacetylated octamers, the deacetylase inhibitor, Na-butyrate [37,38] was present during cell growth and all protein purification steps.

Octamer concentrations were measured by the Bradford assay [39] and by absorbance at A_{230} using histone standards as references. Purity and stoichiometry were confirmed on 15% SDS–PAGE gels. Acetylation levels were estimated using triton-acid–urea (TAU) gels [40]. Octamers are stored at -80 °C in HSB (50 mM sodium phosphate, 2.5 M NaCl, 0.5 mM PMSF, pH 8.8).

2.2.2.2. H3/H4 tetramers. Histone tetramers were prepared by a modified method of Luger et al. [41]. Lyophilized human H3 or H4 histone (Upstate Cell Signaling Solutions, Lake Placid, NY) were gently dissolved at 2 $\mu\text{g}/\mu\text{l}$ in unfolding buffer (7 M guanidium hydrochloride, 20 mM Tris–HCl, pH 7.5, 10 mM dithiothreitol) at 4 °C for 2 h. H3 and H4 were then combined in volume ratios that would give equimolar stoichiometry of each histone and dialyzed against three changes of 1 L of refolding buff-

er (2 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) for at least 3 h to overnight per change.

2.2.3. hSwi-Snf

The human Swi-Snf (hSwi-Snf) ATP-dependent nucleosome remodeling complex was kindly provided by Gordon Hager and was satisfactory when prepared as described in Wang et al. [16], except it was necessary to reduce the BSA:hSw-Snf ratio to 4:1 in order to obtain clean enough images. Some BSA presence was necessary to maintain remodeling activity [16].

2.2.4. Chromatin reconstitution

We typically study arrays that are subsaturated, for example, 4–8 nucleosomes on templates that would saturate at 12 nucleosomes (Mouse Mammary Tumor Virus promoter, 5S rDNA), in order to be able to distinguish individual nucleosomes (highly loaded arrays tend to compact, cf. [8]). A typical sample for reconstitution will contain 2.5–4 μg of template DNA plus the amount of histone octamer needed to produce the desired octamer:DNA ratio, in a total volume of ~ 30 μl . For a given run, multiple samples (usually three) containing a progression of octamer:DNA ratios will be reconstituted at the same time and each individual sample will be imaged, to determine the one(s) most appropriate for the proposed experiment. To make up such a set of samples, a solution with the highest octamer:DNA ratio to be used is made up. That solution is diluted with a solution containing the final [DNA] but no histone to get the lower octamer:DNA ratio samples. Each solution is 1 \times in TE buffer (pH 8) + 2 M in NaCl. This empirical approach seems to produce the range of occupation levels expected for the range of histone inputs and to cope most reliably with run-to-run variability.

Each ~ 30 μl sample is transferred to a dialysis bag (Spectra/Por 10 mm Flat Width tubing, 6000–8000 MWCO p#132645, prepared in convenient lengths (10–20 cm) as described [34], clamped shut and step dialysis reconstitution is carried out in 1 L solutions of NaCl, TE, pH 8, at 4 °C (with gentle stir bar mixing) as follows: 1.6 M NaCl for 3 h/1.3 M NaCl for 3 h/1.0 M NaCl for at least 4 h/0.8 M NaCl for 3 h/0.6 M for at least 4 h and finally against 1 mM EDTA for at least 6 h. Unfixed nucleosomal arrays show a significant and occupation level-dependent loss of nucleosomes when analyzed in the AFM [7,43] so after the step dialysis, the chromatin samples (while still in the bags) are fixed by dialysis against 0.1% glutaraldehyde (EM grade, Electron Microscopy Sciences, Hatfield, PA, use a freshly opened vial each time) in 1 mM EDTA for at least 6 h, then the bags are transferred to a new container and dialyzed for at least 12 h against a liter of 1 mM EDTA solution (pH 8). A few microliters of each sample is run on a 3.5% polyacrylamide gel to verify reconstitution. The samples are stored in microfuge tubes on ice at 4 °C.

2.3. Topographic AFM imaging

2.3.1. Making the surface

Aminopropyltriethoxysilane (APTES)-derivatized mica is a surface that is often used for AFM studies of nucleic acids [20] and chromatin [6,7]. In 2002, we began using APTES-mica derivatized by glutaraldehyde, called GD-APTES mica [15,22]. This surface is far more reliable than APTES-mica and is suitable for DNA, proteins or chromatin (cf. [17,18]). The reactive aldehydes are thought to form stable adducts with lysine residues, probably those from the projecting N-terminal histone tails of nucleosomes.

The mode of attachment of molecules to GD-APTES permits repetitive scanning of the same field of molecules. Repetitive scans enable one to assess the role of the scanning process itself on sample molecules (cf. [16]) and to add reagents such as NaCl [8,15], ATP [16,17] or antibody-blocking peptides [18,43] to alter the environment of the sample while still monitoring the same individual molecules before and after reagent addition. The latter ability allows one to track changes on individual molecules during a process. The degree of GD modification (the GD concentration used) can be varied from nanomolar to millimolar [15]. Higher concentrations make a surface with more sites of attachment as well as a rougher surface. Lower GD concentrations allow more freedom for sample molecules on the surface. We have typically used $\sim 1 \mu\text{M}$ GD concentrations. At these modification levels, the DNA in nucleosomal arrays can be progressively loosened and ultimately completely removed from arrays by the addition in situ of NaCl (Fig. 4). Also, striking changes in protein and DNA topology (cf. Fig. 5), including complete DNA release from nucleosomes [17], as well as major changes in histone composition [43], can be produced by the in situ activation of ATP-dependent nucleosome remodeling complexes. Thus, surprisingly, although chromatin molecules are attached to a GD-APTES surface strongly enough to allow repetitive scans, the DNA and even the histones maintain a good deal of freedom.

2.3.1.1. Making AP-mica. The setup for producing AP-mica, the first stage in making GD-APTES, is shown in Fig. 6. Clips from which mica strips (1 in. \times 1 in.) will be

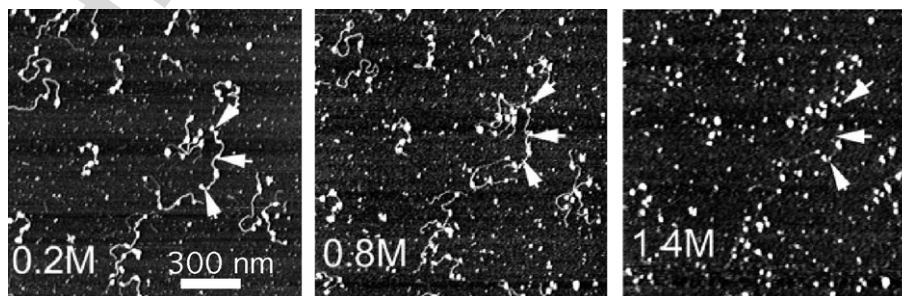


Fig. 4. NaCl can completely remove the DNA from nucleosomal arrays bound to GD-APTES. These three images are taken from a larger series obtained by repetitive imaging of the same field of molecules deposited on GD-APTES as NaCl concentration was increased in 0.2 M increments in situ, in a flow-cell linked to the AFM. Arrows identify one particular MMTV nucleosomal array; others are visible in the field. Complete DNA release from these arrays becomes significant at $[\text{NaCl}] > 1.2 \text{ M}$. (Reprinted from Biophys. J. 83 (2002) 3623, with permission.)

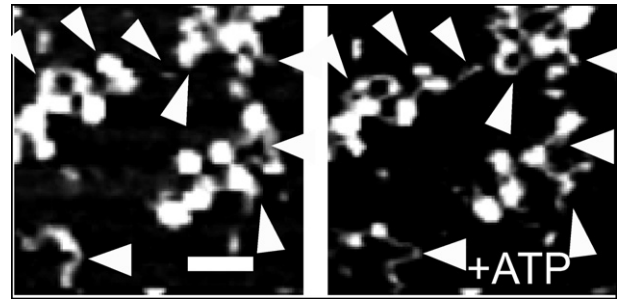


Fig. 5. Nucleosome remodeling produces significant changes in DNA and histone organization on nucleosomal arrays bound to GD-APTES. This pair of images shows the same set of MMTV nucleosomal arrays before (left image) or after (right image) in situ addition of ATP to activate human Swi-Snf. The remodeling complex was incubated then deposited with the chromatin arrays. Both DNA and histone changes are observed. Some of the more significant changes are marked by white arrows. The scale bar is 50 nm. (Reprinted from Biophys. J. 87 (2004) 1967, with permission.)

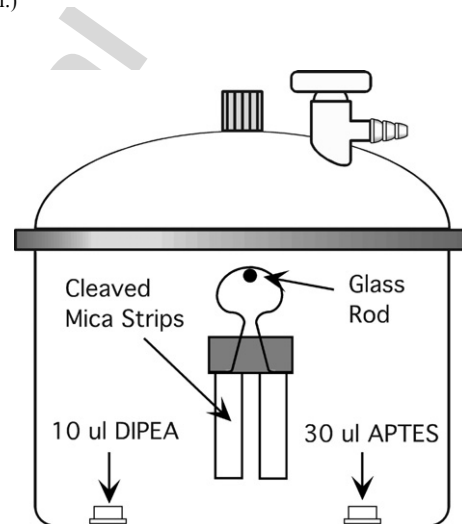


Fig. 6. Making AP-Mica. Mica strips are clamped in metal clips suspended on a glass rod of appropriate length (to nest snugly across the upper part of the dessicator). The reagents DIPEA and APTES (see text) are placed in small containers on the bottom of the dessicator (see text for details).

hung are suspended on a glass rod of a length that will fit snugly across the top of a glass dessicator (2.5 L capacity, no dessicant, with a valve in the cover) that is clean and

dry. Two small containers (cf. the tops of 1.5 ml microfuge tubes) are placed in the bottom of the dessicator and the dessicator is purged with ultra pure argon through the cover valve until air and moisture are removed (~ 2 min). Sheets of mica (Ashville-Schoonmaker, Newport News, Va) are stripped on one side with tape until smooth and immediately placed into the dessicator. The dessicator is purged again with argon for ~ 2 min. The dessicator cover is partially opened and 10 μ l of DIPEA, *N,N*-diisopropylethylamine (99%, distilled, Sigma–Aldrich) and 30 μ l of APTES (99%, Sigma–Aldrich, St. Louis, MO) are carefully pipetted into the two containers on the bottom of the dessicator. It is important to avoid spilling either component in the dessicator. The cover is replaced, the dessicator is purged with argon for ~ 3 min and then sealed off, leaving the mica exposed to APTES vapor for 1 h (although exposure times can be varied between 30 min and 2 h with no apparent effect on the process). After this exposure, the APTES container is carefully removed from inside the dessicator, the dessicator is sealed and purged with argon. The treated mica (AP-mica) can be stored in the sealed dessicator until needed (with the remaining DIPEA). AP-mica is best when used within 1 week.

APTES has been used both as received and after redistillation. Redistillation seems to be necessary only if the APTES is older than ~ 2 months or has been exposed to air for periods of hours. It is important that the dessicator and especially the seal be kept clean of spilled materials. Alcohol should be used to wipe out the dessicator before each new run.

2.3.1.2. Glutaraldehyde functionalization of AP-mica. Two hundred microliters of a 1 mM–1 nM glutaraldehyde (grade I, Sigma–Aldrich) solution in water is pipetted onto AP-mica immediately upon its removal from the storage dessicator described above and allowed to incubate for 12 min. Fresh vials of glutaraldehyde should be used each time GD-APTES is made. The mica is rinsed with ultra-pure water and chromatin samples are deposited on the mica immediately (see below).

2.3.2. Deposition and imaging

2.3.2.1. Deposition. A few microliters of a reconstituted chromatin sample (~ 40 nM in DNA) is diluted 10-fold in 1.0 mM EDTA, pH 7.5, to make a chromatin stock solution (to keep near the instrument, for example). For a deposition, the stock solution is diluted roughly 20-fold (final ~ 0.2 nM in DNA) in deposition buffer (10 mM NaCl/5 mM NaH_2PO_4 , pH 7.5) and pipetted carefully onto rinsed (as above) GD-APTES surface and the sample allowed to adsorb for 40 min. The surface is then rinsed with 1 ml of H_2O three times and the sample is mounted into the SPM liquid flow cell (Molecular Imaging, Phoenix, AZ) and imaged immediately.

For nucleosome remodeling studies, hSwi-Snf is added to the diluted chromatin and the two components

incubated in deposition buffer (10 mM NaCl/5 mM NaH_2PO_4 , pH 7.5) for 25–30 min then deposited as above.

2.3.2.2. Imaging. Virtually all of our imaging is carried out in solution, using a Macmode PicoSPM (Molecular Imaging) equipped with triangular, magnetically coated, Type-IV Si_3N_4 cantilevers (Molecular Imaging) with a spring constant of 0.1 N/m (“soft” cantilevers). Measurements are performed at ~ 9 kHz driving frequency and 5 nm of amplitude with 8% amplitude reduction. The scanning rate is 1.78 Hz. Note: if one is imaging chromatin or DNA in low ionic strength solutions (< 200 mM), hard cantilevers, i.e., those with spring constants > 2.0 N/m, can also be used, if desired. For imaging in solutions with $[\text{NaCl}] > 200$ mM, soft tips must be used.

We routinely image in a flow cell linked to the AFM (Fig. 7). This allows one to alter the sample environment, adding solutions of NaCl, peptides or ATP, for example, by gently pushing the “input” syringe and simultaneously pulling the “output” syringe at the same speed (Fig. 3). If this is done carefully, reagents can be added while still keeping the same individual molecules within the scanned image (cf. Figs. 4 and 5). For chromatin remodeling studies, the sample is scanned twice before activating the remodeling complex, to assess the frequency of scan-induced changes, then once after activating the complex (by ATP addition), to assess remodeling-induced changes (see [16,17]). In our studies, we typically waited 30 min after adding ATP before scanning again but images are acquired at a rate of ~ 5 min per image so rescanning can be done at those intervals.

2.4. Recognition imaging—identifying specific types of proteins in complex samples

2.4.1. Antibody preparation and tip modification

In order to identify specific proteins in a compositionally complex sample, an antibody against the protein of interest can be attached to the AFM tip. During the scanning process, the usual AFM topographic image is generated simultaneously and in exact spatial registration with a

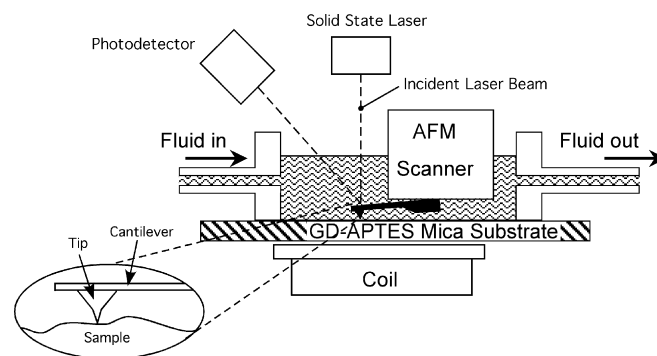


Fig. 7. Diagram of an AFM/flow-cell. The flow-cell linked to the AFM, which we used in our studies, is schematically illustrated, with the tip-sample interface enlarged below left.

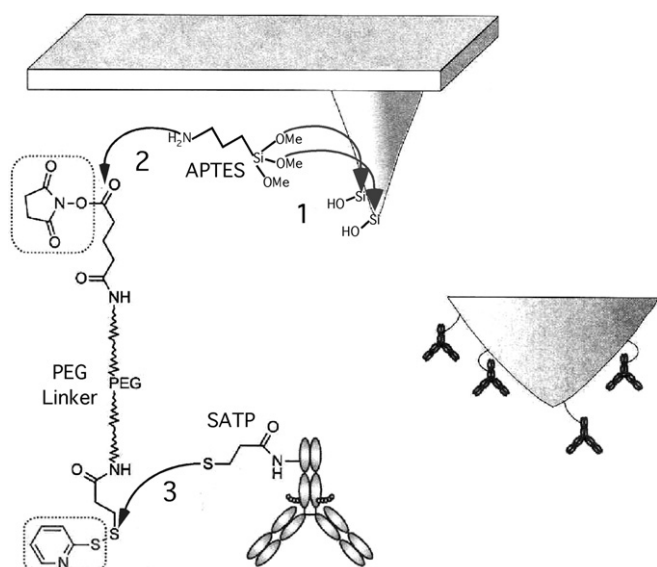


Fig. 8. Attaching antibodies to an AFM Tip. The AFM tip is the conical downward projection at the top of the illustration. To attach the antibody (inverted Y at lower center), the tip is first derivatized with APTES (step 1), then a bifunctional PEG crosslinker is used to link the modified tip to SATP-modified antibody (steps 2 and 3). See text for details. The drawing to the lower right illustrates the fact that multiple antibody molecules can become attached to a single tip. The number actually attached to a given tip is unknown.

“recognition image”, which identifies the locations of antibody–antigen binding events and thus the locations of the protein of interest in the field [18]. The two images can be electronically superimposed to obtain an accurate map of specific protein locations relative to the topography (Section 2.4.3). The protocol involves attaching an antibody modified by SATP addition (see below) to an APTES-modified AFM tip via a bifunctional PEG linker ([42]; Fig. 8).

2.4.1.1. Modifying the antibody with SATP. A PD-10 desalting column (8.3 ml bed volume) is washed with 30 ml buffer A (100 mM NaCl, 50 mM NaH_2PO_4 , 1 mM EDTA, pH 7.5). Then 200 μl of antibody stock solution (1 mg/ml) are diluted to a total volume of 500 μl with buffer A and put over the PD-10 column. The column is batch-washed 9 times with 500 μl buffer A per wash, collecting the eluate after each addition. The A_{280} is read to determine in which tube(s) the protein resides. Fractions containing significant [protein], usually 7 and 8 in our hands, are pooled.

A 10-fold molar excess of SATP (*N*-succinimidyl 3-(acetylthio)propionate) in DMSO (15 mM stock solution) is added to the washed antibody and the solution is incubated for 30–60 min under argon, stirring with a magnetic bar. Two PD-10 columns are washed with 30 ml buffer A, then 500 μl of SATP-modified antibody solution are put on each PD-10 column and the columns are washed as above (9 times with 500 μl buffer A per wash) and the fraction(s) with significant [protein] collected. The SATP-antibody is stored in 100 μl aliquots at -70°C .

2.4.1.2. Modifying AFM tips with APTES. Type IV (soft) tips (see above) are cleaned in a UV-cleaner (Boekel Industries, Feasterville-Treose, Pa) for 15 min to get rid of any organic contamination on the tips. The tips are placed in a Petri dish at the bottom of a desiccator and modified with APTES just as in preparing AP-mica (described above). After the treatment process, the APTES is removed and the treated tips (AP-tip) are stored in the sealed desiccator until needed.

2.4.1.3. Attaching the crosslinker. One milligram of bifunctional PEG crosslinker [42] and 5 μl of triethylamine are mixed in 1 ml CHCl_3 . AP-modified tips are placed into the solution, and treated for 2–3 h. The tips are washed with CHCl_3 and dried with Argon. The succinimide group attaches to the amino group on the AP-tip (Fig. 8).

2.4.1.4. Linking SATP-modified antibody to AFM tips. Next, the PDP group (2-(pyridyldithio)propionyl group) on the linker is attached to the SATP on the modified antibody (Fig. 8). The PEG linker-bound tips (see above) are incubated in 50 μl SATP-antibody, 25 μl of NH_2OH -reagent (500 mM $\text{NH}_2\text{OH}\cdot\text{HCl}$, 25 mM EDTA, pH 7.5) and 50 μl buffer A (100 mM NaCl, 50 mM NaH_2PO_4 , 1 mM EDTA, pH 7.5) for 1 h; then the tips are washed once with buffer A and once with PBS buffer (150 mM NaCl, 5 mM Na_2HPO_4 , pH 7.5). The antibody-tethered tips can be stored in PBS buffer at 4°C and are good for 1 month.

2.4.2. Imaging with modified tips

For recognition imaging, samples are deposited on GD-APTES, just as for topographic imaging (Section 2.3). Scanning with antibody-modified tips should always be carried out with Type IV soft (0.1 N/m spring constant) tips. Imaging amplitude is set between 2.0 and 2.5 V. Our imaging was done on a PicoPlus AFM with a Picotrec recognition imaging attachment (Molecular Imaging, Phoenix AZ) with 6–8 nm amplitude oscillation at 9 kHz, imaging at 70% set point and scan speed at <1 Hz. For testing specificity of the recognition reaction, 50 μl of a 30 $\mu\text{g}/\text{ml}$ solution of a peptide antigenic to the antibody (usually the one that was used to elicit the antibody response) is flowed into the cell and the sample rescanned. If recognition is specific, the presence of the peptide will block the recognition to a very high degree.

Note: soft cantilevers with low frequency (6–9 kHz) should be used for recognition imaging. Stiffer cantilevers (higher frequency) do not produce good recognition images. Also, scan speeds >1 Hz should be avoided as they increase the leakage of topography into the recognition image.

It is very important to check the specificity of antibodies used for recognition imaging studies. We have noted a number of non-specific reactions, i.e., antibodies that recognize non-antigens, sometimes quite strongly, in both AFM recognition imaging (including force curves) and standard ELISA assays (cf. [19,43]). Recently, we used phage display technology to produce a more specific anti-

body [19]. Unfortunately, antibody specificity remains a major limitation of the AFM recognition imaging technique. The use of nucleic acid aptamers may provide a solution to this problem [32].

2.4.3. Analyzing recognition images

The recognition signal is generally quite noisy but recognition data can be processed in the following way: first, the recognition image is filtered with a median filter (Adobe Photoshop) set to average signal response over approximately 6 nm, the length of the tether we use. In a 1 μm square image containing a 512 pixels a line (i.e., approximately 2 nm/pixel), the median filtering is set to a value of three pixels. The background noise level is established by measuring the distribution of pixel heights close to but not on a recognition spot. The distribution of pixel heights across the nearby recognition spot is then measured by taking perpendicular line traces across the spot and the two distributions are compared in order to establish a cutoff height at which the recognition signal exceeds the noise level. Recognition events thus identified are marked with a colored spot. When each event has been marked, the layer containing the colored spots is overlaid (electronically) on the topographic image; this will allow the two types of images to be placed in exact registration.

3. Concluding remarks

The AFM techniques described above provide a number of unique opportunities for chromatin studies, as well as studies of many other biological systems. AFM is a single molecule technique that is well suited to the study of moderately large biological complexes; it thus provides information for a size range of molecules for which traditional biochemical approaches are limited. The ability to image repetitively the same set of molecules in aqueous solution allows *in vitro* studies of processes occurring on individual single molecules under physiologically relevant imaging conditions, i.e., avoiding artifacts associated with dehydration. For example, the ability to reimage the same single molecules after changing the sample environment by adding NaCl (nucleosome stability), effector molecules like ATP (nucleosome remodeling) or reaction modulators (peptide blocking of recognition imaging) provides a novel opportunity to monitor reactions of individual molecules and, by using recognition imaging, to be able to follow changes involving specific factors in multicomponent systems. These approaches should be broadly applicable and capable of providing insights into a broad range of biological questions.

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