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Atomic Force Microscopy of Chromatin

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1. Introduction

Atomic force microscopy or scanning tunneling microscopy (AFM/STM) is a powerful single molecule tool for the visualization of biological materials at sub-nanometer resolution. AFM is versatile because it can directly measure physical properties due to its sensitivity at picoNewton force scales, thus enabling dissection of molecular forces. STM/AFM has been considered revolutionary since its discovery 32 years ago, and a mere 7 years after its invention, Binnig and Rohrer were awarded the Nobel Prize in Physics, along with Ruska, inventor of the first electron microscope. In subsequent years, AFM has evolved fast, with the range of applications for STM/AFM expanding to encompass all physical sciences. Despite its popularity in physics, engineering, material and chemistry sciences, it has remained a less prominent tool for biologists, who are much more familiar with techniques like electron microscopy (EM), X-ray crystallography or nuclear magnetic resonance (NMR). The relatively low cost of the microscope, the ease of sample preparation, and the lack of any requirement for staining, freezing, excessive denaturation or preservation, should make AFM an ideal tool for laboratories interested in imaging and manipulating biological samples down to macromolecules at the nanoscale. In this chapter, we will discuss how AFM can be an effective high-resolution technique for structural and molecular interaction studies in biology.

2. Biophysical tools to characterize biological structures

Biophysical techniques like AFM, EM, X-ray crystallography and NMR are commonly used to determine ultra-structure of macromolecular complexes. The choice is often related to the availability of the tools as well as the skills required to operate them in a given laboratory. All of these techniques have distinct advantages and disadvantages for imaging of biological specimens, which should be considered before making a choice (Table 1).

2.1 Electron microscopy

EM is the preeminent and most mature high-resolution microscopy (Ruska, 1980) which uses a focused beam of electrons to generate a magnified and detailed image. Since the wavelength of electrons is orders of magnitude smaller than that of visible light, this method

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Technique	Electron microscopy	X-ray cristallography	NMR	AFM
Resolution	For SEM: 1 nm For TEM: 5 Å	0.2-3.5 Å	2-3.5 Å	1-5 nm (lateral) 0.1 nm (vertical)
Advantage	Magnification (max 50 000 000X for TEM)	Atomic resolution	Measurements in solution	Measurements in solution
Disadvantage	Sample preparation Expensive equipment	High quantity of purified crystal Time of preparation	Purification of molecule Time of acquisition	Immobilized sample
Observation	Fixed sample under vacuum	Crystal	Sample in solution	Sample in ambient air or in solution

Table 1. Comparison of biophysical techniques commonly used in biology.

is not limited by the diffraction limit of visible light (200 nm). Therefore, its resolution approaches Angstroms. However, the use of focused beam of electrons at high voltage (typically 40-100 kV) is destructive, and requires elaborate sample preparation and mounting techniques. To obtain a homogeneous and straight beam of electrons, EM is always performed under high vacuum, which requires elimination of liquids from the sample to be visualized. Nucleoprotein complexes have very low electron density (and thus are “invisible” by standard EM). Thus, visualization of such material requires fixation using glutaraldehyde, dehydration by drying or ethanol substitution, followed by shadowing or staining with heavy atom-containing reagents (e.g. gold, palladium, platinum, uranyl acetate, *etc.*). Heavy metals diffract electrons strongly, providing meaningful contrast so that sub-molecular details in nucleoprotein complexes can be revealed.

Two types of EM were developed, whose use depends on the sample and the purpose of imaging: transmission electron microscope (TEM) and scanning electron microscope (SEM). TEM is used for thin samples to generate two-dimensional, electron density maps of transmitted electrons. A collection of such images at a series of sample rotations can be used for 3-dimensional (3D) reconstruction of internal structures of a cell or macromolecular complexes (electron tomography). In contrast, images generated by SEM result from the capture of diffracted electrons, giving 3D details of the surface of the sample, typically used for cells, organelles, and other large structures.

Advances such as cryo-EM permit the visualization of samples embedded in a liquid lattice, requiring imaging at sub-zero temperatures (Lepault et al, 1983; Taylor & Glaeser, 1974). This technique lacks the high contrast offered by more traditional EM techniques involving fixation, shadowing and staining, and consequently requires extensive post-processing and image reconstruction from hundreds of identically oriented particles. However, cryo-EM does afford a native view of particles, and is a powerful tool for analysis of materials at very high resolution.

2.2 X-ray crystallography

X-ray crystallography is the highest resolution technique currently available for the determination of ultra-structure (Chiu, 1993). This method consists of illuminating a crystal composed of highly ordered particles created from *in vitro* ultra-pure components which must be all aligned in precisely the same orientation within a crystal lattice. A beam of X-rays is focused on the crystal to determine the 3D organization of atoms by subsequent analysis and molecular modeling of X-ray diffracted patterns. The major disadvantage of this method is the sample needs to be crystallized, requiring milligram quantities of complexes folded correctly at very high concentrations in non-physiological buffers. Since X-rays are destructive, each crystal can only be imaged once. While this technique is still the gold standard for obtaining precise location for every atom and molecule in a complex at Angstrom resolution, the complexes exist in a static conformation. Moreover, it is still very challenging to obtain useful crystals for classes of molecules like membrane proteins and intrinsically disordered proteins.

2.3 Nuclear magnetic resonance

NMR, like X-ray crystallography is used to derive 3D structure and dynamics of molecules (Chiu, 1993). Highly purified complexes in solution are exposed to a multidimensional magnetic field, exciting specific magnetic resonance of active nuclei in their environment. The resonance signatures, called chemical shifts, give the distances between atoms which enable the 3D reconstruction of the molecule. Time required to acquire and to analyze the signature is a major disadvantage of this technique. The resolution of the obtained structure depends on the size of the macromolecule with a limit to about 100 kDa. NMR relies on previously obtained structural models of the major parts of the complex, upon which the resonance shift can be mapped. One advantage of NMR is its ability to characterize intrinsically disordered proteins, which is not possible with X-ray crystallography.

2.4 Atomic force microscopy

In AFM, images are topographic maps of samples attached to a solid substrate (Binnig et al, 1986). They are obtained by scanning the surface of the samples with a nanometer-size tip etched at the end of a flexible cantilever (figure 1). Two unique capabilities distinguish the technology: (i) the ability to both image and perform co-localized force spectroscopy for the determination of a range of sample properties (mechanical, electrical, molecular recognition) and (ii) the ability to probe and image samples in ambient air conditions or under physiological fluids. These are accomplished by the tip-deflection of the cantilever being continuously monitored by a focused laser beam with accuracy of about 1-2 Å. The stiffness of the cantilever is estimated (the softest commercially available cantilevers have nominal stiffness around 0.005N/m), the force sensitivity is in a range of picoNewtons, which allows force interactions at single molecule level. A number of imaging modalities have been developed to accommodate the nature of the sample and the purpose of the investigation. The simplest one, called contact mode, has the cantilever contacting and scanning the sample with a preset small deflection while a feedback loop maintains the deflection constant (Figure 1). The other most common modality has the cantilever oscillating with preset amplitude near its resonance frequency and is named tapping mode (or non-contact mode or amplitude modulation feedback) (Figure 1). When the cantilever approaches the

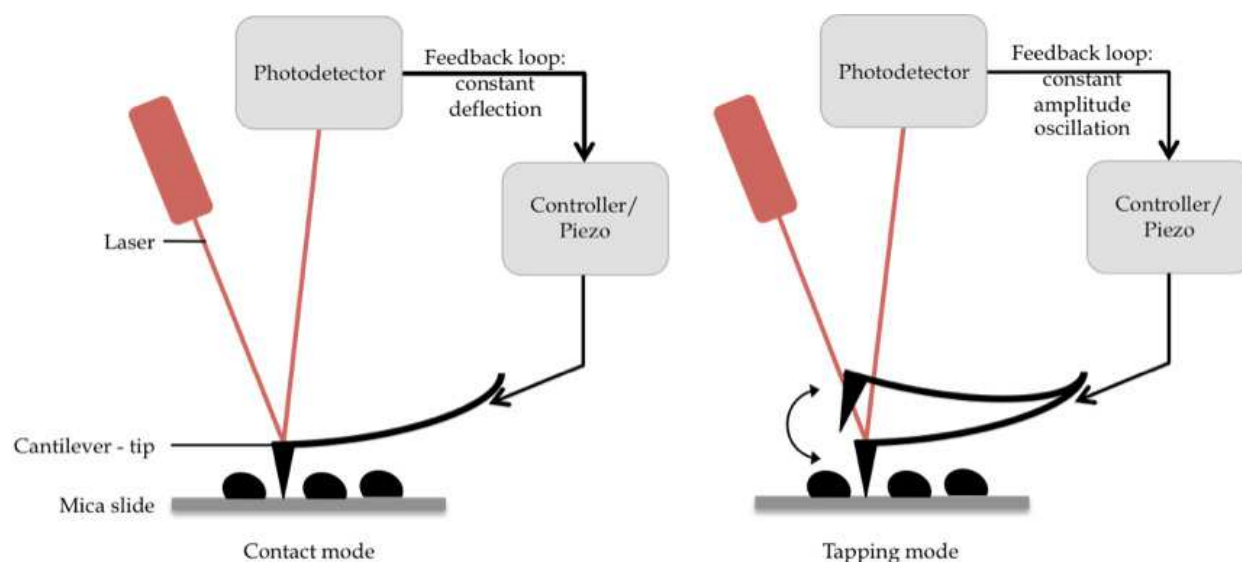


Fig. 1. Principle of atomic force microscopy.

In contact mode (left), the tip contacts the sample surface with a small cantilever deflection and the feedback loop maintains that deflection constant; this is equivalent to a constant force. In tapping mode (right), the input to the z-piezo-actuator is oscillatory and the photodetector output signal is analyzed into amplitude and phase of the cantilever oscillation. The feedback loop maintains the oscillation amplitude constant. The phase shift between input and output depends on sample properties.

sample, probe-sample interaction forces lead to a drop in amplitude and a small shift in resonance frequency. The feedback maintains the reduced amplitude of oscillation constant during the scan. To maintain the feedback parameter constant, the piezoelectric actuator that controls the motion of the cantilever has to follow the sample topography and hence generates the topographic image of the sample.

A great advantage of this method is that minimal sample preparation involved. AFM is normally performed under gentle physico-chemical conditions (physiological hydration, temperature, salt concentration) in air or in buffer, thus allowing biological samples to retain their native form during the experiment. More challenging, sometimes, is the attachment of the certain types of sample to the substrate; attachment has to be firm enough to permit stable imaging. Both substrate properties and sample solution composition are important in this respect.

3. Choice of AFM instrument modality and conditions for biological sample studies

The mode in which the AFM will be used depends entirely on the sample and on the objectives of the study. Biological samples, from tissues, and cells, and down to single molecules, present very different properties and require different handling.

The first issue to decide is whether imaging will be performed in ambient air or under fluid. For hydrated tissues (e.g. cartilage), biological membranes (e.g. supported lipid bilayers),

and live cells, the choice of fluid environment is obvious. Because experimenters are interested in the functionality of biological samples under physiological conditions, fluid environment is also a better choice for molecular recognition studies (i.e., using tips coated with an antibody that recognizes its corresponding antigen on the sample) and for molecular pulling or force curve spectroscopy (for the study of the folding properties of proteins). One disadvantage of using fluid is the higher noise level compared to ambient air. So, if samples do not absolutely require the use of buffers, such as stably folded proteins, nucleic acids (DNA - Deoxyribonucleic acid or RNA - Ribonucleic acid), or nucleoprotein complexes, acquisition of data can be performed in air. It is generally well accepted that even after drying a significant level of hydration contained within molecules maintains samples in their native structure (Cantor & Schimmel, 1980; Garcia de la Torre, 2001). For both types of imaging, air or fluid, the most commonly used surface for the attachment of biological samples is freshly cleaved mica which is atomically flat, inert, and presents the most appropriate properties for AFM imaging.

A second choice, which is mainly dependent on the physico-chemical properties of the sample, is whether AFM will be performed in "contact" or "non-contact/tapping" mode. As briefly discussed above, these are the two basic modes for imaging. Imaging under fluid or air can be performed in either mode and the choice will depend on the firmness of attachment to the mica plate and on the stiffness of the sample. For example, supported lipid bilayers are usually quite robust and can equally well be imaged in contact and in tapping modes. On the other hand, live cells are usually rather soft and labile, requiring gentler forces such as those applied in tapping mode. For imaging in air, contact mode is most challenging due to the high capillary forces between the adsorbed water layers on tip and hydrated sample. Given the usual sensitivity of soft biological samples, imaging in air is always performed in tapping mode.

For imaging nucleic acids or chromatin in air, it is best to use rather stiff cantilevers in tapping mode. Commonly available tips made of single crystal silicon with resonance frequency in the range of 300 kHz, stiffness in the tens of N/m and tip radius of around 5-7 nm typically work well. For the highest resolution, the use of super-sharp cantilevers with a tip radius in the 1-2 nm range will enhance the acquisition. However, ambient humidity should be maintained at 60% rh to reduce tip-sample adhesion due to excessive surface hydration and the probability of tip contamination. In all cases, gentle tapping is recommended to avoid sample damage and tip contamination.

For these same types of samples in fluid, tapping mode is performed with much softer cantilevers. Softer cantilevers are also necessary for molecular recognition studies since the binding forces to be measured are in the range of picoNewtons. Such levels of force sensitivity can only be provided by very soft cantilevers. For example, the detection of 1 nm deflection resulting from 10 picoNewton force can easily be detected by cantilever stiffness of 0.01 N/m. It should be noted that imaging resolution under fluid will be lower than in air, because of higher noise, and because softer silicon-nitride cantilevers are less sharp than ones made of silicon. Stable tapping with the softest cantilever in the market (stiffness 0.005 N/m) may be challenging and often, a slightly stiffer cantilever (0.06-0.12 N/m) may give better results. Despite the two orders of magnitude stiffness differences among the cantilevers used under fluid, their resonance frequencies are all in the range of 10 kHz, primarily determined by the fluid mass displaced by the oscillation of the cantilever.

The quality of imaging is determined by the parameters described above, and by scanning parameters. A critical parameter is the free tapping amplitude, namely the amplitude of the cantilever oscillation when the tip is far from the sample and free of interactions. For samples like DNA and chromatin, this amplitude should be kept at $\sim 5\text{--}30\text{ nm}$, with lower amplitudes used for more fragile samples maintained under fairly low humidity. Even more critical is the amplitude set-point, which corresponds to the amplitude reduced by tip-sample interaction forces when tip reaches the sample. The set-point determines the tip-sample forces during scanning, and in tapping mode, a feedback loop attempts to keep this set-point constant. The speed of the scan should be kept low enough ($\sim 1\text{--}5\text{ }\mu\text{m/sec}$), so that the feedback loop is given adequate time to work efficiently. The feedback gain parameters characterize the speed at which the mathematical control algorithms are attempting to follow the topography of the sample. These parameters are critical, but instrument and modality dependent. One can try increasing the gains as long as the feedback stays stable. The amplitude set-point also determines the force regime and magnitude of the tip-sample interaction forces. During sample engagement, as the cantilever approaches the sample, the interaction force first goes from zero through the long-range attractive van der Waals region, and if pushed closer to the sample, enters the repulsive region, wherein there is actual contact during the downward leg of the oscillation (Figure 2). For a given tapping frequency and set-point, the operating regime is also determined by the elasticity of the sample, but for single molecular complexes such as chromatin, it may be assumed that the sample is rather stiff, similar to the mica plate. Under these conditions, it is desirable to stay in the attractive region which applies lower forces to the sample and produces reliably accurate and high quality images. This is generally accomplished by choosing a set-point slightly lower than the free oscillation amplitude (90–95%). Tip-sample forces shift the cantilever resonance to lower frequencies causing a change of the free amplitude. Staying within the attractive regime is ensured by setting the oscillation frequency slightly lower than resonance of the tip. Upon engagement, the amplitude of oscillation decreases, reducing the chances of entering the repulsive regime.

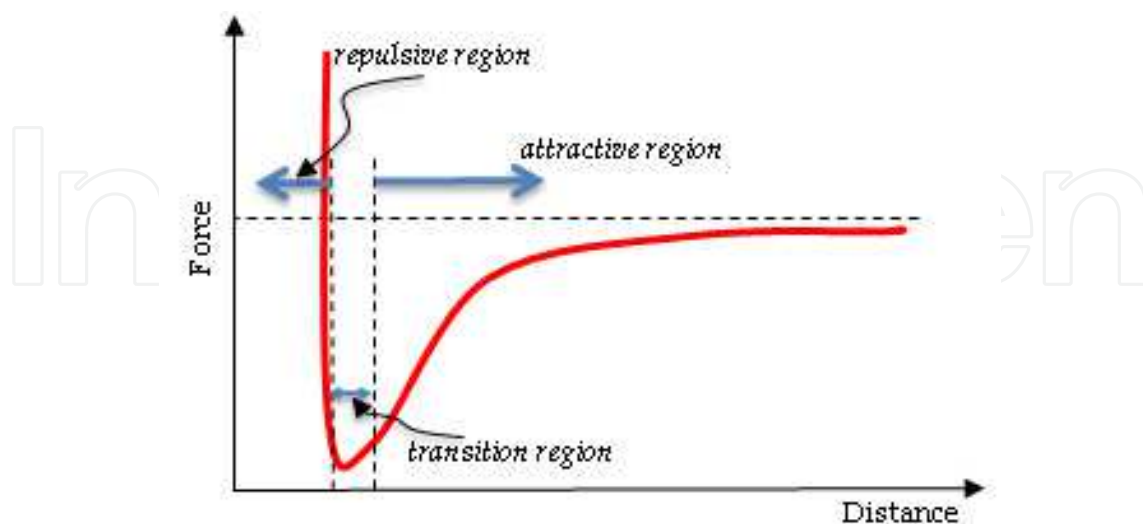


Fig. 2. Scanning force regimes in tapping mode.

Depending on the amplitude of free oscillation, the amplitude setpoint and the oscillation frequency relative to the resonance of the cantilever, the tip-sample force can be attractive or repulsive.

4. Sample preparation

The eukaryotic genome is a complex macromolecule, presenting a range of folding from 2 nm-wide DNA to 1.4 μm -wide chromosome. The nucleosome is the fundamental repeating structural unit of the chromosome and consists of 147 base pairs of DNA wrapped around an octamer of two copies of each histone proteins H2A, H2B, H3 and H4. Linker histone H1 stabilizes this structure through its binding to DNA. The distribution of nucleosomes along the DNA molecule leads to the formation of an 11 nm fiber, called “beads on a string”, which can be folded into higher-order structures ranging from 30 nm to 100 nm fibers in the interphase nucleus, to quasi-crystalline chromosome during mitosis (Li & Reinberg, 2011). Chromatin structure is also dynamic, since it is regulated by reversible modifications such as DNA methylation, post-translational modifications of histones, and interactions with chromatin-binding factors (Kouzarides, 2007). Mechanisms involved in chromatin folding and biophysical characteristics of each level of genome organization have been extensively studied using AFM technology.

In contrast to relatively rigid samples encountered in solid-state or polymer physics, biological samples must be maintained in an appropriate environment that will protect them from degradation. For example, proteins are sensitive to proteases, and DNA or RNA will be cleaved by nucleases naturally present on human skin. In addition, the best environments for biological samples could be inappropriate for standard AFM manipulation and special modifications, and trade-offs have to be designed. Nevertheless, AFM still allows a higher flexibility than other techniques. Indeed, samples can be analyzed in air or in liquid, and in real time, for instance, to follow the modification of chromatin condensation, or in response to the addition of chromatin-binding protein. Below we will discuss appropriate use of sample preparation and buffers that best preserve structural information.

4.1 Mica slides preparation

Mica is a naturally occurring sheet silicate mineral with a hexagonal pseudo-crystal structure arranged in 1 nm thick layers. After cleaving using adhesive tape or a sharp scalpel, mica sheet presents an atomically flat surface, which is highly negatively charged, hydrophilic, chemically inert, and very stiff in the transverse direction. Thus, fresh cleaved mica constitutes an ideal surface for the attachment and topographic imaging of individual macromolecules. For AFM, grade 1 muscovite mica is preferred for its high purity and ease of cleaving.

The negatively charged mica offers strongly adsorbing support for positively charged samples but not negatively charged molecules, such as DNA. In addition, ionic conditions of the sample solution may alter attachment. In the presence of significant quantities of monovalent ions (e.g. Na^+ or K^+) charge screening prevents DNA from attaching to bare mica (Pastre et al, 2006; Pastre et al, 2003). Divalent ions (e.g. Mg^{2+}) presumably bridge negative charges on the sample and the mica, and are often and reliably used. However, in many cases the use of divalent is not recommended for functional reasons and chemical modification of the mica surface is necessary. The most common modification involves the use of silanes to produce a positively charged and hydrophobic mica surface with exposed amine groups. Several protocols for mica silanization, using generally 1-(3-aminopropyl)silatrane (APS) or 3-

aminopropyltriethoxysilane (APTES) have been developed (Figure 3) (Lyubchenko et al, 1992; Shlyakhtenko et al, 2003). The simplest method uses direct deposition of APS solution (300x dilution of 50 mM stock in ultra-pure water) onto the mica and incubation for a brief period (~30 minutes) before rinsing and drying the modified surface. An extension of these methods that also works reliably entails the treatment of silanized mica with 0.1% fresh-prepared glutaraldehyde for 5 min at room temperature, followed by careful rinsing with ultra-pure water and drying (Wang et al, 2002).

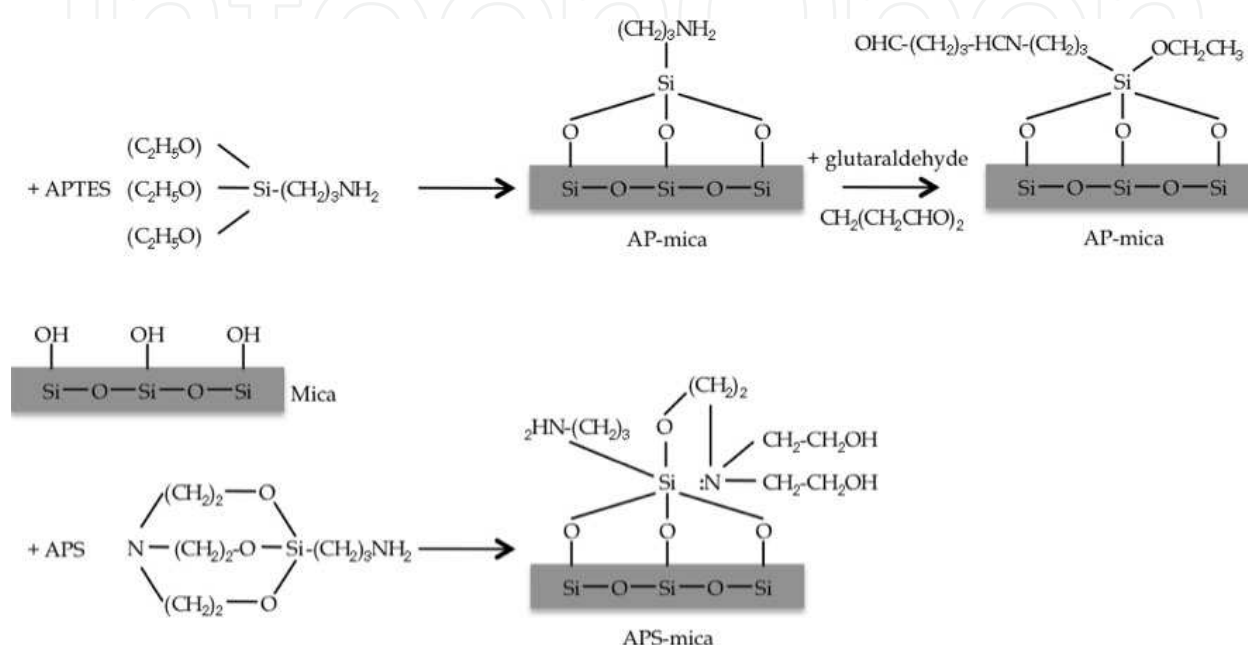


Fig. 3. Schema of modified mica surface by APS or APTES followed by glutaraldehyde treatment.

4.2 DNA preparation

DNA is a long polymer, whose the sub-unit is a nucleotide composed of a base, a sugar and a phosphate group. Two DNA strands are usually entwined in a double helix stabilized by hydrogen bonds between bases on the different strands. DNA can present different conformation: relaxed or coiled, or complex forms during cellular process such as replication or recombination, which may be visualized on AFM (Barattin & Voyer, 2011; Hamon et al, 2007; Hansma & Laney, 1996; Hansma et al, 1995; Lyubchenko et al, 1992; Shlyakhtenko et al, 2003).

DNA analysis takes place in a nuclease-free environment. All buffers used for the purification and the loading of DNA on the mica slide, must be sterile. In a typical protocol, 20-50 picograms of DNA is diluted in 5 mM HEPES buffer pH 7.5, 2 mM $MgCl_2$, and deposited on the fresh cleaved mica for 5-10 minutes. APS-mica itself is a very good substrate for DNA under physiological conditions, in the absence of divalent ions (Shlyakhtenko et al, 2003). Slides are gently rinsed three times with ultra-pure water to remove salts before drying in a stream of inert argon for analysis in air, or with the appropriate buffer to remove unattached objects for analysis in fluid.

4.3 *In vitro* and *in vivo* chromatin preparation

AFM is a useful tool for the characterization of chromatin structure by the extraction of information like the height, volume, position and movement of nucleosomes, the determination of the DNA wrapping handedness around the core histone complex, the association of chromatin-associated factors and changes in chromatin folding. Preparation of chromatin has to be performed in appropriate conditions of salts and temperature to best conserve the native state. Both chromatin extracted from nuclei (referred to as “*in vivo*” chromatin) or chromatin reconstituted *in vitro* from purified recombinant components can be analyzed equally well by AFM, allowing direct comparison. Here we described protocols used in our laboratory to examine chromatin structure by AFM.

4.3.1 Native chromatin extraction from various tissues/cell lines

The following protocol of native chromatin extraction has been adapted successfully in our lab for a range of samples including human cancer cell lines (such as HeLa, DLD1, SW480), non-carcinoma cells (HEK293), human tumors, mouse tissue, as well as *Drosophila* S2 and Kc cells (figure 4) (Dalal et al, 2005; Dalal et al, 2007; Dimitriadis et al, 2010; Wang et al, 2008).

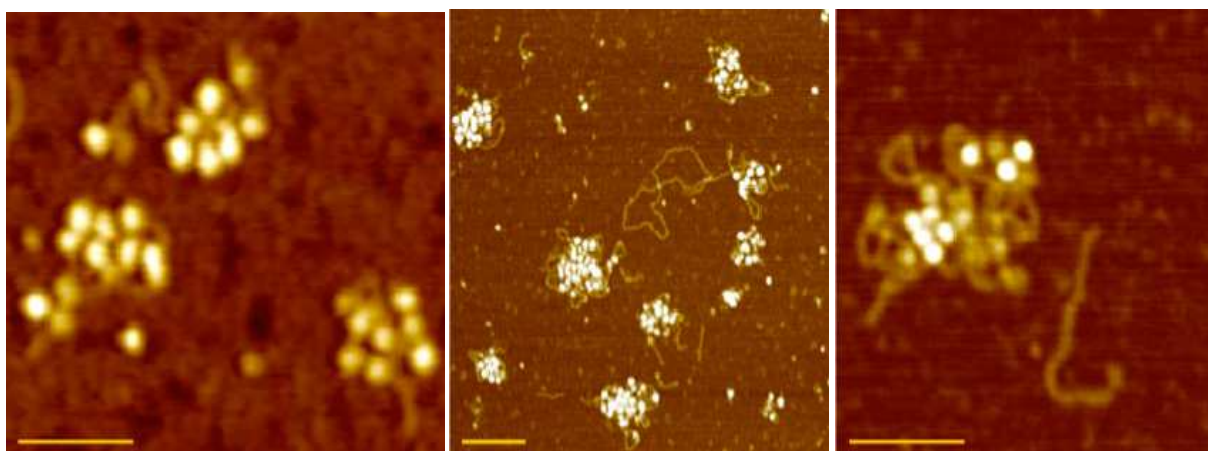


Fig. 4. Examples of topography images of native chromatin extracted from HeLa cells (unpublished data; scale bars 100 nm, Nanoscope, non-contact mode in air).

1. Tissues are disrupted and homogenized by Dounce (no. A) strokes in standard chromatin buffer (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM Tris, pH7.4). For cell lines, cells are harvested from flasks by trypsinization.
2. Released cells either from tissue or flasks are washed three times with 1X PBS, 0.1% Tween.
3. Nuclei are extracted with TM2 Buffer (20 mM Tris pH8.0, 2 mM MgCl₂, 0.5 mM PMSF) supplemented with 0.5% Nonidet P40 (Sigma Cat#74385), and rinsed once with TM2 buffer.
4. Chromatin is digested by Micrococcal nuclease (Sigma Cat#N3755) at 0.2 units/mL final in the presence of a final concentration of 2 mM CaCl₂ at 37°C, and the reaction is stopped by addition of 10 mM EGTA. Incubation time depends on the size of the array expected, typically ranging from 30 seconds to obtain oligo-nucleosomal arrays to 10 minutes to obtain mononucleosomes.

5. After centrifugation 10 min 800 rpm at 4°C, the nuclear pellet is resuspended in low salt buffer (0.5X PBS, 5 mM EGTA, 0.5 mM PMSF) or high-salt buffer (0.35 M NaCl-supplemented 1X PBS, 5 mM EGTA, 0.5 mM PMSF) and incubated over night at 4°C on an end-over-end rotator.
6. Soluble extracted chromatin fibers are separated from remnant nuclei by spinning down the pellet by centrifugation 10 min 8000 rpm at 4°C.
7. Chromatin is deposited on APS-mica pre-treated with Mg^{2+} for 10-30 minutes. The mica is rinsed gently before drying in a gentle stream of inert argon. For fluid imaging, the mica is rinsed twice with 1X PBS to remove background unattached materials, and a thin layer of 1X PBS is added to the sealed imaging chamber prior to imaging. Care must be taken that the liquid does not dry while the AFM tip is engaged.

4.3.2 *In vitro* chromatin reconstitution by salt dialysis

Purified histones can be obtained by the expression of recombinant histones in *Escherichia coli*, followed by denaturation in urea and re-folding (Luger et al, 1997; Workman et al, 1991). An alternative popular method is the purification of native histones by hydroxyapatite extraction from chicken erythrocyte or HeLa nuclei (Bloom & Anderson, 1978; Stein, 1989). For chromatin reconstitution, DNA, such as negatively supercoiled plasmids or DNA fragments, are diluted at a final concentration of 200 mg/mL in 2 M NaCl, 10 mM Tris pH8.0, 1 mM EDTA and then mixed with purified histones at ratio ranging from 0.7-0.9 mg of histones/mg of pure DNA in 2 M NaCl, 10 mM Tris pH8.0, 1 mM EDTA, 0.5 mM PMSF. After 30 min incubation on ice, the sample is dialyzed 2-4 hrs at 4°C successively against 1/0.8/0.6/0.15 M NaCl diluted in 10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM PMSF. Supercoiling and nuclease assays are recommended to ensure that correct chromatin deposition has occurred. As for native chromatin, the sample is directly deposited on APS-mica pre-treated with Mg^{2+} for 10 min and mica slide is rinsed gently either before drying in a stream of inert argon or incubation in the appropriated buffer for liquid mode imaging.

4.4 Chromosome preparation

AFM has also been used for observation and structural analysis of bigger macromolecules like intact metaphase chromosomes (figure 5). The following protocol was developed for different cell lines such as HeLa cells (Hoshi et al, 2004; Ushiki & Hoshi, 2008).

Cells are treated with 0.1 mg/mL final concentration of colcemid for 12 hrs at 37°C. Cells are washed twice with 1X PBS before trypsinization. Trypsin activity is stopped by addition of complete medium when cells begin to lift off. Medium, PBS and trypsinized cells are collected in a conical tube and submitted to centrifugation 5 min 1000 rpm. Pellet of cells is resuspended drop-by-drop in 2 mL pre-warmed hypotonic buffer (75 mM KCl) and then additional 40 mL is added. After incubation at 37°C for 20 min, 5 drops of freshly prepared fixation solution (3:1 methyl alcohol/glacial acetic acid or Carnoy's solution) are added and chromosomes are harvest by centrifugation 5 min 1200 rpm. The chromosomes are washed three times with fixation solution and finally conserved at 4°C in this solution. Chromosomes are spread on fresh mica slide and observed after evaporation of the fixation solution. This protocol may be used for fluid analysis as described by Ushiki *et al.* (Ushiki & Hoshi, 2008).



Fig. 5. Example of topography images of metaphase chromosomes extracted from HeLa cells (unpublished data; AFM: Agilent 5500, tapping mode in air).

5. Usual applications of AFM in biology

The major application of AFM in biology is the visualization of topography and the characterization of material properties via phase or other measurements. From the analysis of topography, information such as the height and volume of molecular complexes may be measured, allowing determination of stoichiometries and of 3D arrangement of oligomeric complexes. Phase imaging, namely the map of the phase difference between the piezo excitation and the cantilever tip response, informs of sample surface properties, like elasticity, charge and hydrophobicity. Although not quantitative, phase offers a possible measure for distinguishing regions on a sample and for molecular recognition when they cannot be differentiated by the topography (Hansma et al, 1997; Nagao & Dvorak, 1999; Zhao et al, 2010).

However a more robust approach for molecular identification can be achieved by probing antigen-antibody interactions with tips chemically functionalized to carry specific antibodies or other molecules that have specific binding partners on the sample. Consequently, AFM is a powerful technique for the structural study of complex macromolecular structures precisely because it can simultaneously provide visual, physical, electrostatic and chemical insights. Another important feature is the acquisition of real-time and kinetics sample information with the use of high-speed AFM (HS-AFM), which is able to acquire numerous images in a short period of time. Thus, DNA, chromatin and chromosome have been extensively studied using this technique, examples of which are provided below.

5.1 Topography analysis

5.1.1 Alternative DNA structure imaging

DNA is a flexible macromolecule which could present different conformations such as canonical B or alternative A and Z conformations, but also cruciform or triple helices, both observed during, and involved in, DNA replication, transcription and repair (Lebrun &

Lavery, 1997; Mills et al, 2002). AFM has been used to study the structure and the function of these conformations. Thus Shlyakhtenko *et al.* described continuous transition between two forms of cruciforms (Shlyakhtenko et al, 2000; Shlyakhtenko et al, 1998). The first cruciform presents a square planar structure with a 180° angle between the hairpin arms, whereas the second form displays an “X” conformation with bent main DNA strands. The transition between both forms may be performed by the recombinant protein RuvA, which specifically binds and unfolds cruciforms to convert them to an extended planar conformation. Further study has revealed the dynamic of cruciform structure like Holliday junction (Mikheikin et al, 2006). This transition between both cruciform forms involving the coordination of arm movement for the parallel orientation of arm and the resolution of the structure, was also suggested by X-ray crystallography and FRET (Förster resonance energy transfer) (Hohng et al, 2004; Khuu et al, 2006). Interestingly, negative DNA supercoiling is important for the stabilization of the folded conformation.

DNA sequence may be involved in disease development like Huntington's, a neurodegenerative disorder, which results from the expansion of the CAG trinucleotides beyond 300 repeats in the Huntingtin gene (Finkbeiner, 2011). The analysis of DNA containing high number of CAG repeats by AFM revealed unusual and unexpected structures of the nucleic acid at this locus, structures like hairpins and loops, which may contribute to the neurodegeneration process *in vivo* (figure 6) (Duzdevich et al, 2011).

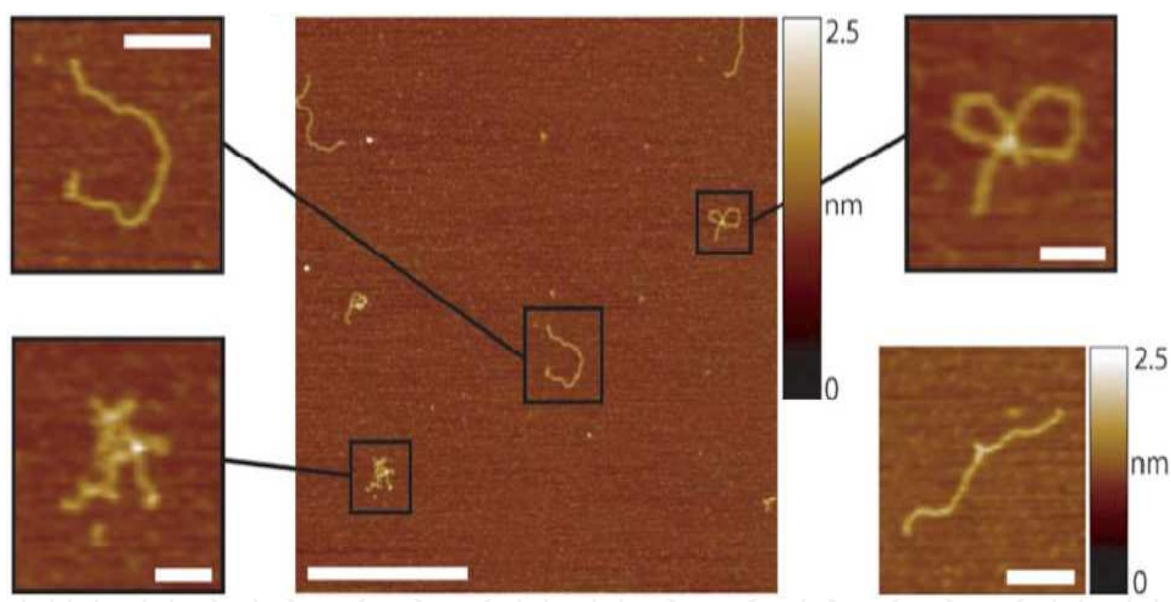


Fig. 6. DNA structure of CAG trinucleotides repetitions.

AFM study of CAG repeats fragments revealed linear normal DNA (upper left; scale bar 100 nm) and unexpected DNA structure such as unorganized (lower left; scale bar, 50 nm), folded (upper right; scale bar, 50 nm), or with protrusion (lower right; scale bar 100 nm). Inset, middle; scale bar 500 nm. From (Duzdevich et al, 2011) doi: 10.1371/journal.pone.0017119.g002.

5.1.2 Chromatin fiber folding

The formation of the 10 nm chromatin fiber has been explored using AFM. Particularly, the influence of DNA length and bending upon the efficiency of *in vitro* chromatin

reconstitution using purified histones has been examined (Hizume et al, 2004). Results show that relaxed circular or positively supercoiled plasmids are not competent to form chromatin *in vitro*, whereas longer DNA with negative supercoiling strongly promotes the formation of nucleosomes. In addition, the efficiency of *in vitro* chromatin reconstitution using linearized DNA is lower than with circular plasmids. These observations confirm previous biochemical and biophysical studies, which showed that the negative supercoiled double helical nature of DNA was the form most preferred by nucleosomes (Germond et al, 1975; Luger et al, 1997; Simpson et al, 1985). This conformation also supports chromatin condensation and separation of DNA strands during replication.

5.1.3 Histone and higher folding

The importance of the N-terminal histone tail in chromatin structure has been also investigated (Hizume et al, 2009). *In vitro* chromatin reconstitution using tail-less histones is efficient, but interactions between histones are weaker than using full histones. Importantly, 30 nm chromatin fiber could not be formed after addition of linker histone H1, suggesting the role of histone tails, in collaboration with H1, is primarily to effect higher-order chromatin folding. These AFM-based results were independently confirmed by biophysical sedimentation studies (Gordon et al, 2005; Kan et al, 2007; McBryant et al, 2009).

5.1.4 Histone modifications and chromatin dynamics

A key regulator of chromatin dynamics is the post-translational modifications of histones. Ten different types of modifications have been described to date, including major types such as phosphorylation, methylation, and acetylation, and less common types such as ubiquitination, biotinylation, glycosylation, ADP-ribosylation and SUMOylation (Kouzarides, 2007). Acetylation is usually associated with more accessible and transcriptionally active chromatin, whereas methylation has been linked to chromatin condensation and transcriptional quiescence.

The role of biotinylation in chromatin structure was revealed to be related to higher condensation compared to non-biotinylated nucleosome using AFM as a tool (Filenko et al, 2011). This phenotype results from an increase of DNA turn around the octamer with 1.75 and 2 turns for non-biotinylated and biotinylated nucleosomes respectively (Figure 7). This result provides a mechanistic basis for a previously described role of biotinylation in gene silencing (Hassan & Zemleni, 2008). Thus, AFM is a sensitive tool to reveal subtle modifications of chromatin condensation.

5.1.5 Study of chromatin-binding protein function in DNA pathways

AFM was also used to characterize the role of chromatin-binding proteins in complex cellular pathways. These proteins may play a role as regulator of chromatin folding, like histone variants and post-translational modifications. They participate either in the relaxation or the condensation of chromatin. Several investigations focused on Parp-1 (Poly-ADP-ribose Polymerase-1) whose binding to single and double strand breaks triggers the decondensation through the synthesis of ADP-ribose polymer and initiates DNA repair (Hakme et al, 2008). Parp-1 is also involved in DNA transcription through its binding to alternative DNA structure at gene regulating domains (Soldatenkov et al, 2002). To better

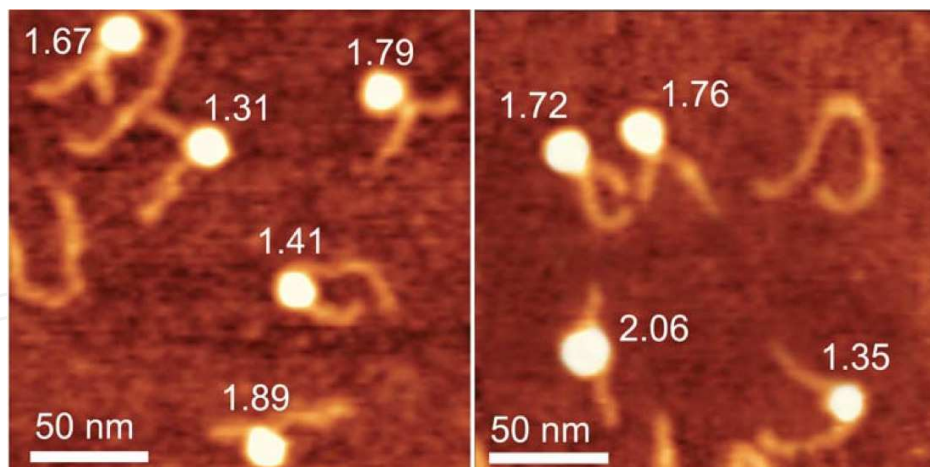


Fig. 7. Modification of *in vitro* nucleosome structure in response to biotinylation of histone H4. The effect of the post-translational modification of histone H4 (biotinylated) is analyzed by the determination of the number of DNA turns around histone octamers. On left, non-biotinylated nucleosome. On right, nucleosome containing biotinylated histone H4. From (Filenko et al, 2011) doi:10.1371/journal.pone.0016299.g003.

understand Parp-1 function in this pathway, the dynamic of chromatin structure, in the presence or absence of this factor, was investigated by AFM (Wacker et al, 2007). Condensation of *in vitro* reconstituted chromatin is observed in presence of Parp-1 in a concentration-dependent manner, like after addition of histone H1. The absence of N-terminal tail of histones does not affect this Parp-1 property. Further study showed that only the DNA binding domain of Parp-1 is required for chromatin compaction, whereas the addition of NAD⁺ (nicotinamide adenine dinucleotide), substrate of Parp-1 for the synthesis of ADP-ribose polymer, reversed the chromatin condensation. Together, these data suggest a cooperative role of Parp-1 DNA binding domain and catalytic domain in regulation of chromatin dynamic. Again, AFM has proven a powerful tool for the investigation and confirmation of molecular mechanisms. Indeed, in *Drosophila melanogaster* and in mammals, Parp-1 is localized at silenced genes loci (e.g. Hsp70), which are expressed by the activation of Parp activity in response to the addition of NAD⁺ or heat shock (Ouararhni et al, 2006; Tulin & Spradling, 2003).

Transcription has been extensively studied by AFM. The assembly of the bacterial RNA polymerase (RNAP) machinery and its progression on DNA after flow-through of nucleotides, were followed by AFM (Guthold et al, 1994; Kasas et al, 1997). Later, Crampton *et al.* analyzed the mechanism of RNAP collision, which results from discontinuous progression of RNAPs along DNA. Authors showed that collided RNAPs stayed associated with DNA with the retrogression of one of them (Crampton et al, 2006). These works demonstrated the interesting possibility of the *in vitro* study of processes in complex biological protein machinery by AFM.

Another example of DNA pathway analyzed by AFM is the V(D)J recombination, essential for the establishment of a large repertoire of adaptive immune T cells (Gellert, 2002). This mechanism consists of DNA fragments rearrangement in T cell receptor gene by endonuclease activity of RAG1/2 complex. These proteins recognize specific DNA motifs where they catalyze double-strand breaks and lead to the formation of a DNA loop to

facilitate the next step of ligation. EM and AFM analysis of purified RAG1/2 complex binding to specific recombination motif revealed the stoichiometry of the nucleo-protein complex and its structural organization (Figure 8) (Grundy et al, 2009).

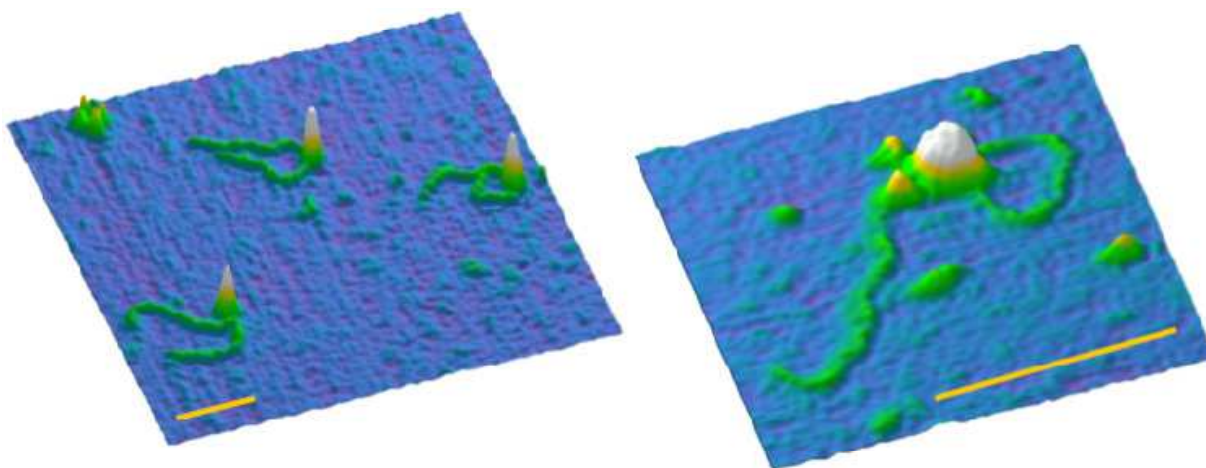


Fig. 8. Visualization of RAG1/2 complex loaded on DNA recombination motif by AFM. On the left, topography image reveals the 3-D arrangement of the complex. On the right, a tetramer of the proteins RAG1 and RAG2 bind two recombination signal sequences on DNA and lead to the formation of a DNA loop. (scale bars 100 nm). From (Grundy et al, 2009) doi: 10.1016/j.molcel.2009.06.022.

5.2 High Speed AFM

Beyond the standard commercial instruments, several configurations and capabilities have been developed and are being implemented offering new possibilities. The most powerful of these is the advent of HS- or video AFM. The major disadvantage of traditional AFM is the time required to acquire a high-resolution image. The speed for the acquisition is kept low (typically 1-2 lines/sec) to allow time for the feedback to follow the topography faithfully without affecting the sample or reducing the quality of the final image. Consequently, the time scale for obtaining an image ranges from a few minutes to several hours, which is far too slow for probing molecular kinetics, such as the movement of molecules, or the interaction between an enzyme and its substrate. To resolve this issue, HS-AFM was developed (Manalis et al, 1996).

5.2.1 Strategy of HS-AFM development

Different strategies have been explored for the buildup of HS-AFM. Humphris *et al.* conceived a new laser system, which focuses onto the back on the tip for a direct measurement of the height, whereas the laser deflection at the extremity of the tip is used for the feedback loop only (Humphris et al, 2011). In addition, VideoAFM (infinitesimal, Oxford, U.K.) has also been developed, wherein the increase of scan-time speeds results from quartz oscillation, and which can magnify the images at 1000X (Zhao et al, 2009). Another breakthrough strategy utilizes miniaturized cantilever with high resonance frequency (0.5-2 MHz) enabling faster response time (Ando et al, 2001). A scan stage was added to enhance spatial and temporal resolution for a capture of 10 images per second (1000 X 600 nm - 192 X 144 pixel).

5.2.2 Chromatin analysis by High-Speed AFM

The accessibility of DNA in the context of chromatin and the dynamic of nucleosome on a millisecond time scale were investigated using HS-AFM (Miyagi et al, 2011). Remarkably, a long-held speculation of how nucleosomal DNA can be accessed, which posits spontaneous wrapping/unwrapping at the entry and exit DNA of the nucleosomes was found to be accurate of single nucleosome oscillations (Li et al, 2005). Indeed, AFM studies revealed that spontaneous nucleosome unwrapping in the absence of ATP or any chromatin remodelers can occur *in vitro*, on a time scale of less than 1 min. Furthermore, the study was able to identify kinetic steps in the process. First, the nucleosome was observed to slide along the DNA molecule until it was closer to its ends. Then, a transient state of unwrapping was observed, relaxing the DNA-octamer interactions, followed by the asymmetric unwrapping, which was revealed by the length increase of only one DNA arm but not the other.

Studies of nucleosome dissociation using HS-AFM also showed a second potential and alternative mechanism (Miyagi et al, 2011; Suzuki et al, 2010). Nucleosomes may translocate reversibly along the DNA molecule through a process of sliding (Miyagi et al, 2011). Interestingly, complete eviction of nucleosome by one-directional sliding mechanism happened if a non-denaturing detergent (i.e. CHAPS) was added in the medium. This mechanism occurred by translocation steps of around 10-15 nm (3/4 turn of DNA). In agreement with these observations, nucleosome may be evicted from chromatin fiber in two different forms. Suzuki *et al.* observed that nucleosome may dissociate in one step from the octameric form or sequentially in two equal small subunits, probably two tetramers (Suzuki et al, 2010).

In vitro chromatin assembly in presence of the histone chaperone NAP1 and the ATP-dependent motor protein ACF is still studied by HS-AFM (Torigoe et al, 2011). Nucleosome formation is performed in different steps with first a rapid formation of DNA-histones intermediate, before the wrapping of DNA around the octamer to form the nucleosome. This second step is slower and ATP-dependent. The binding of histones onto DNA is initiated by the deposition of H3-H4 and may require NAP1 but the histone chaperone does not stay associated.

The ability of HS-AFM to analyze kinetics is already established. EcoP15I is a type III restriction enzyme, which recognizes two inversely oriented non-palindromic sequences and generates double strand break. EcoP15I cutting activity depends on the presence and recognition of two restriction sites separated by 3500 base pairs. AFM analysis demonstrated that to reduce this distance and generate the break, a large DNA loop is formed between the two sites (Crampton et al, 2007). The acquisition of 1-3 images per second revealed the ATP-dependent translocation of EcoP15I along plasmid at a rate of 79 base pairs/s, leading to the accumulation of supercoiling. Similar experiment with the restriction enzyme EcoRII highlighted the importance of HS-AFM as tool to characterize the mechanism of search of second restriction site by these enzymes and their translocation along the DNA molecule (Gilmore et al, 2009).

5.3 Elastic and other material property measurement

The unique feature of AFM is that it can both image and measure forces at high resolution. The latter offers the possibility to measure material properties at the micro- and the nano-

scale. One of the first applications of AFM was the measurement of elastic properties of microscopic biological structures, such as single cells and inhomogeneous tissues at microscopic scales. This is done by indenting the sample with the AFM probe and acquiring cantilever deflection *vs* displacement curves. Then, with mathematical models of contact mechanics, the elastic modulus of the sample (Young's modulus) is estimated (Dimitriadis et al, 2002; Lin et al, 2007a; Lin et al, 2007b). AFM is the only method that can map elastic properties at high resolution in ambient or under physiological condition. These elasticity maps can be correlated with topography to gain insights into the architecture that leads from the nanoscale to the macroscale properties of a tissue.

5.4 Receptor-ligand interaction

5.4.1 Strategy of coating

The AFM is also used for the chemical recognition of molecules. The technique requires the pre-coating of the AFM probe with a specific antibody against a protein of interest. Such functionalized probes are then used to scan the sample and map the corresponding target antigen. During the topography scan, the binding of the antibody with its antigen will be detected by the modification of the cantilever oscillation, generating a parallel image of antigen-antibody interaction hits (Hinterdorfer & Dufrene, 2006; Kamruzzahan et al, 2006; Raab et al, 1999; Stroh et al, 2004). The success of the recognition depends on the choice of this specific antibody and the quality of its coating.

Several protocols for covalently attaching such molecules to AFM probes exist in the literature, ranging from simple physisorption to complex chemistry that first attaches a spacer molecule to which the antibody is then covalently bound (Barattin & Voyer, 2011). Examples of chemical systems are glutathione/ glutathione S-transferase, or Ni-NTA (nickel-nitrilotriacetic acid) -His6/GST-His6-tag-protein (Gamsjaeger et al, 2004; Schmitt et al, 2000; Yoshimura et al, 2006). The spacer gives the antibody freedom of movement to accommodate the fixed position and orientation of the antigen. A challenge to recognition is non-specific binding which requires carefully planned controls.

5.4.2 Examples of receptor-ligand analysis in the context of chromatin

This technique has been already used in the chromatin field, such as for the recognition of proteins within the centromeric histone variant nucleosome (Wang et al, 2008). In this study, the direct identification of CENH3 confirmed that CENH3 nucleosomes display mainly a tetrameric structure. This method of recognition was also used to follow assembly/disassembly of nucleosome during remodeling. Swi-Snf is an ATP-dependent nucleosome remodeling complex whose function was analyzed by recognition imaging using specific antibody against histone H2A (Bash et al, 2006). Incubation of *in vitro* reconstituted chromatin with Swi-Snf led to the release of histone H2A, suggesting that remodeling requires partial disassembly of the nucleosome.

As previously mentioned, the specificity of the antibody is a major factor of success for recognition. Lin *et al.* tested another system of recognition using DNA aptamers, to reduce cross-reactivity and enhance detection (Lin et al, 2007c). These nucleic acid molecules display similar properties to antibody such as their characteristic folding complementary to the structure of their target epitope (Bunka & Stockley, 2006; Colas, 2008). However, their

small size and their nucleic composition facilitate their design and synthesis. DNA aptamers against histone H4 were used successfully with a high efficiency of recognition (85% compared to 48% for a commercial antibody) and limited cross-reactivity with the other histones. While this exciting alternative strategy has not been used in chromatin field, it continues to be investigated for the selection of strong affinity aptamers by the method of AFM-SELEX (Systematic Evolution of Ligands by an Exponential enrichment) (Miyachi et al, 2010).

6. Next generation AFM for the study of specific molecules in biological context *in situ*

An exciting new generation of AFM instruments is being developed and made available to the research community, which combines the power of structural analysis by AFM with simultaneous molecular identification of specific targets using immunofluorescence/confocal microscopy. Instruments now exist which combine AFM with epi-fluorescence, TIRF microscopy (total internal reflection fluorescence), high resolution Raman and infrared spectroscopy.

For chromatin studies, epi-fluorescence and TIRF are equivalent, since the chromatin samples will have to be immobilized to the mica surface. The combination of high resolution fluorescence microscopy with AFM (FIONA-AFM) allows the identification and the localization of fluorescent-tagged molecules such as proteins on topographic image. The strength of this technique was evaluated by the analysis of the interaction of the protein complex UvrA-UvrB, involved in the initiation of nucleotide excision repair pathway, with DNA, and the binding of RNAP on *Escherichia coli* DNA (Ebenstein et al, 2009; Fronczek et al, 2011). Both studies showed the correlation between optical and AFM signals, providing a proof of principle for combining structural details of multi-molecular complexes with dynamic information yielded by fluorescence.

Raman and infrared spectroscopies (Fourier Transform-Infrared Spectroscopy or Ft-IR) are vibrational techniques used for chemical identification. They give similar but complementary information since they use different regions of the spectrum. Standard Raman or FT-IR spectroscopies are diffraction limited. Therefore the resolution of Raman spectroscopy is in the 1 μm range while for FT-IR it is in the 5-10 μm range. Raman spectroscopy has the additional disadvantage of a weak signal requiring large samples and long acquisition times. Their combination with AFM is relatively recent but the technological advances hold great promise for label-free, single molecule identification. The sub-diffraction resolution of Raman spectroscopy is achieved by using the AFM tip, coated with Au, Pt or Ag to achieve Raman signal enhancement by orders of magnitude (Anderson & Pike, 2002). More recently, Neascu *et al.* demonstrated enhancement of over 10^9 to achieve single molecule sensitivity (Neascu et al, 2006). Applications in biology are starting to appear more frequently and are expected to expand further in the future.

In the infrared range, a novel idea directly couples AFM with IR spectroscopy in a new photothermal technique called photothermal induced resonance (PTIR) (Yeo et al, 2008). A pulsed, tunable infrared laser is directed to the sample through a prism at a total reflection angle. Infrared absorption by the sample excites the cantilever through the contacting tip into resonance. Analysis of the ring-down signal of the cantilever maps the IR absorption

spectrum. The method has demonstrated a resolution below 50-100 nm and applicability to a fluid environment (Dazzi et al, 2007; Dazzi et al, 2008).

7. Conclusion

As we have seen above, AFM is a versatile and powerful tool for biological studies. Several protocols are now perfectly established to characterize nucleoprotein complexes such as chromatin, or nucleic acid molecules (DNA or RNA). Applications range from observation of native complexes undergoing dynamic change to precise measurement of static physical dimensions. The panel of potential available studies results from the different modalities of the AFM by itself (e.g. non-contact/tapping vs. contact mode; ambient air vs. fluid; high-speed) and its combination with other biophysical method such as Raman spectroscopy. Newer applications permit combination of fluorescence imaging to single molecule measurement, thus finally bridging the resolution gap between light microscopy and electron microscopy. In consequence, the fast evolving nature of the technology continuously yields new applications for AFM researchers. Pre-existing AFM applications, and yet to be developed new tools, will continue to qualitatively and quantitatively enhance our capacity to study of dynamics and structure of biological materials.

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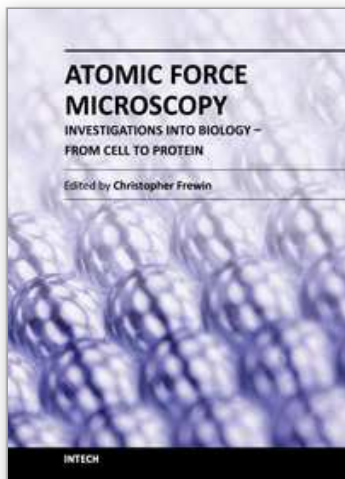
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The atomic force microscope (AFM) has become one of the leading nanoscale measurement techniques for materials science since its creation in the 1980's, but has been gaining popularity in a seemingly unrelated field of science: biology. The AFM naturally lends itself to investigating the topological surfaces of biological objects, from whole cells to protein particulates, and can also be used to determine physical properties such as Young's modulus, stiffness, molecular bond strength, surface friction, and many more. One of the most important reasons for the rise of biological AFM is that you can measure materials within a physiologically relevant environment (i.e. liquids). This book is a collection of works beginning with an introduction to the AFM along with techniques and methods of sample preparation. Then the book displays current research covering subjects ranging from nano-particulates, proteins, DNA, viruses, cellular structures, and the characterization of living cells.

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