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Review

Atomic Force Microscopy as a Tool in Nanobiology Part II: Force Spectroscopy in Genomics and Proteomics

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Abstract. *We present possible applications of Atomic Force Microscopy (AFM) as a force spectroscopy tool in genomics and proteomics. AFM applications in these fields have opened new opportunities for studying the mechanical properties of biomolecules and their interactions in their native environment, as well as in determining the binding affinity of DNA proteins in dependence with the target DNA sequence for further correlative studies on physical affinity and biological relevance of the controlled gene. Furthermore, force spectroscopy is a powerful analytical tool to investigate structural and functional features of biomolecules. Altogether, these tools have revealed useful application in genomics and proteomics.*

Genomics have contributed greatly to the understanding of the molecular basis of disease and to the development of new therapies and drug design. In the post-genome era, proteomics aim to translate the nucleic acid information archive into an understanding of how the cell actually works and how disease processes operate. The most relevant information archive to clinical applications and drug development involves the elucidation of the information flow of the cell: the program of protein pathway networks.

There are several reasons to use the atomic force microscope (AFM) in the field of proteomics. First, the AFM provides a detailed look into the overall structure of proteins. Information on size, shape and subunit composition of the protein can be recorded and, on a functional base, it is possible to follow molecular interactions, *e.g.* bio-assembly processes (1,2). Second, protein surface interactions, the protein surface binding process and how it affects its biological functionality, can be investigated by AFM techniques (3,4). The results can be used for the development of new biosensors and immunoassays.

Considering the recent advances in experimental genomics and proteomics, we emphasize the use of AFM as a force spectroscopy tool. When used as a sensor, AFM opens the possibility of enormous change in our ability to analyze and interpret complex biological processes, allowing, for example, the detection and elucidation of protein-DNA and protein-protein interactions.

AFM as a sensor

Understanding the force (derivative of energy with respect to distance) that drives specific molecular interactions is a challenging task in molecular and structural biology, because it is the most direct means of obtaining information about how the interaction energy between two different structures is distributed in space. Such specific interactions result in multiple weak, non-covalent bonds formed between defined portions of the interacting molecular partners.

The development of the Atomic Force Microscope (AFM), as a Scanning Probe Microscopy (SPM) family member, has opened new perspectives for the investigation of surfaces at high lateral and vertical resolution (5). During the last decade, AFM was proposed to study inter- and intra-molecular interactions forces in biological macromolecules (Figure 1), mainly due its precision and sensitivity to probe surfaces in physiological environments with molecular resolution and with forces down to the pico-newton range (5,6). Thus AFM is a useful tool to evaluate and to characterize mechanical properties of biological samples such as: topography, elasticity and adhesive properties. Furthermore, due to its ability to operate at least as well in

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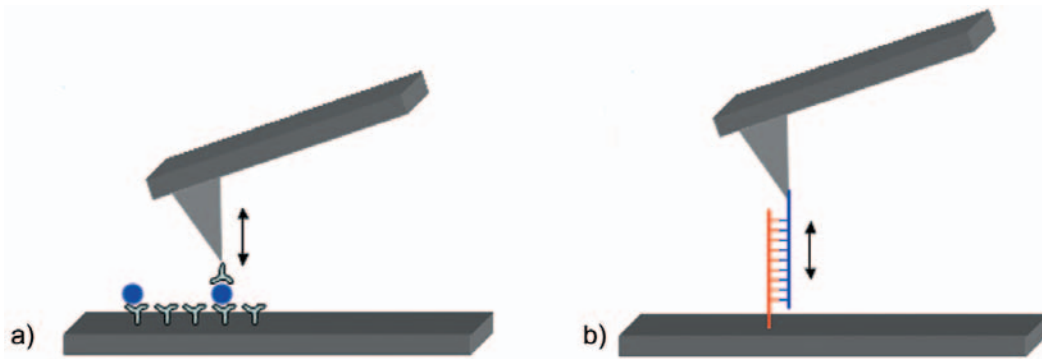

Figure 1. *Schematic set up of force spectroscopy experiments: By immobilizing a molecular partner either over AFM tip and substrate AFM can test a) intermolecular interactions between receptor and ligand; and b) complementary nucleic acid binding between DNA strands.*

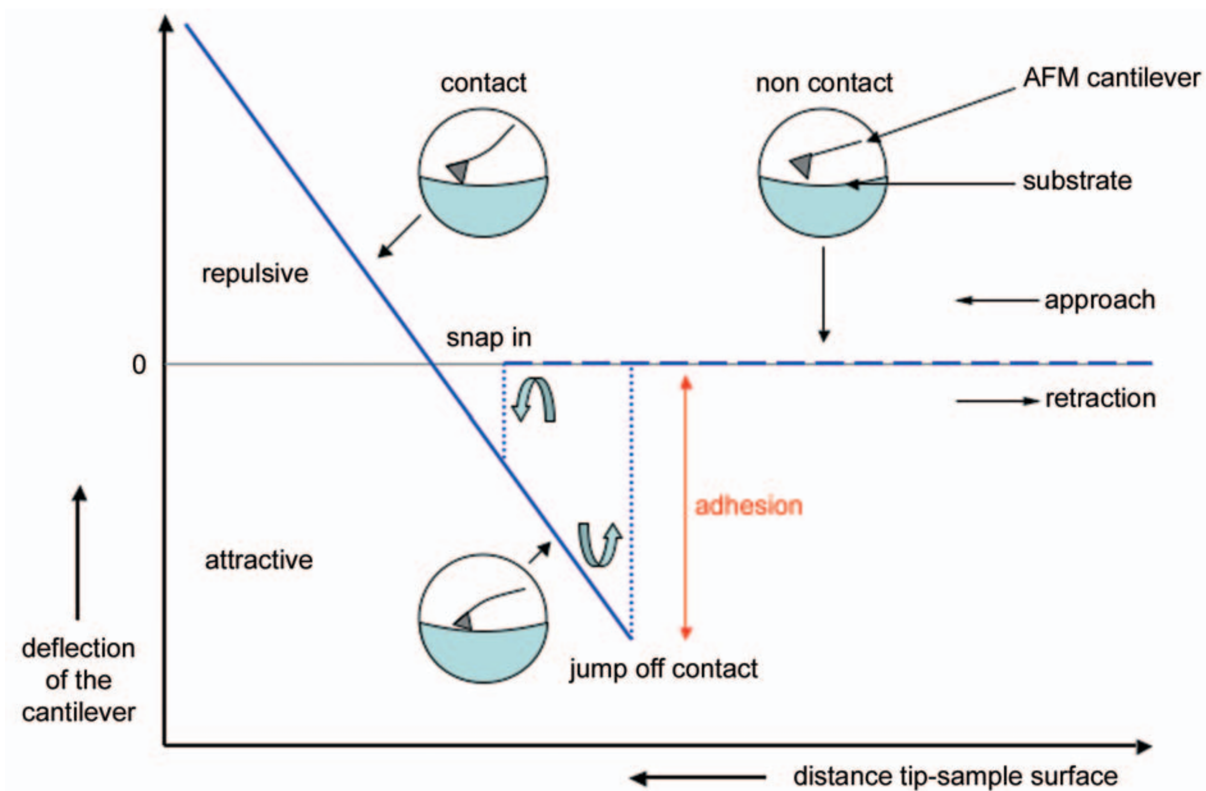

Figure 2. *Force contact curve generated by an AFM: The deflection of the cantilever is shown as a function of the tip-sample surface distance. In the beginning of the approach cycle the AFM tip is not in contact with the surface; then, the AFM tip is being pushed into the surface resulting in a bending of the cantilever. In the retraction cycle the tip is being withdrawn from the surface, the tip adheres to the sample surface and, after the jump-off contact from the surface, the tip is brought to the non-contact position again.*

liquid as in air, AFM opens the possibility of probing biological sample interactions in aqueous buffer *i.e.*, under conditions, close to their native environment.

When recording a force curve the AFM tip starts far from the sample surface, where no interactions between either exist. As the tip-sample separation is reduced beyond a certain point, attractive dispersion and electrostatic forces between the two surfaces begin to interact, causing the flexible cantilever to bend towards the sample up to a certain point, where strong repulsive forces bend the cantilever outwards. At each distance, the cantilever bends until its elastic (restoring) force equals the tip-sample interaction force and the system is in equilibrium. The resultant plot of the cantilever deflection *versus* the separation between the

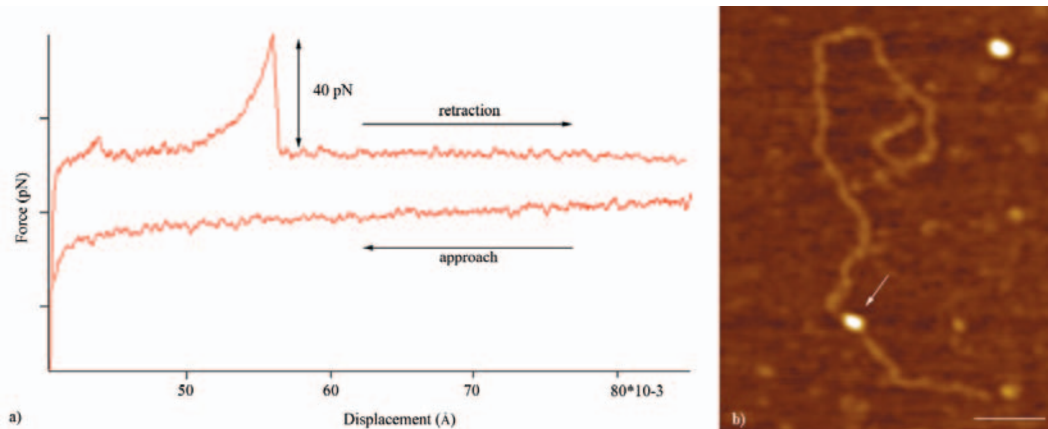

Figure 3. *a) Force-distance curve: The protein LexA was covalently immobilized onto an amino group pre-treated glass slide via carboxymethylamylose and DNA (recA sequence) was covalently bond to the AFM tip, also via carboxymethylamylose. An unbinding event could be identified at a certain distance from the point of contact due to the length of the polymer linker (carboxymethylamylose) and the stretching of this linker prior to the jump off contact. b) AFM images of LexA-DNA complex at a single molecule level. The specific binding of LexA to the DNA (see arrow) was achieved by incubating DNA (16 nM) with LexA (34 µM) for 30 min at room temperature. The DNA fragment contained the recA operator. Imaging was performed in dynamic mode under ambient conditions; bar: 100 nm.*

tip and the sample is the force curve. The AFM combines high force sensitivity in the pico-newton range (7) with high lateral resolution, often better than a nanometer, which is in the molecular range. A typical force *versus* displacement curve generated by AFM is shown in Figure 2.

Inter- and intra-molecular interactions

For quantitative force adhesion measurements, the partners in the molecular recognition reaction have to be immobilized on both the substrate surface and the AFM tip (Figure 1a). Furthermore, the specific interactions must be compared to the background of non-specific probe/surface interactions, which may be as high as, or even higher, than the biological interactions. This is done by using non-functionalized probes or surfaces for comparison, by blocking the interactions between the immobilized molecular partners with free ligands in the medium, or by changing the pH or the salt concentration in the medium.

Concerning intermolecular interactions, biotin/streptavidin (or avidin) has been used as a model system for ligand-receptor adhesion studies, because of its high affinity and the availability of structural and thermodynamic data (8,9). The unbinding forces of antibody-antigen complexes measured by means of AFM force spectroscopy correlate with the spontaneous association (k_{on}) and dissociation (k_{off}), and the dissociation constant ($K_D = k_{off}/k_{on}$), which describes the equilibrium behavior (10).

Furthermore, single molecule AFM experiments measuring coupling of stretch and twist movements, overstretching mechanics and base pairing forces provided an intimate look into the biophysical details of DNA. Direct measurements of the unbinding forces between single strands of DNA used covalently bound DNA oligonucleotides to the AFM tip and surface, respectively. The adhesive forces between complementary 20 base pair strands are in the nano-newton range, and the intra chain interaction resulting from the molecule's elasticity showed a long-range cohesive force behavior (11,12). Sequence-dependent mechanical properties of DNA measured by unzipping experiments showed that the transition from B- to S-DNA conformation occurs at significantly lower forces (65 piconewtons) in poly (dA-dT) compared to poly (dG-dC) oligonucleotides (13). Using individual double-stranded DNA molecules attached between an AFM tip and a gold surface, the mechanical stability of overstretched DNA was described (Figure 1b). In the case of Lambda phage a DNA B-S transition was observed followed by a second conformational transition, during which the DNA double helix melts into two single strands (14).

A major focus of current genomics research is the detection of single-nucleotide polymorphisms (SNPs) within known gene sequence, as well as in the whole genome (15). Such mismatched base pairing was detected using a gold-coated AFM cantilever functionalized with thiolated 20- or 25-mer probe DNA oligonucleotides and exposed to target oligonucleotides of varying sequence in static and flow conditions, providing a distinct positive/negative signal for easy interpretation of oligonucleotides hybridization. The association of SNPs with disorders is expected to be particularly useful in identification of cancer and neural

degenerative disorders such as Alzheimer's disease and is also related to the individual variations in drug responses (16).

Recently, dynamic studies of DNA-protein interactions came into the forefront of biophysical research. Single molecule force spectroscopy showed the specificity of the interaction between ExpG protein and the promoter regions in the galactoglucan biosynthesis (*exp*) gene cluster. Moreover the binding mechanism involved with respect to its thermal off-rate and additional molecular parameters, describing the energy landscape of the separation process, was characterized (17). Our group has shown the specific interactions between LexA repressor protein, derived from the SOS repair system of *Escherichia coli*, and two specific DNA binding motifs, *recA* and *yebG* (a topographic AFM image of the complex *recA*-LexA formation is shown in Figure 3b). A typical force curve is shown in Figure 3a. This experiment showed a pronounced feature in the energy landscape along a force-driven pathway between the rupture force and the off rate (k_{off}) value for each operator (Costa *et al.*, 2004 in preparation).

Furthermore AFM force spectroscopy can be used to acquire detailed information in the three-dimensional structure of chromosomes. These studies are important to understand the mechanisms of histone movement for transcription, replication and repair of DNA. To determine the forces necessary to break the bonds between the DNA and histone octamers in the nucleosomal particles, AFM experiments were carried out by stretching single chromatin fibers bound to the AFM tip (18).

In the post-genomic area the understanding of protein folding plays an important role and has wide-ranging impact in structural biology. AFM also allows the study of intramolecular interactions like protein-folding mechanisms, especially unfolding of multi-domain protein molecules or individual protein domains. The unfolding and stretching of each domain creates an individual peak in the force curve, leading to a characteristic saw-tooth pattern. Single molecule force measurements of individual titin immunoglobulin domains showed an unfolding by forces in the range of 150 to 300 pN, and dependent on the pulling speed. After relaxation, refolding of immunoglobulin domains could be observed (19). Using recombinant constructs from different titin parts, the distribution of mechanical stability along more than 200 Ig and fibronectin III domains in titin could be demonstrated (20). It has been shown that the force required for the mechanical unfolding spectrin repeats is between 25 to 35 pN. In other words, the unfolding forces of the α-helical spectrin domains are five to ten times lower than those found in domains with β-fold, like in the immunoglobulin or fibronectin III domains, where the tertiary structure is stabilized by hydrogen bonds between adjacent strands. This demonstrates that the forces which stabilize the coiled-coil lead to a mechanically much weaker structure than multiple hydrogen-bonded β-sheets (21).

The combination of high resolution AFM imaging with force spectroscopy provides an insight into the interaction forces between the individual protomers of the hexagonally packed intermediate (HPI) layer of *Deinococcus radiodurans*. Individual protomers were sequentially stretched, unfolded and removed from a bacterial surface layer until an entire bacterial pore formed by six protomers was unzipped (22). Besides the possibility of single molecule force spectroscopy, the AFM can also be used as a manipulator. This was demonstrated with purple membrane patches, from *Halobacterium salinarum*, where individual molecules of the membrane protein bacteriorhodopsin could be localized, unfolded and extracted from the lipid membrane (23). Further, the influence of pH and local mutations on the stability of individual structural elements of bacteriorhodopsin against mechanical unfolding has been analyzed. The polypeptide loops act as a barrier for unfolding and contribute significantly to the structural stability of bacteriorhodopsin (24).

Cellular adhesion mediated by biological macromolecules and their respective ligands plays an essential role in a number of diverse biological phenomena including inflammation and cancer metastasis. How the adhesiveness of receptor-ligand interactions is controlled by the affinity of the individual receptors to single ligands is not well understood. Using single molecule force spectroscopy, the tensile strength and off-rate of single P-selectin molecules binding to single ligands on intact human polymorphonuclear leukocytes and metastatic colon carcinomas were probed *in situ* and compared to the overall adhesiveness of these cells for P-selectin substrates (25). Characterizing the biochemical and biophysical properties of functional P-selectin ligands on carcinomas will provide guidelines to engineer novel therapeutic agents that will selectively block ligand function and thus interfere with metastatic spreads.

Force-distance curves have also been utilized to identify cell partners that interact specifically in certain biological reactions. Cell-cell adhesion mediated by specific cell-surface molecules is essential for multi-cellular development. By functionalizing AFM tips with whole cells of a given type and studying their interaction with monolayers of other cell types, it was possible to identify the cell type in the uterine epithelium that interacts specifically with cells in the embryo during implantation (26). The cell-cell adhesion mediated by specific cell-surface molecules has been investigated using a glycoprotein layer, contact site A (csA) as a prototype of cell-adhesion proteins. csA is expressed in aggregating cells of *D. discoideum*, which are engaged in development of a multicellular organism (27,28).

Viscoelastic properties of biomolecules

AFM has also become a powerful tool to measure the viscoelastic properties of biological structures and macromolecules. Whenever the effective stiffness of the

cantilever and the biological sample on the surface are of comparable size and the AFM tip is pushed into the sample, the sample is indented. At the time when the stress (deformation force) and the strain (the amount of deformation) are linearly related, the deformation of the material is elastic and the material will regain its original form upon relaxation. The depth of indentation can be used to perform local elasticity measurements of Young's elastic modulus (the mechanical resistance of a material while elongating or compressing). The capability of the AFM to provide information on the elastic properties of biological structures has been used to study different types of differentiated cells and organelles (29) and human chromosomes (30). The elasticity of human chromosomes was determined in neutral and alkaline pH but also in acetate buffers (30).

The behavior of soft biological samples is different to a hard surface, indicative in the force retraction curve. Whereas lift-off occurs quickly on hard surfaces, it may be considerably slowed down in the case of soft biological samples. For example, the lift-off speed has be used to estimate the viscosity of the sample and the elasticity of lysozyme adsorbed on mica (31).

Force mapping

Individual curves can be assembled into a force-volume providing a three-dimensional, laterally resolved description of the forces within the sample (29). A force-volume can be used to produce sample/surface maps reflecting different properties of the surface as adhesion, viscosity, elasticity, *etc.* Using this approach a map of antigenic sites on a surface by molecular recognition of an antigen by an antibody tethered to an AFM tip has been acquired (32).

The question as to whether cell division is driven by cortical relaxation outside the equatorial region or by cortical contractibility with the developing furrow alone has been approached by monitoring spatially-resolved changes in the cortical stiffness with time. Here force-mapping was used to track dynamic changes in the stiffness of the cortex of adherent cultured cells along a single scan-line during methaphase to cytokinesis (33).

Conclusion

The Atomic Force Microscope (AFM) as a force spectroscopy tool in genomics and proteomics opened new opportunities for studying the mechanical properties of biomolecules and their interactions in their native environment. Those tools have shown useful applications in genomics and proteomics studies by determining the binding affinity of DNA proteins dependent on the target DNA sequence for further correlative studies on physical affinity and biological relevance of the controlled gene. Further, force spectroscopy is a powerful analytical tool to investigate structural and functional features of biomolecules. AFM used in programmable DNA sensors (34) and protein biochips (35) opens promising applications in the new field of nanobiotechnology.

Acknowledgements

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