Biological Applications of the AFM: From Single Molecules to Organs

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ABSTRACT: The application domains of the atomic force microscope have increased dramatically in recent years. We present a short review of the contributions of this microscope to biology. These are illustrated through the study of different samples, starting with the imaging of single molecules all the way up through the length scales, and ending with imaging of tissues. So that nonbiologists can appreciate the significance of these studies, special attention has been paid to a description of the samples and to point out the motivation of these studies and their implications for the field of medicine. © 1997 John Wiley & Sons, Inc. Int J Imaging Syst Technol, 8, 151–161, 1997

I. INTRODUCTION
The atomic force microscope (AFM), also known as the scanning force microscope (SFM), was developed 10 years ago [12] and is an instrument which probes the interaction forces between a sharp tip and the surface of a sample. The ability of this microscope to achieve high resolution (subnanometer) in liquids and to probe the mechanical properties of the sample at a nanometric scale make this instrument increasingly interesting for the study of biological specimens. Consequently, the number of AFM articles published per year in this field has grown exponentially [48]. Every conceivable type of biological material has been explored using the AFM (for reference, see review articles [18,19,79,82,86,111,122,126]). This review presents selected biological applications to illustrate the microscope’s potential in the field of biology.

In the first part, we outline the general working principle of the instrument and its different imaging modes. AFM applications can be classified in different ways. We have chosen to divide them into imaging and nonimaging categories. One advantage of the AFM is the ease with which data can be acquired over a wide range of length scales. When discussing imaging, we will present some applications of the AFM at different magnifications, from single molecules to organs. After this, we will discuss the nonimaging category, which includes measurement of forces involved between single molecules, measurement of the micromechanical properties of the sample, and detection of protein motion. Finally, we will discuss the future of AFM in biology.

II. WORKING PRINCIPLE
The principle of the AFM is simple: a sharp tip fixed at the end of a flexible cantilever is raster-scanned over the surface of a sample. As the tip interacts with the surface, the cantilever deflects and its deflections are monitored and used to reconstruct the topography of the sample [122,145]. Surfaces can be imaged nondestructively because the interatomic spring constant of the sample is on the order of 10 N/m, in comparison with typical contact mode AFM cantilevers, which have spring constants in the range of 0.01–1 N/m. The cantilever is a macroscopic spring that has a lower spring constant, meaning that the applied force can be kept well below the force which would disturb the atoms from their sites, while still achieving measureable cantilever deflections [122]. If the microscope is operated in liquids or in vacuum, high resolution is more readily achieved, since the strong capillary forces, due to the thin liquid film present on all samples in air, are absent.

III. THE SCANNER
The precise positioning of the sample relative to the tip is one of the practical reasons why AFM can achieve subnanometer resolution on hard samples. Scanning is accomplished using piezoelectric translators, with a precision on the order of angstroms.

In most AFMs the sample is positioned on top of a four-segment piezoelectric tube and is scanned under a fixed tip. The mechanical components of the microscope are designed to be rigid and compact, insuring the high mechanical stability required for atomic resolution imaging. Figure 1 depicts the typical architecture of such an AFM. For biological applications it can be useful to observe the sample simultaneously with an optical microscope, hence, free-standing AFMs mounted on the top of an inverted optical microscope have been developed [113]. In this architecture, the sample is fixed, and the tip, which is mounted on a piezo tube, moves about the sample [51,138]. The disadvantage of this architecture is poorer mechanical stability, limiting the resolution of the instrument. However, since the sample is
However, the optical lever technique depicted in Figure 1, which uses a laser beam reflected off the very end of the lever, is now the most commonly used [4,99]. As the cantilever is displaced, the reflected beam changes its direction. These changes in direction are detected using a two-segment photodiode. A four-segment photodiode can be used to monitor torsional motions of the cantilever and thereby determine frictional forces between tip and sample. The deflection of the laser spot at the photodetector is amplified in such a way that a displacement of 0.1 Å of the cantilever corresponds to a displacement of 3 ± 10 nm at the photodetector, which is large enough to generate a measurable voltage [96,99,123]. The other detection methods are mostly used for specific laboratory experiments and are not implemented in the commercially available microscopes.

VI. THE DIFFERENT IMAGING MODES

A. The Force Curve. A force curve contains information about the force interaction between the tip and the sample surface as a function of relative tip–sample distance. Analysis of these data, assuming models for tip shapes, can give information about the mechanical properties of the sample [16]. A typical force curve is depicted in Figure 3. This curve is obtained by stopping the scan and approaching and retracting the tip from the sample while recording the deflections of the cantilever. As the tip approaches the sample, the cantilever remains undeflected until it comes close enough to the sample surface for the tip to experience attractive forces such as van der Waals. These forces bend the tip and lever down toward the sample, until they are balanced by the repulsive forces generated from the overlapping electron orbitals between the tip apex and the sample. If the piezo continues to push the cantilever into the sample, the cantilever will deflect in an upward direction. The extent of this deflection will depend on the stiffness of the sample. This is reflected in the shape of the force curve: the harder the sample, the more linear the curve will be after the contact point [144]. The contact point can be defined as the point at which the repulsive and attractive forces are balanced so that the cantilever is touching the surface but is undeflected (Fig. 3). On retraction, the cantilever deflection can retrace a similar curve as the scanner pulls the tip away from the surface. If the microscope is operated in air, the presence of a thin water film on the surface of the sample dramatically increases the adhesion force. Capillary forces hold the tip in contact with the surface, bending...
A Lennard–Jones-type potential. The AFM will operate in this mode if the tip is operated at the repulsive or attractive modes, depending on the part of the surface friction on the sample [26,29,31,55,98,108]. The twisting motion of the cantilever is detected by replacing the two-segment curve the AFM tip held.

Figure 3. Force curve of tip–sample interactions. This figure depicts a typical force curve for samples imaged in air or in liquids. The tip approaches the sample (1) and contacts it at the end of the first horizontal part of the curve. The downward motion of the tip continues until the cantilever deflects upward upon contact with the sample [segment (2) of the curve]. The shape of the lever in this part of the force curve is represented in black on the upper-left portion of the graph. Once the motion of the tip is inverted, the cantilever retracts and follows path (3). Depending on the adhesion forces, the tip will be released at a different moment during the retraction process. If the adhesion is low, as in liquids, the lever follows more or less the same path as during the approach. If the adhesion is high, the tip is kept in contact with the sample longer and follows the descending gray path of the curve. The deformation of the lever during this process is depicted on the lower-left part of the graph in gray. Once the adhesion is overcome, the tip jumps to its "normal position" along the vertical path of the gray curve and continues to retract without being influenced by the sample [segment (4)].

B. Force Mapping. This type of imaging consists of recording force curves in an array of points across the sample. In this way, the sample is probed over a range of forces, and images at any desired contact force can be reconstructed. The force curves can be analyzed to highlight differences in the attraction, adhesion, repulsion, or different mechanical properties across the image. This imaging mode was first developed in 1994 [10,116,119] and is currently available on commercial instruments [28].

C. Contact Mode. Depending on the sign of the interaction between the tip and the sample, the AFM can work in two different modes: repulsive (contact) or attractive (noncontact). Contact mode imaging is obtained if the tip is operated at the repulsive part of a Lennard–Jones type of potential as shown in Figure 4. This is the imaging mode that was developed first and still one of the most widely used. Data can be collected in two ways with this mode. In the so-called constant force mode, as the tip raster scans the sample, a feedback loop keeps the cantilever deflection constant. In this mode, the tip approximately maps out the topography of the sample and, as its name suggests, keeps the force on the sample constant. Variations in the compressibility of the sample means that a true reconstruction of surface topography at constant force is not possible. In the constant height mode, the cantilever is held at the same height during the scan with the feedback off. The topography of the sample is obtained by monitoring the cantilever deflection. The advantage of this imaging mode is its height sensitivity in the z axis; however, the force applied to the sample increases with increasing cantilever deflection and can cause damage to the tip and/or the sample.

D. Noncontact Mode. In this case, the cantilever is oscillated at or close to its resonant frequency at a distance of 1–10 nm above the sample. Long-range attractive forces (Fig. 4) induce changes in the amplitude, phase, and frequency of the lever and are used by the microscope to keep the tip sample distance constant during the scan [97,108,146]. The forces involved in the noncontact mode are much lower than those in contact, generally about $10^{-12}$ N compared with $10^{-9}$ N. It is therefore possible to image even the softest samples without damage. However since the tip–sample distance is relatively large, a lower resolution is obtained than in contact mode. Stiff cantilevers must be used to avoid the tip snapping down to the surface. Since the forces involved are much lower, the signal detection is also more difficult. For all of these reasons, application of this imaging mode to biological samples is still rather limited [14,35,101].

E. Tapping Mode. In this mode [110], the cantilever is also oscillated above the sample, but, in contrast to the noncontact mode, the tip periodically touches the surface [151]. It is a compromise between the noncontact and contact modes. The principal advantage of the method is the reduction of the lateral forces which can move or damage the sample. This mode has recently been adapted for imaging in liquids and is particularly suitable for imaging biological samples weakly adsorbed to surfaces [50]. However, the vertical probe force may have an absolute value comparable with that in contact mode, especially in liquids, where the adhesion force is absent [147]. Therefore, biological samples are still deformed although not moved laterally by the tip. However, because of the reduction of the lateral forces, this mode is becoming increasingly used for imaging biological samples in air and liquids.

F. Friction Mode. By monitoring the torsional motion of the lever during a contact mode scan, one can detect changes in surface friction on the sample [26,29,31,55,98,108]. The twisting motion of the cantilever is detected by replacing the two-segment
photodiode with a four-segment one. However, this type of cantilever motion can also be induced by surface corrugations, and therefore, only on a flat surface is the image contrast generated merely by frictional forces [1]. In contact mode, artifacts in topographic information can be caused by changes in friction.

**G. Other Modes.** A few other imaging modes exist, such as the force modulation mode [8,93,115], which records the topographic and mechanical properties simultaneously, which has been applied on some biological samples such platelets [115] myocytes [130], and magnetotactic bacteria [33]. Phase detection is another mode in which contrast is generated from changing tip–sample interactions and is related to the mechanical properties of the sample [91,92]. As yet, a full explanation for the generation of phase contrast has not been put forward. Another useful mode is the ultrasonic force mode introduced by Kolosov and Yamanaka in 1993 [76] and successfully applied to DNA [148]. These newer modes will be increasingly used in the future, since they enable one also to reduce topographic artifacts. However, for now, the large majority of biological AFM publications concern contact mode and tapping mode imaging in both air and liquids.

**VII. IMAGING APPLICATIONS IN BIOLOGY**

**A. DNA.** DNA is the cell’s master repository of genetic information. This molecule consists of two strands, each containing an unbranched polymer composed of only four different bases: adenine (A), thymine (T), guanine (G), and cytosine (C). Each DNA base of one strand is linked to a complementary base on the other strand by hydrogen bonds in such a way that A can stranded (ds) reovirus RNA on silanated mica and found lengths nine (A), thymine (T), guanine (G), and cytosine (C). Each difficulty of imaging ssRNA on an atomically flat surface such as mica. Lyubchenko et al. [87,88] measured the lengths of double-stranded (ds) reovirus RNA on silanated mica and found lengths ranging between 0.2 and 1.8 μm. The molecules exhibited convoluted shapes and in some cases compact structures. Hansma et al. [49] studied an ssRNA homopolymer poly(A), which showed two conformations in AFM: short and lumpy; or longer, less lumpy, and straighter. The sizes of the short molecules ranged from 100 to 300 nm, consistent with expected molecular lengths of 400–600 bases. The longer molecules appeared to be ds molecules of poly(A).

DNA has been imaged on several surfaces in air [124] and in a variety of solutions [47,60]. In 1992, Bustamante et al. obtained 8–10 nm resolution images in air, on circular double-stranded DNA [17,137]. When the DNA sample was immersed in alcohol, adhesion forces were reduced, enhancing the specimen stability and improving the reproducibility of the technique [44,45,88]. Imaging in water [90] and aqueous buffers [46] has been much more challenging because of the weakened DNA adsorption to the surface. The problem has been circumvented by the discovery that divalent cations play a critical role in DNA adhesion [46,135,137] to mica surfaces and by the development of tapping mode in liquids. However, the highest reproducible spatial resolution was obtained by depositing DNA on a cationic lipid bilayer and imaging in contact mode under liquid. This technique allowed the right-handed helix of double-stranded DNA to be resolved, which requires a resolution of 2–3 nm [126]. Recently, DNA structural changes in response to protein binding have been investigated using the AFM [121]. This type of study is important, since it is believed that structural changes of DNA play an important role in the transcription process (see next section). Another spectacular achievement was the observation of DNA motion on mica surfaces in solution and its digestion by enzymes [11,18,45].

**B. RNA.** The expression of genetic information represents a complex hierarchy of sequential, interrelated steps in the progression from gene activation to protein production necessary for an organism’s survival. Each component of this reaction mixture relies on the other for survival. The first and most studied ingredient is DNA, which serves as a template for the production of RNA in a process known as transcription. RNA holds the sequence of many thousands of proteins, and thus serves as a template for the production of proteins in a process called translation.

RNA differs from DNA by a single base [thymidine (T) is exchanged for uracil (U)] and contains a ribose instead of a deoxyribose sugar residue to make up the molecule’s backbone. These seemingly small changes dramatically alter the structure of RNA compared to DNA. RNA is usually single-stranded (ss) and can adopt complex secondary structure depending on the extent of intramolecular base pairing. Ternary complexes containing linear DNA, nascent RNA transcripts, and RNA polymerase have been imaged using AFM [46,121,149]. More recently, transcription has been observed in real-time imaging using tapping mode AFM [41,68] in which DNA processively translocates through an RNA polymerase molecule adsorbed to a mica surface.

Surprisingly, few data exist to date on the observation of the structure of RNA using AFM, most probably because of the difficulty of imaging ssRNA on an atomically flat surface such as mica. Lyubchenko et al. [87,88] measured the lengths of double-stranded (ds) reovirus RNA on silanated mica and found lengths ranging between 0.2 and 1.8 μm. The molecules exhibited convoluted shapes and in some cases compact structures. Hansma et al. [49] studied an ssRNA homopolymer poly(A), which showed two conformations in AFM: short and lumpy; or longer, less lumpy, and straighter. The sizes of the short molecules ranged from 100 to 300 nm, consistent with expected molecular lengths of 400–600 bases. The longer molecules appeared to be ds molecules of poly(A).

Heteropolymer ssRNA was observed with AFM and is shown in Figure 5 [60]. This figure depicts nascent RNA molecules produced from stalled ternary complexes of Escherichia coli RNA polymerase with a small circular template [25] adsorbed onto mica and imaged in air after transcription was allowed to proceed in the AFM fluid cell. RNA molecules up to 2 μm can be observed. Many molecules seem to wrap around each other, possibly forming interstrand base pairing. More studies are needed to delineate the structure of ssRNAs with AFM.

**C. Proteins.** Proteins are synthesized by small cellular organelles termed ribosomes. Ribosomes read the messenger RNA and assemble proteins by linking amino acids according to the RNA sequence in a process called translation. Each living organism is constituted of thousands of different proteins having specific functions such as transport, defense, regulation, catalysis, motion, or maintenance of structure. The precise way proteins accomplish their functions is far from being completely understood, and the AFM is a welcome additional tool to achieve this extraordinarily difficult task. A few representative proteins from each of the aforementioned categories have been studied using the AFM. These studies are difficult for two main reasons. Proteins are...
easily deformed under the tip, and in many cases it is not a trivial task to adhere molecules onto a flat surface strongly enough so that they are not swept away by the scanning tip. Several approaches have been developed, such as crosslinking to the surface [66,67,139], cooling down the sample [43], or modifying the protein, by adding to its structure a specific amino-acid sequence, so that the protein can bind to another molecule already anchored to the surface [63]. Some proteins can form two-dimensional protein crystals [20,56,67,78,81,125], which increases their lateral stability. Using these techniques numerous proteins have been observed [82], sometimes with submolecular resolution [78,102,104,125]. Larger protein strands can often be accessed using very simple sample preparation methods. Figure 6 depicts a case in which collagen, an assembly of filamentous protein, has been deposited on a Millipore® filter and imaged in air. Collagen is the most abundant protein of vertebrates and is the major stress-bearing component of the connective tissue, occurring virtually everywhere in the human body, and has previously been studied with AFM by several groups [5,9,22,39,107,120,129].

**D. Viruses.** Viruses are parasitic entities composed of the two previously described constituents: nucleic acids (DNA or RNA) and proteins. The most complex viruses are surrounded by a lipid bilayer and a glycoprotein envelope. Since they can not multiply without a host organism and they lack a metabolic apparatus, viruses are not considered to be alive. It is well known that viruses can infect bacteria and plants as well as humans. Viral diseases varying in severity from pox to the common cold have plagued mankind since the beginning of history. Their structure can be readily studied using electron microscopy as well as AFM [64,89,149]. The AFM resolution on these type of samples is, for the moment, more or less comparable with electron micro-

**Figure 5.** Tapping-in-air image of RNA produced on a mica substrate. Rolling circle complexes composed of small circular ssDNA and RNA polymerase were adsorbed onto mica, and transcription was allowed to proceed on the surface. The sample was dried and imaged in air. The arrow denotes an active transcript attached to a nascent RNA polymer.

**Figure 6.** Collagen fiber deposited on a Millipore filter and imaged in air using the tapping-mode AFM.

**Figure 7.** AFM image of a bacteriophage T4 virus. The head, tail and tail fibers of the virus are resolved by the AFM (by Ikai et al., 1993 [61], reprinted with kind permission by *FEBS Lett.*).
AFM image of kidney cells infected with pox virus. African green monkey kidney cells were infected with pox virus having a size of 200–300 nm. At 19 h following the infection, a virus is imaged leaving the cell at the end of a microvillus (by Horber et al., 1992 [58], reprinted with kind permission by Scann. Microsc.).

inaccessible to any other instrument at such high spatial resolution.

E. Bacteria. Bacteria are unicellular living organisms belonging to the group of the prokaryotes. Procaryotic organisms differ from eucaryotic organisms (e.g., algae, humans, etc.) in several ways. The most noticeable one is that their genetic material is not enclosed within a nucleus. The average size of a bacteria is around 2 µm, but some of them can reach astonishing lengths, such as the several-hundred-micrometer-long Spirochetes. It has been well documented since Pasteur’s time that numerous types of bacteria are pathogenic. The list of diseases induced by these organisms continues to grow, and several pathologies which previously were thought to be induced by noninfectious agents happen to have bacterial etiology. Examples are the stomach ulcer and possibly arteriosclerosis. According to some recent studies, Alzheimer’s disease may also be linked to bacterial infection [105,106]. Figure 9 is an AFM image of one of these bacteria which may play a role in Alzheimer’s.

Here again, the AFM resolution in air is similar to that obtained by the electron microscope. The advantage in using an AFM is the fact that the sample preparation is very fast and simple [72]. However, bacteria preparation for AFM imaging in liquids is a more difficult task, and as far as we know only a few groups have imaged such samples [37,131].

F. Cells. Cells are the smallest living units of organisms such as fungi, plants, and animals. The human body contains at least 200 different types of cells. A typical eukaryotic cell is isolated from its exterior by a bilayer lipid membrane and contains several different organelles among which the nucleus (housing the genetic information necessary for replication and survival) is the biggest one. Cell sizes can vary from 7 µm in diameter for red blood cells to almost 1 m in length for some nerve cells. Cells were among the first biological samples to be imaged by AFM [21]. In the beginning, only fixed cells (nonliving) were studied [115]. Later, it appeared that living cells could be imaged, too, and several interesting dynamic features have been observed on their surface [7,15,38,57,71,80,128,130,132]. It also appeared that several internal features can be observed by the AFM [52,74,128]; however, the exact contrast mechanism which makes this possible is not very clear [53,79]. Observing living cells with the AFM is not a trivial task; their softness and roughness seriously limits resolution. In addition, in some cases it can be difficult to anchor them onto a flat surface. One way to overcome this problem involves blocking round-shaped cells into the mouth of a micropipette or a Millipore® filter and to image the protruding part [57,59,73]. An additional problem is the tip-induced perturbation of the cell. It seems that the scanning tip is a stimulus to the cell and can induce some observable reactions. Monika Fritz and collaborators monitored the activation of platelets and suspected the scanning tip to be the activator of the process [32]. The resolution of the AFM on whole cells fits somewhere between the optical and the electron microscope. The AFM advantage, however, is that it simultaneously can record some of the mechanical properties of living cells.

G. Tissues and Organs. A tissue is composed of a group of cells having more or less the same shape and sharing the same function. The human body contains muscular, epithelial, connective, and nervous tissues. An organ is a functional unit composed of the four different tissues, i.e., kidney, brain, etc. Tissues and organs are the privileged study domain of the optical microscope; however, in some cases, the AFM has been used to study the morphology of some organs as well as some phenomena inaccessible to the traditional optical microscope such as the attack of tooth enamel in acid solutions [70]. Some other organs were studied by AFM such the kidney [136] and the cornea [36]. One limitation in the use of the AFM for the observation of tissues is that the accessible range of movement for the tip in the vertical (z) axis is restricted to 15 µm, prohibiting observation of extremely rough structures. Here again, the sample preparation is a difficult task and no standard preparation methods exist [13,69,77].

VIII. NONIMAGING APPLICATIONS IN BIOLOGY

A. Sensing Force Interactions with the AFM. In addition to imaging, the AFM can be used as a force sensor to estimate the bond strength between different biological molecules such li-
gands and receptors. The AFM is well adapted to these type of studies, as demonstrated by reports of measurements of interactions as weak as a single hydrogen bond [54]. The principle of these experiments involves attaching one type of molecule to the tip and binding another kind on the substrate. The tip is then approached to the surface to let the two molecules interact. By pulling the tip back, the cantilever deflects as a function of the binding strength between the two molecules. This system has been successfully applied to probe biotin–streptavidin [30], avidin–biotin [103], cell-adhesion proteoglycans [23], antigen–antibody [24], and complementary DNA strands [83] interactions. The principle of such experiments is illustrated in Figure 10.

These studies are motivated by the fact that molecular recognition mediated by ligand-receptor binding is a key process in every living organism, and the forces involved in it are largely unknown [84].

B. Determining Mechanical Properties at the Nanoscale. Testing the micro- and submicromechanical properties of biological samples is also within reach of the AFM. Viscoelastic properties of several biological materials have been obtained, from cells [6,7,114,115,130] to organs [133], using this instrument.

C. Monitoring Protein Dynamics. To accomplish their enzymatic function, many proteins change their conformation while reacting with their substrate. This motion in some cases involves changes in length of several angstroms for a time scale which can extend for seconds and can, in principal, be detected by AFM. The study of such motions is the key to understanding the chemical and physical processes involved in enzyme reactions. Recently, the activity of lysozyme has been detected with AFM [118] and a method for detecting single protein motion has been developed [134]. Previously, several other dynamic processes involving single proteins have been observed using the AFM such as antigen–antibody complex formation [143] and immunoglobulin adsorption [85] and fibrin polymerization [27].

IX. THE FUTURE OF AFM IN BIOLOGY

Having outlined some of the most recent and exciting results published, we hope the reader now has a feel for the prospects...
of AFM in biology. The benefits that AFM brings to biology are its ability to obtain information on structure, dynamics, mechanical properties, and force interactions, all at the nanometer scale. We see future research efforts diverging into three major categories: 1) determining high-resolution structure, 2) probing mechanical properties and determining force interactions, and 3) monitoring biological processes and dynamics. Researchers are currently working on ultrahigh-resolution studies of proteins and DNA. Methods include using cryo-AFM [43] to increase sample rigidity and stability, and a good understanding of the system studied to produce samples with optimum potential for high-resolution imaging. Researchers will continue to functionalize the AFM tip and, in Hermann Gaub’s words, go “fly-fishing” for molecules on a surface to measure mechanical properties of single molecules and individual bond strengths. These ideas may ultimately be advanced to use the AFM as a biosensor in the medical profession to screen samples for a particular chemical or biological agent. Finally, the ability to acquire movies (sequential images) under aqueous environments will be used to follow biological processes, dynamics and macromolecular assembly occurring in real time.

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