Atomic force microscopy and other scanning probe microscopies
Helen G Hansma and Lía Pietrasanta

The highlight of the past year is the unfolding and refolding of the muscle protein titin in the atomic force microscope. A related highlight in the intersection between experiment and theory is a recent review of the effects of molecular forces on biochemical kinetics. Other advances in scanning probe microscopy include entropic brushes, molecular sandwiches and applications of atomic force microscopy to gene therapy.

Address
Department of Physics, University of California, Santa Barbara, CA 93106, USA

Abbreviations
AFM   atomic force microscopy/microscope
SFM   scanning force microscopy/microscope
SICM   scanning ion conductance microscopy/microscope
SPM   scanning probe microscopy/microscope
STM   scanning tunneling microscopy/microscope

Introduction

Scanning probe microscopy (SPM) continues to be an innovative and rapidly growing field of research. Of the many SPMs, atomic force microscopy (AFM) is currently the one that is being used most for biomedical research. Therefore, our review mostly covers references to AFM, also known as scanning force microscopy (SFM).

AFM is also being used for applications in which no scanning is required, where the atomic force microscope tip is used as a sensitive force sensor. These AFM applications are particularly interesting and are discussed here. As these applications do not always use scanning and usually sense forces more in the molecular than in the atomic range, the name 'force microscopy' [1] is probably more descriptive for this research than AFM or SFM. Of the other SPMs, scanning tunneling microscopy (STM) and scanning ion conductance microscopy (SICM) will be mentioned briefly.

The field of biological AFM is continuing to grow rapidly, as shown by the citations in Medline (Figure 1). As AFM matures from being a novelty to being a practical research tool, an increasing number of papers that use AFM do not mention AFM in their titles (Figure 1). The breadth of the AFM literature in the past year includes a mention even in the *American Journal of Psychiatry* [2], which features an image of β-amyloid fibrils, a pathology characteristic of Alzheimer's disease. Reviews of AFM and SPM [3,4••,5–13] have appeared in periodicals in such diverse areas as radiation oncology and nephrology. The *Journal of Structural Biology* published a special issue devoted to SPM (July 1997).

A new journal, *Probe Microscopy*, was launched in 1997 as a forum specifically devoted to the science and technology of SPM. AFM and SFM have been also newsworthy items in *Science* and *Nature* in the past year [14••,15•–17•,18••,19].

An introduction to AFM is covered well in a recent issue of *Current Opinion in Chemical Biology*, which describes and illustrates the design and mode of operation of AFM [4••]. The AFM images sample surfaces by raster-scanning a sharp tip back and forth over the surface. The tip is on a cantilever that responds to height changes on the sample surface in a way that generates a topographical map of the surface. We build on this excellent introduction by presenting some of the many advances in SPM that have occurred since that review was written. (The accompanying images are from AFM research in the authors' lab).

Atomic force microscopy imaging
Proteins
Entropic brush
A clean space around neurofilaments was the clue that the sidearms of neurofilaments might be in constant thermally driven motion and therefore function as an entropic brush. This model has been supported also by force–distance measurements in the AFM that show a weak repulsive force, extending >50 nm from the core of the filament [20••].

Cold myosin
Cryo-AFM has revealed differences in the lengths of the myosin tails between phosphorylated and nonphosphorylated myosins that have not been detected with electron microscopies [21•]; for a commentary, see [22]. The cryo-AFM reduces thermal motion of molecules by imaging samples in a liquid nitrogen vapour.
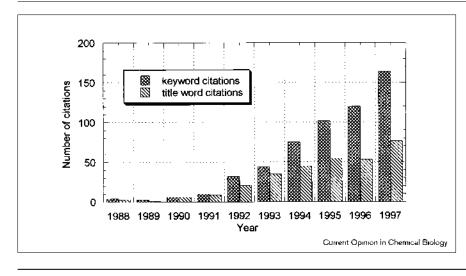
Laminin flexing its arms
Laminin, a major basement membrane protein, shows a variety of conformations of its cruciform structure in the AFM, when dried samples are imaged in air [23•] (Figure 2a). Sequential images of an individual laminin molecule in aqueous solution (Figure 2b) show the flexibility of the laminin arms as they move and bend [23•]. Imaging biomolecules and processes in aqueous solutions continues to be difficult. In view of this, it is interesting to note that many enzymes are also active in organic solvents [24•], which introduces a new possible direction for AFM imaging of processes in fluids.

Two-dimensional protein layers
It is exciting to see the growth of two-dimensional (2D) protein crystals, high resolution images of these crystals and crystal defects all in the same paper [25•]. The protein reported in [25•], annexin V, crystallized on supported lipid bilayers. Yet another report of a high quality protein

Figure 1

Citations in the Medline database to 'atomic force microscop#' continue to grow rapidly. Results are shown for keyword searches and title word searches. 'Atomic force microscop#' is the best search request for retrieving articles about AFM/SFM without also retrieving unwanted references.

crystal has come from Mueller *et al.* [26•], again showing force-dependent conformation changes. Both surfaces of the 2D crystals of a bacteriophage head–tail connector were imaged, showing 12 subunits of the wide connector domain, with a right-handed vorticity.
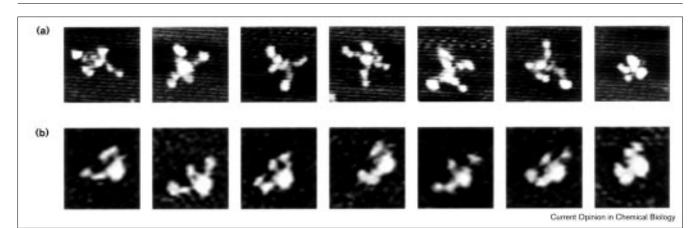
DNA–protein interactions
Transcription is a central biochemical process in gene expression that is still not fully understood. Two groups [27,28•] have investigated the mechanisms that regulate the initiation of transcription in bacteria, providing new insights for understanding common mechanisms of all transcription. Rippe *et al.* [28•] visualized and analyzed, by choosing appropriate conditions, the structure of various intermediates in the transcription process, using *Escherichia coli* RNA polymerase.σ^{54} (RNAP.σ^{54}). The distribution of bending angles under different conditions suggested that the transition from the RNAP.σ^{54} closed complex to the open complex was accompanied by an increase of the DNA bending angle associated with the changes in the spatial relationships between the DNA and the protein.

The challenge of imaging biochemical processes in liquids in real time is to have the molecules bound to the surface to be imaged by the tip and, at the same time, to have them free to move relative to each other. Recent improvements in deposition (cations) and in imaging conditions (tapping mode in fluid) under liquid have made it possible to visualize the RNAP.σ^{54} activity. A collaboration of our lab with another [29••] has detected the activity of RNAP.σ^{54} in sequential AFM images by observing the translocation of double-stranded DNA along RNAP.σ^{54} after the addition of ribonucleoside 5′-triphosphates. We have obtained a few intermediate images of the transcription process.

Figure 2

The 900 kDa protein, laminin, in the AFM, showing (**a**) conformations adopted by this cruciform protein in air, and (**b**) motions of the laminin arms in aqueous buffer. Images in (a) are 140 nm × 140 nm. See [23•].

The repair of double-stranded breaks is critical to normal development of the immune system and the maintenance of genomic integrity. The exact role of the DNA-dependent protein kinase (DNA-PK) and Ku (a nonhistone DNA-binding protein) in this process remains under investigation. Several groups have examined the binding interactions of Ku protein with DNA [30] and the behavior of Ku and DNA-PK in association with linear DNA using AFM [31,32].

Allison *et al.* [33•] have reported the successful mapping of intact DNA molecules by direct AFM imaging. They have demonstrated the accuracy and the resolution of the technique by mapping the five *Eco*RI restriction sites on bacteriophage λ DNA. Using a mutant *Eco*RI endonuclease that site-specifically binds, but does not cleave, DNA, it is possible to measure the distances between enzyme molecules bound to λ DNA. Images are presented where mutant endonuclease molecules can be clearly seen bound to six restriction sites on a 35 kb cosmid isolated from mouse chromosome 7. This technique is potentially applicable for the high-resolution identification of any protein–nucleic acid interactions of small or large genomic fragments.

Other protein–DNA studies using AFM include the binding of the wild type human tumor suppressor protein p53 to supercoiled and linear DNAs by combining agarose gel electrophoresis with AFM [34], and the study of the regulation of gene transcription [35].

We are investigating the centromere-bound kinetochore complex in *Saccharomyces cerevisiae*. This specialized chromosomal structure enables accurate chromosome segregation during cell division (Figure 3; L Pietrasanta *et al.*, unpublished data).

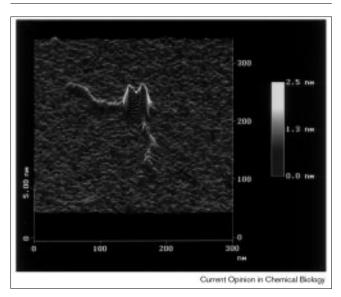### New applications for atomic force microscopy: gene therapy

In the intersection of two growing fields, AFM is being used as a tool in the development of gene therapy. AFM has been used to observe the extent of DNA condensation with polycations for receptor-mediated DNA uptake [36,37]. In related work, DNA condensation into toroids ('donuts') has been observed to be induced by positively charged surfaces, without any polycation in the solution [38]. AFM has also been used for measuring the size of DNA-containing liposomes for seeking correlations of transfection efficiency with liposome radius [39,40]. AFM is valuable for this research because differences can be visualised between DNA complexes that are successful for gene therapy and DNA complexes that are not.

Other imaging modes
Molecular recognition
Tip–sample interactions can be used to map surfaces by molecular recognition when the tip is coated with a

Figure 3

Current Opinion in Chemical Biology

AFM image of centromere DNA binding factor (CBF3)–centromere (CEN)-bound DNA complexes deposited on freshly cleaved mica and imaged in air by using tapping-mode AFM. In tapping mode, the cantilever is oscillated with amplitudes of 5–10 nm and with frequencies at or near its resonant frequency. The AFM tip 'taps' the sample gently and prevents the large lateral forces that can damage or move the sample in the conventional contact mode. Image size is 300×300 nm. The z dimension (height) is indicated by the grayscale bar to the right; the mica surface is at half-maximal height. Image is displayed as a line plot at a 60° tilt angle in order to emphasize topography. A multisubunit protein complex, CBF3 binds to a short and conserved CEN DNA sequence. The DNA fragment (914 bp) contains a single binding site for CBF3 located 489 bp from one end (L Pietrasanta, unpublished data).

molecule such as biotin and scanned over a surface patterned with a molecule such as streptavidin, which is known to bind biotin [41]. Molecular recognition is a powerful capability of AFM that is becoming possible only slowly. Modified tips are available commercially [42]. These tips are coated with various molecules involved in molecular recognition.
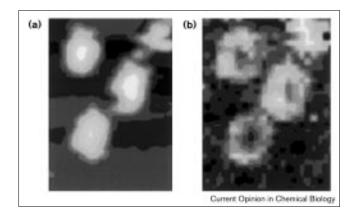
Force mapping
Forces between the tip and the sample can be mapped over the sample surface, revealing patterns of elasticity, adhesion, electrostatic forces and so on. Force mapping has been applied recently to synaptic vesicles (Figure 4), revealing hard centers in the vesicles [43] and to chicken cardiocytes, revealing patterns of stress fibers [44].

Non-imaging atomic force microscopy
Molecular forces in single macromolecules are being measured without the use of scanning, using AFMs and modified AFMs. Koshland [45] says that we are in the era of pathway quantification in our understanding of cellular metabolism and are "entering the era of 'how much?'" With the atomic force microscope it is possible to measure how much force is required to separate or unwind macromolecules.

Figure 4

Cholinergic synaptic vesicles in aqueous buffer. **(a)** The height image shows that vesicles are tallest at their centers. **(b)** The 'force map' or 'force volume' image shows a pattern of cantilever deflection in which the centers of the vesicles and the mica surface are both stiffer than the peripheries of the vesicles. The stiffness of the vesicles' centers is increased by adding 5 mM $CaCl_2$ to the aqueous buffer. Image sizes are 0.32×0.43 μm. See [43].

Forces can affect biochemical kinetics in such enzymes as molecular motors. For example, if an enzyme undergoes a conformational change during the course of its enzymatic reaction, its activity will be altered by exerting a force on it along the direction of this conformational change. Thus AFM can be used to examine the mechanisms of coupling between force and enzyme activity in single macromolecules [46].

Titin unfolding
Titin is a huge protein that provides most of the elasticity of relaxed striated muscle. Titin unfolding with the AFM reveals a force profile with prominient 'sawtooth' waves spaced precisely 25 nm apart as successive immunoglobulin domains are denatured. This denaturation or unraveling requires a very high force sustained for only a short distance to initiate the process. Unlike a classic spring, the titin spring exhibits an enormous hysteresis: the force on the ends of the molecule must be reduced almost completely before the immunoglobulin domains can refold. These results provide a new picture of the function of titin in muscle. Originally thought to simply position the myosin band in the center of the sarcomere, titin is now believed to provide a large reservoir of extra length during muscle stretching ([47••]; for a commentary, see [18••]). Titin can also be stretched with laser tweezers but not with such high forces as with AFM [48,49].

The results of these force measurements are, in turn, tested with molecular dynamic simulations; but the latest molecular dynamic simulations of biotin–avidin dissociation with a force constant similar to the AFM cantilever are much faster than AFM dissociating experiments and show much larger rupture forces [50].

Molecular sandwiches
Another use of non-imaging AFM is the measurement of height fluctuations in a potassium channel with an AFM tip [51•]. This is a new way to probe the activity of single ion channels. The potassium channel on the AFM tip is lowered onto the mica surface and height fluctuations are measured as physiologically stimulating fluids flow past the tip.

Membrane potential and atomic force microscopy
The integration of electrophysiology and AFM is a promising area that has been slow to develop. What is happening to the membrane potential of a cell while it is being scanned with an AFM tip? This technically difficult issue has not yet been resolved. Another approach is to probe the motions of voltage-gated ion channels, such as the *Shaker* potassium channel of *Drosophila*, which have a polypeptide segment that moves into the extracellular space during membrane depolarization. Such movements have been probed with an AFM cantilever on the surface of a cell attached to a patch pipette for applying changes in the membrane potential. Cells moved outward 0.5–15 nm in response to depolarization regardless of whether the *Shaker* potassium channel was present; in cells containing the *Shaker* potassium channel, however, this outward movement correlated with the magnitude of the holding potential [52].

Other scanning probe microscopies
Scanning tunneling microscopy
An unexpected development in STM of macromolecules is the ability of STM to image biomolecules on an insulating surface — mica — in a humid environment [53]. Furthermore, STM is useful for imaging conformations of adsorbed porphyrin molecules on different single-crystal metal surfaces and as they pass through metastable states on the way to their final state of adsorption [54].

Scanning ion conductance microscopy
SICM has been used to image the patterns of ion conductance on the surface of protein layers from the nacre, or mother-of-pearl, of the abalone shell. This has brought about a new paradigm for the growth of this remarkably strong biomaterial. The new paradigm is that the mineral layers of nacre are aligned because the mineral can grow through pores in the protein layer, producing a single crystal connected by mineral bridges between the layers rather than by heteroepitaxial growth of the mineral on each new protein layer ([55•]; for a commentary, see [19]). Developers of an SICM tip now have a calcium-sensing probe — a tip waiting for a new SPM to be built around it [56]?

Conclusions
As SPM continues to mature, we anticipate that there will be continued advances both in new science with existing SPMs and in the development of new SPMs. New findings will certainly be made in the field of molecular pulling. In

the field of microscope development, new AFMs are being built using short cantilevers that can scan at higher speeds with less thermal noise [57].

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