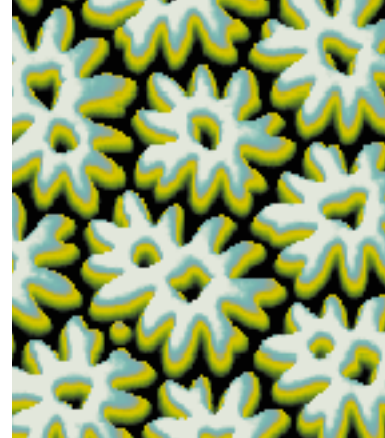


Atomic force microscopy

Alastair Smith



● The principle of the atomic force microscope

Microscopes have historically been tools of great importance in biological science. The atomic force microscope (AFM) is one of a family of scanning probe microscopes which has grown steadily since the invention of the scanning

tunnelling microscope by Binnig and Rohrer in the early eighties for which they received the Nobel Prize for Physics in 1986. The AFM uses a cantilever, usually made from silicon or silicon nitride, with a very low spring constant, on the end of which a sharp tip is fabricated using

semi-conductor processing techniques (Fig. 1). When the tip is brought close to a sample surface the forces between the tip and sample cause the cantilever to bend and this motion can be detected optically by the deflection of a laser beam which is reflected off the back of the cantilever. If the tip is scanned over the sample surface then the deflection of the cantilever can be recorded as an image, which in its simplest form represents the three-dimensional shape of the sample surface. Many variants now exist which use special tips to probe the electric, magnetic (Figs 2 and 3) or thermal properties of surfaces, and even optical spectroscopy is now possible with about 50 nm lateral resolution using scanned probe techniques.

The resolution of AFM depends mainly on the sharpness of the tip which can currently be manufactured with an end radius of a few nanometres. Atomic resolution is easily obtained on relatively robust and periodic samples. Soft samples however, particularly biological samples, provide a more difficult surface to image because the forces exerted by the tip during imaging can cause deformation of the sample. The problems involved with imaging soft samples have been overcome to a large extent by the introduction of tapping mode AFM imaging. Instead of maintaining a constant tip-sample distance of a nanometre or so, the cantilever is oscillated in a direction normal to the sample resulting in only intermittent contact with the surface. This greatly reduces the lateral forces being applied in the plane of the sample which are responsible for most of the damage as the tip is scanned. The AFM is capable of better than 1 nm lateral resolution on ideal samples and of 0.01 nm resolution in height measurement.

There are some significant advantages of AFM as an imaging tool in biology when compared with complementary techniques such as electron microscopy. Not only does AFM achieve molecular resolution but the technique requires almost no sample preparation and, most importantly, can be performed under fluids, permitting samples to be imaged in near native conditions. The fluid may be exchanged or modified during imaging and

therefore there is the potential for observing biological processes in real time. Several instruments are commercially available for around £100K and in general the technology is straightforward to use and occupies only a small table top.

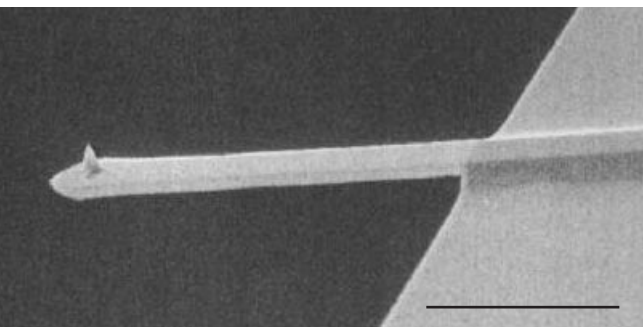
● High resolution imaging

There have been many studies of biological materials using AFM in the few years since its conception, including nucleic acids and their complexes with proteins, two-dimensional protein crystals and individual isolated proteins, membranes and membrane-bound proteins, and living cells.

One of the most extensively studied systems is nucleic acids which have now been imaged with sufficient resolution to measure the pitch of the double helix. Nucleic acids may be deposited on mica quite simply by using a divalent cation to bridge between the negative backbone of the nucleic acid and the negatively charged mica surface. Most recently there have been several reports of AFM imaging of the complexes formed between proteins and DNA which have, for example, provided detailed information about the changes in conformation of DNA in response to protein binding. The one-dimensional diffusion of RNA polymerase along DNA has been imaged in real time and the enzyme was seen to slide along DNA and even hop to neighbouring nucleic acids on the substrate surface. Fig. 4 shows one recent example of AFM imaging of a protein/DNA complex. Tapping mode AFM has been used to image an individual complex of human transcription factor 2 with DNA. It appears that a protein-protein interaction has facilitated looping of the DNA to allow two distal DNA sites to be brought together on the substrate. A goal of some workers is to develop AFM imaging techniques to a point where the DNA sequence can be read; however, this ambitious goal remains elusive.

The highest resolution images are obtained on tightly packed structures such as two-dimensional arrays of proteins because the packing affords a greater mechanical stability to withstand the imaging forces. In cases such as this the resolution of the images is better than 1 nm because the highly regular assembly of proteins also allows averaging to be performed which greatly improves the signal-to-noise ratio. The quality of information in such images has been demonstrated by Engel and co-workers who have reported real-time observation of the central pore of proteins in the HPI layer of *Deinococcus radiodurans* opening and closing. Another example, shown in Fig. 5, is the molecular resolution tapping mode AFM image of the periplasmic surface of OmpF porin from *Escherichia coli* taken in buffered solution. A rectangular unit cell arrangement ($a = 135 \text{ \AA}$, $b = 82 \text{ \AA}$) can be seen each containing two porin trimers with a lateral resolution of about 8 Å.

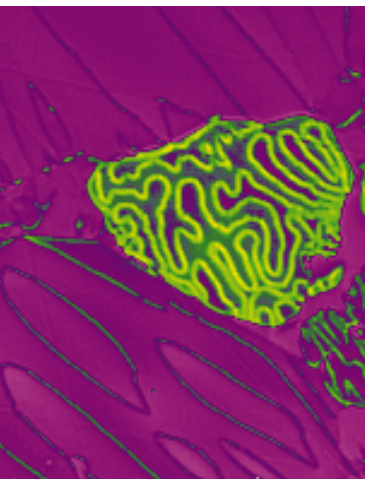
Imaging of living cells is not straightforward because the cell surface is extremely soft despite the structural support of the cytoskeleton. Obviously deformation of the sample during imaging can seriously limit the resolution which can be achieved on cells and although the overall shape can be



ABOVE:
Fig. 1. Electron micrograph of a typical tapping mode AFM cantilever shown 'upside-down' to reveal the sharp tip used for imaging. Bar, 100 µm.
COURTESY DIGITAL INSTRUMENTS, VEECO METROLOGY GROUP

TOP RIGHT:
Fig. 2. Magnetic AFM image of a garnet film showing flower-like magnetic domains.
COURTESY DIGITAL INSTRUMENTS, VEECO METROLOGY GROUP, AND V. FRATELLO

BELOW:
Fig. 3. Magnetic AFM image of a praseodymium-iron-boron permanent magnet.
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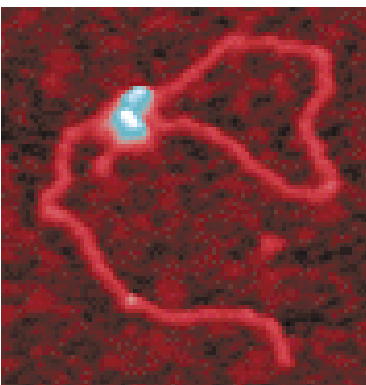


measured easily, surface detail is difficult to obtain. In some cases the nucleus can be clearly seen in the images, which suggests that it is less easily deformed than the cell membrane, and movement of the actin filament bundles beneath the periphery of the cell membrane has been observed.

● Measurement of biological interaction forces

In addition to the potential of the AFM to provide very high resolution images of biological samples and to monitor conformational changes and biomolecular processes in real time under native conditions, the instrument is also capable of manipulating molecules and measuring the strength of biomolecular interactions with piconewton sensitivity.

The forces exerted between the silicon nitride tip and the sample arise principally from van der Waal's interactions. These interactions are rather non-specific in the biological sense but lead to bending of the cantilever which provides the topographical information for the images. However, it is relatively straightforward to modify the tip surface chemically so that its interaction with the sample may be made highly specific. For example the tip may be modified to have a charged surface, one which readily forms hydrogen bonds or may be made very hydrophobic. In these simple cases it is the interaction with the sample which bends the cantilever and therefore the information that is contained in the images has chemical information about the tip-sample interaction. Tip modification is usually achieved using the well known process of thiol self-assembly on gold. The tip is first coated with a thin layer of gold by evaporation, then immersed in a solution of ω -functionalized alkyl thiol molecules. These molecules have a functional head group which will provide the tip with the desired physico-chemical property and an



alkyl chain spacer of about 10 carbons terminated with a thiol group. The sulphur spontaneously forms a covalent bond with the gold coating on the tip and the molecules pack tightly to form a well ordered monolayer with the functional group uppermost, creating a new surface of tailor-made chemistry on the tip.

Tips may also be modified to have biological functionality. For example, antibodies can be tethered to the tip via flexible polyethylene glycol spacers, permitting the antibody to bind to an antigen on a sample surface. Using such a tip, the distribution of antigens over a cell or other surface may be mapped, but it is also possible to pull the antibody-antigen complex apart by moving the tip away from the substrate on which the antigen is absorbed and measure the strength of the interaction directly. The binding event between single pairs of streptavidin/biotin

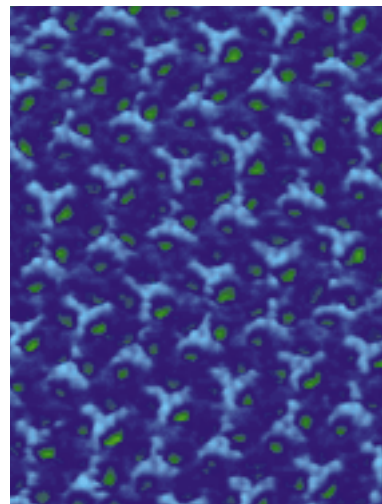
molecules has been observed by several groups and measured to be of the order of a few hundred piconewtons. The resolution of the AFM as a force or binding strength measuring device is of the order of about 10 piconewtons, limited by the thermal noise of the cantilever at room temperature. An exquisite example of the measurement of such biomolecular interactions was reported by Lee and co-workers at the Naval Research Labs in Washington. Two complimentary DNA oligonucleotides, one tethered to the tip and one to a gold substrate, were allowed to interact and then pulled apart whilst the forces required to rupture the double helix conformation were measured.

Perhaps one of the most exciting demonstrations of the potential of the AFM to measure biomolecular interactions has been the recent demonstration by Gaub's group, and others, of the mechanical unfolding of single proteins. Gaub has shown that the forces required to unfold the subdomains of titin, the giant muscle protein, can be measured by AFM. In these experiments the AFM tip is used to pick up part of the vast titin molecule whilst the rest remains absorbed to a gold substrate. The tip is then withdrawn and a series of small tugs on the cantilever can be observed which have been attributed to the unfolding of the immunoglobulin and fibronectin domains. The forces measured are in two phases. First, the extension of unfolded chains which appear to fit well to a worm-like chain model and second, a dramatic step which is identified with the catastrophic unfolding of a domain. This type of measurement, which has initiated a flurry of activity in mechanically manipulating biomolecules, is only the first step in a developing new field of biophysics based on AFM technology.

● The future

The fact that AFM is relatively cheap technology and straightforward to use suggests that it will be accepted quickly as an imaging tool in biology. The ability to perform real-time observations of biological processes under native conditions will be of great interest and as the biochemistry is developed to modify tips in more complex ways to attach proteins, peptides and small organic molecules, then the detailed measurement of biomolecular interactions appears to be an area with very great potential. The simplicity of the instrument also lends itself to integration with other instruments. In the author's laboratory, as in several others, AFM is being combined with laser spectroscopy so that mechanically induced conformational changes can be monitored by fluorescence or infra-red spectroscopy. It seems that AFM is likely to become one of the most widely used high resolution microscopy techniques in the biological sciences.

● *Dr Alastair Smith is a lecturer in the Department of Physics and Astronomy at the University of Leeds. His current research is focusing on the application of laser spectroscopy and scanning probe microscopy to the study of biomolecular structure and dynamics. e-mail d.a.m.smith@leeds.ac.uk*



ABOVE:
Fig. 5. Image of the periplasmic surface of OmpF porin from *E. coli*. COURTESY DIGITAL INSTRUMENTS, VEECO METROLOGY GROUP, AND A. ENGEL

LEFT:
Fig. 4. Tapping mode AFM image of an individual complex of human transcription factor 2 with DNA. COURTESY DIGITAL INSTRUMENTS, VEECO METROLOGY GROUP, AND C. BUSTAMANTE

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