Applications of AFM

Peter Eaton
Paul West

DOI:10.1093/acprof:oso/9780199570454.003.0007

[Abstract and Keywords]

AFM has been applied with great success to an incredibly wide range of scientific and technological fields, and in the final chapter we present a range of applications that show the breadth and depth of the uses of AFM. In particular, the examples have been chosen to illustrate many of the different types of experiments that can be carried out by AFM, as well as a sampling of the wide range of samples that may be studied. The example applications are drawn from materials science, chemistry and physics, nanotechnology and nanoscience, biology, biophysics and biochemistry, and the use of AFM in commodity and high-technology industries.

Keywords: physics, chemistry, materials science, biology, biophysics, nanotechnology, nanoscience, industry, applications, AFM
Although AFM, via STM, had its origins in imaging of metals and semiconductors, it was originally invented in order to be able to extend the possibilities of STM to other samples, in particular biological samples. AFM’s ability to image a wide variety of samples, coupled with its simplicity of operation and the relatively low cost of the instruments, means that AFM very quickly gained acceptance in an extremely wide range of fields. In this chapter we arbitrarily divide the illustrative examples into life, physical, and nanosciences, and industrial applications. However, AFM recognizes no such boundaries, and it is hard to think of any field of study involving a solid surface that the technique has not been applied to. The use of these categories can be useful, if only because AFM users have traditionally assigned themselves to one of these areas. However, as illustrated below, the intense interest in nanotechnology and nanosciences has further blurred the distinctions between these areas. This is partly because by definition just about everything AFM studies is defined on the nanoscale, since almost all AFM images have a resolution greater than 100 nm. The range of samples that have been studied by AFM is staggering. Although the areas shown in Figure 7.1 probably cover over 90% of AFM use, AFM has also been used in such diverse areas as art conservation [384, 385], astrobiology [386], geology [387], and food science [388, 389], amongst others.

The variety of applications of AFM is so large that it’s not possible to even mention them all in one book. Instead we have chosen to highlight in this chapter a few applications in each of the categories mentioned above. Mostly, these examples are chosen to illustrate the capabilities of AFM, in particular the different modes and kinds of experiments that can be carried out, rather than to be exhaustive lists of all the applications of AFM to any one field. In each of the four main sections, the introductory paragraph gives an overview of the main applications within the field, and we highlight important advantages of AFM for the field, and more detailed reviews to guide the interested reader to comprehensive summaries of applications within the field.

7.1 AFM applications in physical and materials sciences

The very first applications of AFM were in surface science, and this discipline is still a heavy user of AFM, as well as STM. The ability of AFM to image with atomic resolution is a key advantage for the physical sciences, while the measurement of electrical, magnetic, or mechanical properties also extends the range of possible applications.

Fundamental applications in physical sciences include imaging of the fine structure of metals and absorbed species on metals and semiconductors; although this is one area where STM is still more widely applied than AFM, some studies show information from AFM can under some circumstances exceed that available from STM. AFM imaging is very suitable for structural studies of inorganic and organic insulators and has been widely (p.140)
Fig. 7.1. Application areas where AFM is most commonly used.

applied [372, 390–392]. Such fundamental studies of pure surfaces which require atomic or near-atomic resolution are often carried out at low temperatures, in vacuum or both. These types of experiments are outside of the scope of most commercial AFM instruments. Structural studies of molecules have mostly focussed on high-resolution imaging of assemblies of molecules packed into crystals [390], because resolution is generally higher when the molecules are arranged in such highly-ordered structures. However, one of the strong points of AFM is that unlike, for example X-ray diffraction, single molecules can also be studied, and the ability to crystallize the sample is not a prerequisite for AFM imaging. Molecular interactions are commonly studied by AFM, both directly by chemical force microscopy [142] and by studying the topography of the complexes they form [393], and this sort of study is greatly aided by the ability to image in different environments, enabling the study of interactions in situ.

The ability of AFM to directly study the sliding of materials over each other (i.e. lateral force microscopy) means the technique is very useful in fundamental work on friction and wear, which is of vital importance in materials science [189, 394, 395]. In fact, AFM is a particularly useful technique in materials science, and other popular applications include the topographic, tribological, roughness, and adhesion/fouling characterization of a wide variety of technologically useful materials [395–397]. Mechanical characterization of materials is also an area where AFM can contribute to their study, especially when studying materials that are heterogeneous on the nanoscale [158, 398–400]. For biomaterials, the modification of the materials by proteins, cells or other biological materials is ideally suited to analysis by AFM due to its ability to image both soft and hard materials [398, 401].

7.1.1 Roughness measurements of high-performance materials
Surface roughness is an extremely important parameter for many material surfaces. Surface roughness can affect adhesion to other materials, optical and electronic properties, surface energy, bioadhesion and other properties [402–405]. Surface roughness is easy to (p.141)
measure with AFM, due to the fact that AFM produces high contrast on relatively flat surfaces, and produces three-dimensional, digital data by default. In contrast, scanning electron microscopy often gives the impression that surfaces with roughness values less than 10 nanometres are extremely smooth and featureless, due to the fact that SEM detects electrons scattered from a few nanometres into the surface. On the other hand, optical techniques are limited in resolution, and require opaque surfaces. AFM has no such restrictions in terms of samples, and gives accurate values for surfaces with roughness down to the level of atomic flatness. In addition, neither SEM nor optical microscopy can directly supply data suitable to measure roughness parameters, but determining roughness parameters from AFM data is extremely simple. Therefore, AFM has become the method of choice to measure nanoscale roughness, and is routinely applied to determine roughness of metals and metal oxides [406], semiconductors [407], polymers [408], composite materials [158], ceramics [409], and even biological materials [398, 410].

An example of the use of such measurements is the imaging of titanium, one of the most commonly used materials for many medical implants. Because of its technological importance, and because the surface texture and roughness of the implant is very important for its performance, there have been many studies of titanium and titanium oxide surfaces by AFM [366, 406, 411-414]. For example, in the study by Cacciafesta et al., a series of titanium surfaces with different treatments were compared by AFM [411]. Some example images, of these surfaces are shown in Figure 7.2. The results showed that roughness varied enormously with surface treatment. Clearly, examination of the images shown in Figure 7.2 can give some important information on its own; for example while the polished sample shown in Figure 7.2A displays many pits, it is (p.142)

---

Fig. 7.2. AFM height images of titanium surfaces subjected to various treatments. Samples were treated by A: polishing with colloidal silica, B: polishing followed by acid etching, C: sandblasting then treating with air plasma, and D: grinding with silicon carbide paper. Adapted with permission from [411]. All images have an X-Y scale of 20 × 20 μm, and a z scale of 6 μm.
Table 7.1. RMS roughness values of the different treatment procedures represented by the images shown in Figure 7.2.

<table>
<thead>
<tr>
<th>Sample (Figure 7.2)</th>
<th>Treatment</th>
<th>$R_q$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mechanical polishing</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>B</td>
<td>Mechanical polishing then acid etching</td>
<td>270 ± 27</td>
</tr>
<tr>
<td>C</td>
<td>Sandblasting and plasma treatment</td>
<td>900 ± 300</td>
</tr>
<tr>
<td>D</td>
<td>Grinding on SiC paper</td>
<td>160 ± 110</td>
</tr>
</tbody>
</table>

considerably flatter overall than the pit-free sample shown as Figure 7.2C. However, the use of the roughness values allows a more quantitative approach to surface texture characterization. As an example, the $R_q$ (also known as rms roughness) values of the different treatment procedures represented by the images shown in Figure 7.2 are shown in Table 7.1. It is clear that the processing of these titanium implant surfaces has a huge impact on their roughness, and this is very likely to be an important factor on their performance. For example, roughness values of the surfaces on implants as measured by AFM can be correlated to the degree of bone contact and bone formation of the implant [366, 414]. Rougher surfaces appear to enhance bone contact.

Measuring roughness is a simple and quantitative way to compare surfaces. However, some practical issues must be borne in mind in order to make accurate comparisons of sample roughness. The general rule of thumb is that all scanning, processing and analysis must be identical in order to be able to compare roughness values. The main parameter that affects the value of roughness obtained is the size of the scan measured, so this must always be the same when comparing roughness of different surfaces. On the other hand, the pixel resolution has much less effect on the data obtained, varying only for extreme values [415]. See Figure 7.3 for illustrative plots that demonstrate the relationship between image size (spatially, and in terms of pixel density), and roughness.

Processing of the data, in particular levelling, will affect the value of roughness calculated. For example, with polynomial line-by-line levelling, as is shown in Figure 7.3, higher orders reduce the roughness value obtained [361]. The plot at the right of Figure 7.3 shows a line scan along a femoral hip replacement implant, subjected to different polynomial flattening treatments. The line profiles are clearly quite different, and the values of $R_a$ show that this is dramatically reflected in the roughness parameters. Depending on the reason for measuring the roughness, some levelling should always be applied, so it must simply be applied consistently. Filters should also be used with caution, as they can drastically affect the roughness results obtained.

In order for roughness results to be useful, measurement parameters including the size of the area measured and image treatment should be specified with the results [408, 416]. It has also been shown that the measured roughness depends on the probe used and its condition [408]. This is because less sharp probes will tend to smooth out the data as they cannot image fine features on the sample surface.
7.1.2 Hardness measurement of polymer films

Stiffness or hardness measurement using AFM is one of the more common non-topographic experiments. Although there are a number of other techniques able to make hardness (p.143)

![Image of data processing parameters on measured surface roughness](image_url)

**Fig. 7.3.** Illustrations of the effects of scanning and data processing parameters on measured surface roughness. Left: the effect of changing the area scanned (image size) on the measured roughness values ($R_a$ and $R_q$). In general smaller AFM scans show smaller values of roughness. Centre: the effect of changing the number of pixels in the image (pixel resolution) on the roughness ($R_q$). In general, there’s a very weak relation between the pixel resolution and the roughness value. Reproduced with permission from [415]. Right: effect of image processing (levelling algorithm) on roughness of line scans on a femoral head implant replica ($R_a$). The higher the order of polynomial applied, the lower the roughness [361]. Reproduced with the kind permission of Dr. James R. Smith, University of Portsmouth, UK. (A colour version of this illustration can be found in the plate section.)

measurements of materials, AFM-based measurements have some unique advantages. AFM-based nanoindentation was compared to measurements with a dedicated instrument in Section 3.2.2. The main advantages of AFM are high force sensitivity (hence high sensitivity to differences in sample stiffness, especially for compliant materials), and high lateral resolution, which means that small features or domains can be selectively probed. For these reasons, AFM-based nanoindentation has been widely applied in the physical and materials sciences to probe mechanical properties of micro- and nanoparticles [186, 278], metals [417], silicon [418], and many other materials [419].

Polymers have been a particular focus of AFM nanoindentation studies [168, 181]. One reason for this is that many composite polymeric materials exhibit nanoscale domains. Examples include polymers with fillers or other added particulate materials, and block copolymers. Measurement of the stiffness of such domains can help to understand their contributions to the overall mechanical properties of the bulk materials. In some cases, materials are added to a polymer specifically to change the mechanical properties, such as adding stiffness, or increasing elasticity [420]. Furthermore, the nature of the interface between the reinforcing material and the continuous polymer matrix are extremely important for the mechanical properties of such materials. AFM-based nanoindentation is ideal to probe the mechanical studies of nanoscale phases, as well as their interfaces. Furthermore, measuring the resistance to mechanical probing of a polymer surface can also help to identify individual phases in a composite material [158, 181]. An example of this is illustrated in Figure 7.4. In this case, the material under study was a commercial
silicone paint, known to include both large (100–1000 nm) CaCO$_3$ filler particles, as well as small (<10 nm) silica particles. Previous work had failed to detect Ca at the surface, which led to the assumption that features seen at the surface, while of similar dimensions to the CaCO$_3$ filler, had some other origin [421]. AFM-based nanoindentation measurements (p.144)

Fig. 7.4. Examples of nanoindentation experiments on a heterogeneous polymer surface. The sample is RTV11, a silicone polymer with calcium carbonate filler particles. The upper graphs show force–distance curves on a flat and a raised region of the RTV11, along with a steel surface for comparison. Below are shown how these experiments may be made in a spatially resolved way. Both topography and indentation images were derived from a map of force–distance curves acquired in a 10 μm square area of the polymer surface. The correlation between the white protruding features in the topography and dark, hard features in the indentation map suggest that these features correspond to the hard filler particles [158].

were made of the polymer surface via a force–curve mapping routine. This technique acquires force curves at user-defined spatial intervals over a user-defined area of the surface. In this case, 100 × 100 curves were acquired over a 10 μm square area, i.e. curves were acquired every 100 nm. Note that even at this low resolution, such an experiment can be quite lengthy – if curves were acquired at 1 Hz, the 10,000 curves would require almost three hours to record. Examples of the force curves obtained are shown in Figure 7.4. It can be seen that there was considerable heterogeneity on the data from different parts of the sample surface. In particular, when measuring curves over the raised features seen on the surface, they were shown to be considerably stiffer than the surrounding polymer matrix, as is seen in the image of indentation distance.
(Figure 7.4, bottom right). Interestingly, a softer region seems to surround each hard particle, possibly indicating some problems at the matrix-filler interface. These softer interphase domains (p.145) were approximately 100–200 nm thick, and this highlights a strength of AFM-based nanoindentation for analysis of polymer composites; namely the direct measurement of mechanical property variation at the nanoscale.

The analysis shown in Figure 7.4 was very simple; the distance the AFM indented into the sample was calculated by comparing the cantilever deflection with that obtained on a stiff surface (the steel curve shown at the top of Figure 7.4). Further details of the mechanical properties of polymers can be obtained, however. Thus, it's possible to obtain parameters of the surface such as Young's modulus ($E$) or sample spring constant, which can be compared to values obtained for bulk materials, or other materials at the nanoscale. However, to obtain such parameters from AFM data, it is necessary to know or to measure the shape of the probe, as well as the spring constant of the cantilever [168]. Methods to measure these parameters were covered in Chapter 2. Furthermore, knowing the shape of the probe, one must model it with an appropriate shape using the measured dimensions, thus the indentation into the surface may be modelled such that $E$ or the sample spring constant can be obtained [419]. AFM-based nanoindentation is a very powerful technique for the in situ characterization of the interfaces of heterogeneous polymer systems, and the nature and extent of polymer mixing, as well as other surface and interface effects in technologically important systems can be probed by this technique [168, 181, 422, 423].

7.1.3 Atomic-resolution imaging of crystal structures

One of the most exciting capabilities of AFM is the extremely high resolution that is possible. Achieving atomic resolution, however, is only possible or even useful under certain circumstances. Most solid materials are made up of a diverse collection of poorly organized molecules, meaning that atomic resolution is almost meaningless. We can really only interpret atomic-resolution images in extremely pure samples. For this reason, atomic-resolution AFM is almost entirely limited to application in the physical sciences. However, despite these limitations, some of the results available from such efforts are truly astonishing, such as the work shown in Chapter 3 from Morita and co-authors enabling discrimination of individual atoms using spectroscopic non-contact-mode AFM [8, 424].

Many atomic-resolution images produced by AFM are produced by non-contact-mode AFM (using FM detection), and often carried out in high-vacuum conditions [425]. Thus, these studies are out of the scope of most AFM systems, which need to be specially adapted for FM detection or vacuum work. However, it should be remembered that with great care, ultra-high resolution is also achievable in ambient/liquid conditions [372]. In order to discuss atomic-resolution imaging, it's important to define what is meant by atomic resolution. True atomic-resolution images allow the discrimination of individual atoms. However, some images have been described as 'atomic resolution' that do not allow this, but instead show the average arrangement of atoms on a surface. Thus, this pseudo-atomic resolution or 'atomic lattice resolution', as we shall refer to it here, does
not allow imaging of individual adatoms, or of atomic vacancies. This atomic lattice resolution is rather simple to achieve with a normal AFM under ambient conditions. The main requirements are a sharp, flexible probe, very fast scanning (>20 Hz), and a flat, very clean surface (which can be easily obtained by cleaving mica or HOPG). Two examples of atomic lattice resolution measured with AFM are shown in Figure 7.5. Both of these images were obtained in contact-mode AFM in liquid (water and ethanol, respectively).

Images such as those presented in Figure 7.5 allow us to visualize the atomic lattice of crystalline materials, and to make some measurements of it, but their use is limited. The ability to obtain true atomic resolution, and apply it to imperfect or mixed systems is much more useful. This can be achieved under UHV conditions [428] (usually though not always in non-contact FM-AFM), and also in liquid [372, 429, 430]. For instance, this technique allows researchers to observe the atomic structure of a large number of metals, semiconductors, metal oxides [26, 431, 432], and of different materials deposited on top of one another [425, 433]. Crucially, with true atomic resolution, all this is possible with (limited) mixing of components, and allows researchers to image holes, dislocations, stacking faults, sub-monolayers, etc. [372, 434], see Figure 7.6 for examples. This technique can even reveal sub-atomic detail, e.g. information of molecular orbitals can be determined in some cases [435].

True atomic resolution is more routinely achieved in STM than AFM [434]. The main reason for this is that in the imaging mechanism of STM, only the atom on the tip which is the closest to the sample will interact via tunnelling. This means that by manufacturing a ‘rough’ tip, an atomically sharp probe can be produced. Such an approach does not work for AFM, because even with a very sharp probe, the less specific nature of the interaction means that atoms much further away from the surface than the last one on the tip will also interact with the surface. However, the highly specific nature of the interaction of STM probes with the surface can give rise to some surprising results. For example, because the STM probes the electron density in the surface orbitals, changing the sample...
bias can make some atoms appear to appear and disappear [436]. A further example is the case of graphite (usually in the form of HOPG). For many years, achieving atomic resolution on graphite has been used as a quality test for both STM and AFM instruments, and it is a very widely studied material by both techniques [428, 437–439]. Some early STM studies appeared to show the expected hexagonal rings of graphite in STM images, but these were later shown to be artefacts caused by double tips [440]. Although in two dimensions, it would appear that all the atoms are equivalent, consideration of the three-dimensional structure of graphite shows that three of the atoms are in one form, named α, and three are in another form, termed β. The α atoms have lower electron density at the surface, and therefore are invisible to STM [441]. Changing the sample bias does not change this. Work by Hembacher et al. was able to show that the ‘missing’ atoms could be seen by AFM [428]. (p.147)

The experiment was carried out using a custom built instrument that allowed simultaneous measurement by STM and AFM. In order to achieve these impressive results, the experiment was carried out under UHV, and at low temperature. Simultaneous AFM and STM measurements made using this technique are shown in Figure 7.7. In this case, atomic-resolution AFM was able to give a more accurate representation of the atomic structure than STM was.
7.1.4 Friction measurement with AFM

Lateral force microscopy (LFM), or friction force microscopy, is most often applied for one of three purposes: fundamental study of tribology of macroscopic or atomic scale systems [442–444], determination of the friction properties of (uniform) materials [395, 445, 446] or characterization of heterogeneous materials based on their frictional properties [447, 448]. Like some other modes of AFM, LFM allows the determination of parameters that may also be determined by other techniques, which may be simpler to apply or easier to quantify. However, the advantage of LFM is the ability to quantify such parameters on the nanoscale, whether for fundamentals reasons (e.g. the study of tribology at the single atom level), or for practical reasons (e.g. measurement of nanoscopic inclusions in a surface).

Applications include studies of monolayers, including the friction between monolayers on the probe and on a sample surface [444, 448], friction-based discrimination of phases in polymer blends [449, 450] and discrimination of inclusions in heterogeneous polymer surfaces [448], and friction studies of carbide coatings for tool coatings [445, 447]. The ability of LFM to discriminate different chemical groups means it can be used to study phase separation in mixed monolayers [451], and is very commonly used to detect directly deposition of features by dip-pen nanolithography or nanografting, because the contrast is often better than in the height image [260, 452].

Practically, frictional measurements with the AFM are made by measuring friction loops. The term ‘friction loop’ refers to the combination of the LFM data from the forwards and reverse directions. Two example friction loops are shown in Figure 7.8. The actual friction measured on the material under study is obtained by calculating the difference between forwards and backwards scans. This calculated value is typically derived in terms of volts, but may be converted to force with the methods described in Section 3.2.3.1. The lateral force measured nearly always depends on the normal force applied (e.g. the set-point), and for many materials the relationship will be linear [448], meaning that a plot of normal versus lateral forces allows the calculation of the useful parameter \( \mu \), the friction coefficient of the material. An example of such a plot is shown in Figure 7.8.
Measuring quantitative frictional properties as described above is an important application of LFM, but what’s really unique about the technique is its ability to distinguish frictional properties of material at the nanoscale. This means that any materials that can be distinguished based on their frictional properties can be differentiated by the technique with the same resolution as contact-mode AFM, i.e. up to atomic resolution. For example, 3 nm resolution was seen in a semiconductor film sample consisting of InP and InGaAs regions [455]. As well as the very high resolution, this example highlights another important aspect of LFM for compositional mapping: many materials of very similar composition can be distinguished. Further example are the differentiation of CdCO₃ and CaCO₃ [456], many different mixed organic monolayer systems [334, 457–459], Si and SiO₂ [460] and many other mixed systems [189]. Figure 7.9 shows an example of how it’s possible to use lateral force microscopy to characterize different materials in a heterogeneous surface based on their frictional properties, in this case, filler particles in a polymer film.

### 7.1.5 Phase imaging to identify surface features

In the first description of phase imaging in AFM [190], it was described how the technique could be used to generate material contrast on a wide range of materials including composite polymer materials, wood pulp, integrated circuits, quartz/silicon surfaces and more. As described in Section 3.2.3.2, phase imaging is sensitive to viscoelastic properties of the sample and to tip-sample adhesion. This means that many materials can be differentiated by phase imaging, although it is not always possible to directly identify the

---

**Fig. 7.8.** Measuring friction. Top left: friction loop on a homogeneous material. The value of ΔV is characteristic of the materials at a set loading force. Top right: another friction loop acquired on a crystal surface shows atomic-scale stick–slip behaviour. Bottom left: plot showing variation in the friction force as a function of the load for a carboxylic acid-terminated tip contacting hydroxyl and methyl terminated SAMs. From the gradient of these plots, we can obtain μ, the coefficient of friction. Bottom right: the value of μ can be compared to other parameters. Here it is plotted versus cos θ, the water contact angle, which varies as the composition of a mixed SAM is altered. Figures adapted with permission from [336, 453] (copyright 2000 by the American Physical Society), [334] and [454].
materials based on their phase signals, and the magnitude of the phase signal is hard to

\[\text{Fig. 7.9. Example of mapping friction changes across a surface to characterize polymer film heterogeneity. The sample is a Mylar D film, including additive particles at the surface. The images show (a) left to right LFM image, (b) right to left image and (c) the subtraction of (b) from (a). The difference in (c) removes topographic effects, and resolves the ambiguity generated by the differences between (a) and (b); the darker colour of the additive particles in (c) shows they have lower friction than the film. Adapted with permission from [461].} \]

\[\text{(p.150) predict, as it depends on many factors, including the way the instrument is set up, and the scanning parameters [462].} \]

Because of its ability to distinguish many materials, phase imaging has been applied to an enormous number of samples; just some examples include differentiation of semiconductor films [196], detection of water in cracks in glasses [463, 464], nanoparticle characterization and counting [217, 465], observation of spherulites in polymer crystallization [466], polymer blend and composite composition [467–469] (see Figure 7.10), protein adsorption to biomaterials [411, 470], self-assembled monolayers [462, 471, 472], and many more systems [473]. Due to its dependence on topography, phase imaging is mostly applied to flat surfaces such as films. However, with care, phase imaging can even be applied to image differences in composition on the surfaces of small features such as microspheres [474] or micro-organisms [475].

Phase imaging is particularly useful for imaging dynamic systems, as phase images collected by high-speed AFM are often of better quality than the corresponding topography images [476]. For example, with IC-AFM scanning at high speed often means the $z$ piezo cannot respond fast enough to allow the probe to fully track the sample surface, meaning that height images will be of poor quality. Thus while scanning at high speeds, topography images can show artefacts that are not present in the phase images [477, 478].
Fig. 7.10. Examples of using phase imaging to characterize heterogeneous polymers. Left: phase imaging is sensitive to crystallinity via viscoelastic properties. In the height image (top left), it's hard to distinguish the lamellae of the polymer due to macroscopic height differences. The phase image (below) shows them much more clearly. Right: example of phase imaging of a nanocomposite. Phase images of silver nanoparticle/SBS nanocomposite films containing: (a) 0.5, (b) 3, (c) 7, and (d) 10 w.t% Ag nanoparticles. Block copolymers such as this are simply characterized by phase imaging. Reproduced with permission from [466] and [469].

(p.151) Phase imaging is also sometimes particularly useful for AFM imaging where the sample is dynamic, i.e. for imaging moving systems [472, 479].

7.2 AFM applications in nanotechnology
Without a doubt, AFM is a vital technique for nanotechnology and the nanosciences. The combination of extremely high-resolution, three-dimensional information, and local property measurement means that AFM is considered one of the most important tools in nanotechnology. AFM is used to make important discoveries in all areas of nanoscience. For many systems with dimensions of the order of 10 nm, AFM-based techniques are the only solution to make dimensional, electrical, magnetic and mechanical measurements with the accuracy required [395, 480–482]. In addition, AFM can be used to alter and even to build nanostructures [249, 266]. The examples chosen for this chapter are just a few important examples of the areas where AFM has been shown to be particularly useful.

7.2.1 Nanoparticle measurement
Nanoparticles are probably the most widespread nanostructures, and over the last 10 years or so there has been an enormous increase in the interest in their production due to their relatively simple preparation combined with unique properties. Many of the unique properties of nanoparticles are directly related to their size, for example the photoluminescence of quantum dots which changes significantly if the dots grow a few angstroms in size [483]. For this reason, it is very important to have a tool to characterize with extreme accuracy the size of such particles. Of course, the AFM is also capable of providing more information than just topography, and many other properties
of nanoparticles have been probed as well [221, 278, 484–486].

AFM can be used to measure an extremely wide range of nanoparticles including different metal nanoparticles [279, 290, 487–490], metal oxide particles [491], many types of composite metal/organic particles [123, 280, 492–494], synthetic polymer particles [480, 495, 496], biopolymer nanoparticles [283], nanorods [497, 498], quantum dots [499] and others [79, 500]. Some AFM images of nanoparticles with different morphology are shown in Figure 7.11. For pure metallic nanoparticles, TEM is often considered the technique of choice, because metallic particles have high contrast in TEM, don’t require coating, and are not affected by vacuum. For such particles, TEM will be just as quick, if not quicker than AFM for measurement of many particles. In Figure 7.11 it is shown how, with care, TEM diameters and AFM particle heights correlate very closely for metallic particles. For hybrid particles with metallic cores and organic coatings, TEM will only show the metallic core whereas AFM will measure both. In these circumstances, therefore, it can be useful to combine the two techniques [280]. However, some particles, such as polymer particles will require extensive sample preparation for TEM imaging (and even then, imaging is indirect), so such particles are better suited to AFM analysis. AFM analysis of coated nanoparticles can even distinguish the thickness of subtly different coatings [493].

Carrying out imaging of nanoparticles for size measurements is exceptionally simple in AFM. Typically, all the analyst needs to do is to deposit a droplet of nanoparticle (p.152)

Fig. 7.11. Examples of nanoparticle imaging. Left: example images of nanoparticles with different morphologies; from top: spheres, nanorods [284] and nanotriangles. Right: results from imaging of gold nanospheres. Top: comparison of AFM height measurements (grey) with TEM diameters (black). Bottom: illustration of effect of repeated scanning on apparent height of individual nanoparticles. Each particle was measured 12 times with no apparent change in height. Adapted with permission from [290]. Copyright (2008) Wiley, reprinted with permission of John Wiley & Sons, Inc.
suspension onto freshly cleaved mica, allow it to dry, and image it. The imaging can be carried out in contact, non-contact or intermittent-contact mode, although in some circumstances contact mode may require more care to avoid sweeping effects. The analysis can then be carried out manually or automatically, although in the authors' experience and as reported elsewhere [290], a 'semi-automated' routine works best, i.e. the analyst identifies the particles, and the software routine measures them. This avoids user-dependent analysis, but allows the removal of other features, such as aggregated particles and detritus which can affect the results. More details about sample preparation for nanoparticle analysis are given in Section 4.1, and the image analysis of particles samples is covered in Section 5.3.

7.2.2 Mechanical measurement of nanotubes

In addition to their electronic and optical properties, the mechanical properties of pseudo-one-dimensional (1-D) materials such as nanotubes, nanowires, nanorods, nano belts, etc. are the subject of great interest. This is because it has been found that many mechanical properties such as yield strength of one-dimensional materials can exceed those of the bulk materials by orders of magnitude [501]. Indeed, it is due to their extremely high

![Bending stiffness tests on carbon nanotubes. Top: two individual lateral force-displacement curves, measured at different points along a tube. The small arrow shows the reproducible buckling force. Bottom: CNT force constant calculated from curves as shown above. As expected, the force constant decreases with increasing distance from the fixed tube end, allowing calculation of the material spring constant. Reproduced with permission from Wong et al. [506].](image)
mechanical strength and stiffness that carbon nanotubes (CNTs) are the most thoroughly commercially exploited nanomaterial [502–504]. However, making mechanical measurements of individual nanofibres is extremely difficult, mainly due to the difficulties in locating the objects and fixing them to the testing devices. AFM is ideal for this task, as it can perform both imaging and manipulation of the nanostructures. Furthermore the high positioning accuracy makes it possible to mechanically probe objects having dimensions of less than 10 nm easily [502]. For this reason AFM-based techniques have been widely applied to mechanical measurements of 1-D nanostructures [482]. For example, carbon nanotubes [502, 505, 506], many metal oxide nanowires [507], and metal nanowires [508], and other nanorods [506] have been studied by this technique. Mechanical measurements on 1-D nanostructures using the AFM can be performed in a number of geometries, making it a very powerful technique [509, 510]. Just two methods are highlighted below.

An example of the most commonly used approach for testing 1-D nanostructures by AFM is the direct nanomechanical testing of single CNTs carried out by Wong et al. [506] illustrated in Figure 7.12. Both multiwall carbon nanotubes and silicon carbide nanorods were deflected, using lateral force microscopy (LFM) while monitoring the deflection of the AFM cantilever, as the side of the probe tip pushed on the tubes laterally. Before this, the tubes were also imaged by normal AFM, so that both the imaging and mechanical testing capabilities of the AFM were used. One of the major difficulties in this sort of experiment is the problem of how to immobilize the nanotubes while leaving a potion free for mechanical testing [482]. Once testing occurs, the data must be further analysed in order to obtain mechanical parameters of interest, and how the fixed end of the rod is immobilized is important here, too. In the paper by Wong et al., tubes were randomly immobilized above gaps in a substrate, and SiO$_2$ was used to fix one end of the rod to the substrate. This process is inherently random, so AFM imaging is required to find suitable tubes. The LFM experiments were carried out along the length of the tubes, and it was noted that the carbon nanotubes appeared to buckle at a certain applied force, which was the same wherever along the tube the force was applied. This is shown in Figure 7.12.

Another way to test nanotube structures is by axial compression [505]. This may be carried out in one of two ways; the first method is via attachment to or growing the tube on, the AFM probe, and then pushing it against a solid surface [511]. The second method involves growing a sparse ‘forest’ of nanotube structures which are grown on or mounted in a substrate, and then uses an AFM probe to compress them by pushing towards the surface [512]. An illustration of the force curves recorder by this technique is shown in Figure 7.13. Application of forces large enough to bend the fibres through almost 180° can be carried out cyclically, as it does not result in bond breakage, due to the remarkable flexibility of both single- and double-walled CNTs [502, 513]. Finally, it should be pointed out that the related two-dimensional material graphene can also be probed by the same and similar mechanical AFM-based techniques, which has produced some spectacular results, notably showing graphene to be the strongest material yet tested [514–516].
7.2.3 Nanodevice construction with the AFM

Ever since Richard Feynman’s famous speech ‘There’s plenty of room at the bottom’, a major goal of nanoscience has been the bottom-up assembly of useful devices [517]. Bottom-up assembly means fabricating nanodevices from small components (e.g. atoms or molecules) rather than the traditional (‘top-down’) approaches of assembling nanostructures by assembly and removal of parts of large components, i.e. lithography.

There are several possible approaches to bottom-up assembly, but these can be broadly grouped into two categories: bulk techniques and nanomanipulation techniques. Bulk techniques are mostly based on careful manipulation of the chemical or biochemical properties of building blocks such that they self-assemble into the desired structures. These sorts of techniques can produce amazing structures, and can produce them in large quantities but the complexity of the devices produced is limited, the manipulator’s control over the devices is very limited, and their structures are usually determined indirectly. Nanomanipulation, on the other hand, involves assembling the tiny building blocks directly, one at a time. This approach has the advantage of finer control over the structures formed, and highly complex structures can be formed; on the other hand, it is highly laborious, and is better suited to experimentation rather than mass-production. AFM-based systems are well suited to this sort of task, due to the possibility to move the probe with sub-nanometre accuracy. An additional advantage of AFM for this task is the ability to use the instrument as a sensor as well as the manipulator.

(p.155)

\[\text{Fig. 7.13. AFM-based axial compression testing of MWCNTs. Top: schematic sketching of the motion of AFM probe and attached nanotube. Bottom: typical result. The grey (upper) and black (lower) traces show loading and unloading response respectively. A remarkable number of features are reproduced in both curves, showing the reversibility of the transitions in CNTs. Reproduced with permission from [505].}\]
There are many ways to perform such manipulations, but usually such manufacture is performed in a semi-manual way, so that the user issues a series of manipulation commands to the AFM, followed by imaging of the results, another batch of commands, etc. One reason for such a laborious manner of working is the fundamental unpredictability of assembly at the nanoscale. Nanoscale objects such as atoms, nanoparticles, etc. do not behave like macro-scale objects, so it is often difficult to predict how they will react to our manipulation [518]. One way to improve the throughput of nanomanipulation is to improve the interface, such that a real-time feedback to the user of the results of their manipulations is possible. There have been various attempts to interface AFMs with alternative sensing systems such as haptic interfaces (allowing the user to feel the sample) [519, 520] or virtual reality (allowing the user to see the sample and/or probe in true 3-D) [520], or combinations of both approaches [521].

For example, an AFM-based nanomanipulator with a haptic interface can be used to move carbon nanotubes onto micron-scale electrodes in order to measure their electrical properties [522]. Similar experiments can be carried out with gold nanoparticles as well [270, 523, 524]. Many other types of nanostructures have also been manipulated in similar ways, particularly various types of nanoparticles and nanorods [270, 498, 525–531]. In addition, various biological nanostructures (e.g. DNA, chromosomes, etc.) have been manipulated using AFM probes [532, 533]. It should be mentioned that advanced control interfaces make the assembly of complicated devices more convenient, but nanomanipulation is possible using any AFM.

Examples of structures that can be manufactured in this way include wires, transistors [523] and electrical contacts. For example, small gold nanoparticles can be aligned into particle chains by AFM pushing on a surface, and then used as seeds for further gold deposition in order to form a fully connected gold nanowire [527]. Some images showing the process and the resulting wire are shown in Figure 7.14.

7.2.4 Nanoparticle–DNA interactions
One of the most important areas of nanoscience is nanobioscience, which can be defined as using a nanotechnological approach to solve biological problems. Within nanobioscience, probably the most widely applied technology in the medical biosciences is that of nanoparticles, which have been used and proposed for a variety of diagnostic [534], imaging [535] and treatment strategies [536, 537]. One reason why nanoparticles have achieved such broad application is that they approximate the sizes of viruses or even of individual proteins, and thus may interact with the targets as would proteins and viruses. These targets include biomolecules such as proteins and nucleic acids (RNA and DNA), and such targets may be reached in vitro, but nanoparticles are also capable of entering cells (as viruses may do) to find their targets [538]. Another reason why nanoparticles are so useful for these sort of applications is that they can be engineered to have multiple properties (such as optical, magnetic, targeting properties) in a facile way [538].

AFM is the ideal technique to observe directly the interaction of nanoparticles (which are usually based on a metal or crystalline semiconductor) with their biomolecule targets; (p.157)

![Fig. 7.15. AFM height images of different structures made by nanoparticle binding to DNA. Left: nanowires made by non-specific (electrostatic) binding to ‘combed’ or straightened DNA; top: sparse nanoparticle coating; bottom: denser coating. Middle: non-specific binding (sugar-DNA phosphate backbone) of glyconanoparticles to DNA [123]. Both DNA and small nanoparticles are visible. Right: probing the action of a biosensor: measuring specificity of binding of DNA-sensing nanoprobe to their targets. Although the nanoparticles are much larger than the DNA, both can be clearly seen. Grey arrows show non-specific binding events, while white arrows show specific binding events. In total, 70% of binding was found to be specific [534]. Reproduced with permission from [547] (left).](image-url)

in fact no other technique can simultaneously observe both species. There have been a number of examples shown where complexes were observed between various nanoparticles and proteins or DNA using AFM [493, 539–545]. The case of oligonucleotide–DNA complexes is one where AFM can perform a special role. This is because DNA molecules have many potential binding sites for nanoparticles; the AFM’s high spatial resolution can be used to distinguish between them. In many examples, the interactions are rather non-specific; the nanoparticles are designed to interact with any DNA sequence. This is particularly useful to build a nanowire using the DNA as a template [542, 546, 547]. However, if the nanoparticles are modified with short sequences of single stranded DNA (ssDNA), they can specifically recognize and hybridize with target
sequences in the DNA (or RNA) target [534, 548]. This can form the basis of a biosensor for molecular diagnosis of disease; the mechanism of action of the biosensor can be determined by observation of the complexes of the nanoparticles with target DNA by AFM [485]. Example images showing this are given in Figure 7.15. This is one of a number of methods of specifically targeting certain DNA sequences with nanoparticles that have been illustrated by AFM [534, 549]. Figure 7.16 shows some examples of imaging nanoparticle–DNA interactions.

7.2.5 Electrical measurements of nanostructures with AFM

One of the most active areas in nanotechnology is the search for new electronic materials that, based on a bottom-up fabrication approach, could be used to replace today's lithography-based electronics. As well as these applied studies, the study of the fundamental optical and opto-electronic properties of nanomaterials is a very important (p.158)

![Fig. 7.16. Electrical measurements of nanowires. Top: characterization of a device consisting of vertically aligned germanium nanowires in an aluminium oxide substrate. The surface was polished and the aluminium selectively etched away to expose the tops of the nanowires which can be seen in the topography image (a). Conducting AFM with a platinum-coated probe was used to measure the current passing though the nanowires, while potential differences of 20 V (b) or 40 V (c) was applied between the probe and the other ends of the wires. All the wires seen in the topographic image are conductive, and some that are not obvious in the topography can be seen in the current images. Bottom: electrical measurements on ZnO nanorods as a function of distance along the rods, and of light flux. Left: AFM height image of a ZnO rod showing the locations where I-V curves were measured. Middle: I-V curves at different places – the response is uniform, indicating no defects. Right: I-V curves measured on an individual nanorod during dark conditions, and during illumination at two different wavelengths, showing the frequency-dependence of the device. Adapted with permission from [565] and [563].](image-url)

area of nanoscience. All these measurements require that electronics contacts can be made to the nanosized components, typically with placement resolution on the order of 1 nm. This is a major experimental challenge.
Conducting AFM techniques are ideally suited to overcome this difficulty of electrical measurements of carbon nanotubes, one of the most important materials for nanoelectronics [550–554]. Directly measuring the properties of individual CNTs is made much simpler by the ability of AFM to position an electrode (i.e. a conducting AFM probe) at any point along the tube desired. AFM can also be used to deliberately introduce defects into pristine CNTs in order to measure the effect on their electrical properties [553, 555–558]. Other nanostructures that have been probed electrically include quantum dots [559, 560], many types of nanowire [561–565], nanowire-based transistors [566], and even electrically active biological nanostructures such as single metal-containing proteins [567–570], and other types of nanostructures [571, 572].

Fig. 7.17. Electrical measurements of carbon nanotubes. Top: charge injection into nanotubes. A: topography of isolated MWNT, and its EFM image before charging (B). After charge injection by the AFM probe (at the arrowed location), the EFM contrast reverses (C). D: a line profile through the EFM images at the point indicated by the white line. Bottom: measurement of the effect of mechanical compression on charging behaviour of SWNTs. Left: AFM and EFM (measured by lifting) images of a SWNT after charge injection by the AFM tip. Right: plot showing charge density \( \lambda \) (red triangles) as a function of compression of the semiconducting CNT by the probe (indicated by the diameter, \( d \), which decreases as the CNT is compressed, green circles). The black squares show the behaviour of a metallic SWNT, illustrating how compression with the AFM changes the behaviour of the semiconducting SWNT to match the metallic behaviour. Adapted with permission from [550] and [558]. Lower figures copyright (2008) by the American Physical Society. (A colour version of this illustration can be found in the plate section.)

AFM has unique advantages for the electrical characterization of carbon nanotubes, specifically the ability to combine high-resolution imaging with electrical characterization both by imaging and spectroscopy of electrical properties, at the single-particle level. An example of the use of AFM techniques in the characterization of carbon nanotube
electrical properties in shown in Figure 7.17. In the upper portion of the figure, the effect of charging on the EFM signal from CNTs is given. The leftmost image shows a standard IC-AFM height image of an isolated MWNT. EFM was carried out in a lifting mode using conducting probes, and the initial result is shown in the second image, which shows a negative frequency shift of the cantilever over the CNT, indicating attractive force between the probe and CNT. Charge was then injected into the nanotube using the AFM probe, by approaching the tube with a bias applied between the sample and the probe. (p.160) The EFM image after this process is shown in the third image. At this time, both the tip and CNT are negatively charged, and thus a repulsive interaction occurs, leading to a positive frequency shift. Line scans over the CNT in the EFM images are shown on the right. It is interesting to observe that although the charge was injected at one point on the CNT (indicated by the arrow in the left-most image), the nanotubes is homogeneously bright in the EFM image, except for a small defect at the end, showing the spreading of the charge through the nanotube. This effect can be used to detect inhomogeneities and defects in CNTs [550].

Another advantage of AFM for this sort of study is that with AFM it is possible to mechanically challenge the nanostructures in addition to electrically changing and probing them. The relation between the electrical properties of CNTs and their mechanical deformation is a topic of intense interest due to potential applications [573]. There are several different ways in which AFM can be used to alter CNTs mechanically, and then measure the effect on their electrical properties [550, 556, 558, 574]. In the example shown in the lower part of Figure 7.17, two types of SWNTs are considered, semiconducting and metallic nanotubes. The semiconducting nanotubes are able to maintain only a small charge in EFM experiments similar to those shown at the top of Figure 7.17. However, upon compressing the semiconducting nanotubes mechanically by the AFM probe (shown in the figure by the green circles, which is the measured diameter of the tubes), the behaviour of the semiconducting CNTs change, and approaches that of the metallic CNTs (shown by the red triangles approaching the black squares). In this example, AFM was used to change the sample, as well as to characterize the effects both in terms of topographical and electrical changes. This allowed the direct observation of mechanically induced semiconducting–metallic behaviour crossover which had been previously only indirectly observed [558, 575].

7.3 Biological applications of AFM
The biological or life sciences constitute without a doubt, one of the most important application areas for AFM. This is evident from the fact that nearly all AFM manufacturers build specialist models of AFM for biological sciences, and there even exists at least one company that only makes AFMs designed specifically for applications in the life sciences. This is despite the fact that any AFM instrument can be used for biological applications. In fact, AFM itself came about partly in order to extend the possibilities of STM to biological samples. Many innovations in AFM technique and instrumentation which are now used in other application areas also came about due to the interests of biologists in the use of AFM, such as IC-AFM, and later the extension of IC-AFM mode to use in liquid [576], and low-noise force spectroscopy.
As a microscopy technique, AFM has several key advantages for biological application. Probably the most important of these is the ability to work under physiological-like conditions. Almost all biological processes occur in liquid, and often depend strongly on the presence of certain salts, and the temperature of the solution. Many biological samples also change their structures dramatically when dried. Therefore the ability of AFM to image and measure samples in buffer solution, at 37 °C, at any ionic strength or pH is of vital importance to many biological experiments. Furthermore, AFM is particularly simple (p.161) to integrate with optical techniques which are very important for many experiments in the life sciences, such as epifluorescence microscopy or confocal microscopy. Combining these advantages with the possibilities that AFM offers to carry out other experiments, such as mechanical probing, molecular interaction measurement by force spectroscopy, etc. means that AFM is an extremely important tool in many biological areas.

For these reasons, the number of AFM applications is enormous. The sections below include only a few selected areas where AFM has proven particularly useful, but more applications in the life sciences have been covered elsewhere [577–579].

7.3.1 Biomolecule imaging

Biomolecules form the basis of life, and understanding the structure, function and interactions of biomolecules has been the key to the incredible progress in the life sciences, and medicine in particular, over the last 50 years. As a technique with sub-molecular resolution and the ability to image soft samples in water, AFM is very appropriate for the study of the huge range of natural biomolecules. The four major classes of biomolecules are carbohydrates, proteins, nucleic acids and lipids.

Of these, probably the least-well studied by AFM is the class of carbohydrates, although even here, a number of different systems have been studied. These include self-assembled monolayers of glycoconjugates [9, 580], glycosylated particles [280, 581], polysaccharides [582, 583] and force spectroscopy of carbohydrate interactions [144, 584].

On the other hand, proteins have been extensively studied by AFM, not just by imaging but also by other measurements, such as electrical and mechanical measurements [320, 567, 585–587]. Due to their importance in disease and biological processes, proteins are one of the most widely-studied classes of molecules, and literature searching reveals thousands of studies of proteins using AFM. A few representative examples will be given here.

AFM is a particularly suitable technique for protein studies, due to the coupling of high resolution with the ability to study samples under physiological conditions, which is necessary because protein structure can be highly sensitive to the nature of the proteins’ environment. However, despite the incredible resolution achievable on flat atomically well-defined surfaces, AFM of single proteins in physiological-mimicking conditions often gives rather low resolution, only revealing sub-molecular features for
very large proteins or multi-domain protein complexes. This is due to the soft, yet tightly packed and globular nature of most protein structures, meaning an AFM image of the outside topography shows few structural details. Indeed, it is extremely challenging to obtain angstrom-level resolution of native proteins under such conditions by any technique because of the fact that the molecules are under constant movement.

However one area where AFM has been used to provide great details is in protein complexes. Such assemblies are often studied by TEM or crystallographic techniques, which suffer in that they study the complexes under of non-realistic conditions, giving AFM an obvious advantage in the fidelity of the data to biological systems [301]. One such protein complex that has been quite widely studied by AFM is the GroEL/GroES chaperonin complex. This complex is of interest because of its role in assisting the protein folding process [588, 589], and has been quite widely studied by both contact and oscillating AFM modes in buffer solutions [99, 590–592]. Sub-molecular resolutions, (p.162) namely distinguishing the seven sub-units of the oligomer can be achieved [99, 590]. The GroES ring acts like a ‘lid’ for the GroEL, sitting atop it under some conditions, and the GroEL without the lid can be distinguished from the entire GroEL/GroES complex [590], allowing the kinetics of the association/dissociation of the two partners to be studied by high-speed AFM [304, 593], see Figure 7.18.

A major class of protein assemblies that have been extensively studied by AFM is that of fibrillar assemblies, which have great importance for human health, due to their implication in diseases such as Alzheimer's disease and their importance in blood clotting [301]. For example, the formation of protein nets and fibrils formed during blood clotting has been observed on mica and HOPG surfaces [594]. Collagen, the most abundant protein in mammals, also forms fibrils. These have characteristic band structures, which are simple to observe by AFM, and this has become a standard specimen for biological AFM [35, 595–599].

Individual protein monomers can also be studied by AFM, although it is sometimes difficult to image isolated molecules without adhering them to a surface [306]. One preparation method commonly used for membrane proteins is to image them in a phospholipid bilayer, which stabilizes them towards imaging, as well as mimicking biological conditions. Proteins in lipid bilayers are covered in Section 7.3.3. Covalently binding proteins to a surface can stabilize them toward single-molecule imaging, although this might affect the protein structure. In some cases, careful control of pH and ionic strength is enough to enable imaging of isolated proteins absorbed onto mica [301, 306].
Fig. 7.18. AFM imaging of single protein complexes. Left: high-resolution images (contact-mode AFM) of GroEL (top) and GroEL/GroES assemblies (bottom). The association of the GroES onto the GroEL adds about 5 nm to the height of the complex, and covers the internal cavity. Right: high-speed imaging (IC-AFM) of GroEL/GroES association and dissociation. The images use a novel 1-D imaging technique to increase speed. The slow scan axis was disabled, and the fast scan axis (vertical) repeatedly scans a line containing various molecules. In the centre image (in the absence of ATP), this leads to continuous stripes along the time axis (horizontal), as little association or dissociation was occurring. When ATP is added (right image), the assembly heights switch rapidly (see line scans below), indicating rapid association and dissociation. In both examples the images were measured in buffer solutions. Adapted with permission from [590] and [304].

(p.163) Increasing the concentration of protein or their aggregates on a surface can lead to increased stability and imaging resolution, as at high surface concentrations the proteins can stabilize each other. The ultimate extension of this is to form two-dimensional crystals at a surface, which can lead to greatly improved imaging quality, and has been shown to allow sub-molecular resolution in some systems [102, 600]. However, despite the impressive resolution this technique is applicable only to some proteins, and removes one of the great advantages of AFM, i.e. single-molecule imaging and relevance to biological conditions [100].

Nucleic acids are highly suited to AFM imaging, and can be imaged in air and in liquid, and by contact, non-contact and intermittent-contact modes [300, 319, 601]. Samples have included single stranded DNA (ssDNA), double stranded DNA (dsDNA), and even the unusual triple stranded DNA [602]. RNA in both the common single stranded form (with tertiary structure) [603, 604], double stranded RNA [605, 606], and ssRNA in an extended configuration have been imaged [479]. Complexes of proteins (usually enzymes that have the nucleic acid as a substrate) with both RNA (anti-RNA antibodies-RNA complexes [605]) and DNA (e.g. DNAse-DNA complexes) have been imaged in air [607].

High-quality images of DNA can be obtained by deposition of a solution onto freshly-cleaved mica, followed by imaging by NC- or IC-AFM. Reproducible and high-resolution images require some way of binding DNA to the mica, because both mica and DNA are negatively charged under common conditions. This is usually done by either treating the mica with a divalent cation solution before deposition, or including such a cation (e.g. Ni^{2+}
or Mg$^{2+}$) in the deposition buffer. The divalent cations are thought to act as a salt bridge [319], and this treatment allows imaging in air (after washing away most of the salts, followed by drying), or in liquid (the imaging liquid must then contain the divalent cation). Alternative methods include treating mica with an amino-terminated silane [300, 302], although caution must be taken not to increase greatly the roughness of the mica by this method, as imaging the DNA well requires a very clean surface. See Chapter 4 for more sample-preparation details.

One great advantage of the electrostatic absorption via divalent cations, is that if imaging in liquid, careful control of the ionic conditions in the imaging buffer can ensure the DNA stays on the surface, while allowing it freedom to move in two dimensions, and even to carry out physiological functions [2, 4, 478, 608]. This has led to some very elegant experiments in which both DNA and proteins are ‘bound’ to a surface well-enough to be imaged by AFM, while being free enough to carry out their interactions in real-time. Of course, such molecules bound to a mica surface are not under true physiological conditions, but no other technique allows molecular biologists real-time single-molecule imaging of these sorts of reactions at all [609]. Some stills from a time-lapse ‘movie’ that can be generated by this technique are shown in Figure 7.19. More applications of AFM to studies of DNA are discussed in the review [610].

The IC-AFM images shown in Figure 7.19 were acquired at a rate of 1 image per second. This imaging rate is remarkably fast for AFM, especially for IC-AFM images of such delicate structures. However a long-term goal for fast-AFM imaging researchers is to improve the speed of IC-AFM data acquisition even further, allowing AFM to probe protein–nucleic acid reactions with high time and spatial resolution [476].

\[(p.164)\]

*Fig. 7.19.* An example of real-time measurements of DNA-protein interactions. These images were obtained with high-speed IC-AFM, and the numbers in each frame indicate the time after imaging began. The images show a complex of EcoP15I, a DNA restriction enzyme, with DNA. The images from 1 to 10 seconds show the DNA loop passing through the enzyme. Reproduced with permission from [2], copyright (2007) National Academy of Sciences, USA.
7.3.2 Bacterial cell measurements

AFM is a highly suitable tool to examine bacteria, and has been widely applied to their study. Bacteria are commonly studied by optical microscopy, which can give an overall idea about gross cell morphology (via a two-dimensional projection), and is also useful for cell-counting studies. In comparison, AFM is slower, and thus is less useful for quantitative cell-counting, but allows measurement of a variety of other cellular properties, particularly by nanoindentation and force spectroscopy experiments [611]. In addition, the greatly increased resolution of AFM allows for the imaging of finer details of cell morphology and sub-cellular features such as pili and fimriae [612]. The three-dimensional information from AFM can also be useful in differentiating morphologies which would look the same in optical microscopy [6]. Various other micro-organisms have been studied by AFM such as spores [178, 613–615], fungi [616, 617], including yeasts [171, 618], viruses [287, 619], and others [620] but here we concentrate on bacteria for the sake of brevity.

Some species of bacteria that have been well-studied include E. coli [169, 621] and various species of Staphylococcus [169, 317, 622], Bacillus [178, 615], Streptococcus [623, 624] and Salmonella [625, 626], see Figure 7.20. Bacteria generally need to be immobilized on a surface for imaging, and a number of different procedures have been used. For studies in air, drying onto a surface, or even flaming can work well, although one needs to be careful of drying artefacts by these techniques [621]. However, it is useful

---

**Fig. 7.20.** Studies of bacterial morphology. Top left: Streptococcus, showing typical linear clusters. Top right: large clusters of Staphylococcus aureus. Bottom left: Salmonella biofilm showing pili-like fimbrial structures. Bottom right: E. coli. All these images were measured in air. Reproduced with permission from [624] (top left) and [626] (bottom left).
to be able to study bacteria in liquid, and usually this requires a more elaborate preparation protocol because it’s necessary that the cells be fixed to the substrate in some way. The most commonly used techniques include the use of poly-lysine (PLL) or polyethylenimine (PEI) coating of glass for chemical capture and using gelatin-coated glass for a soft physical capture [6, 314, 315]. For spherical bacteria, i.e. cocci, physical trapping of substrates with appropriately-sized holes works well, and it can even be possible to observe the cells dividing while immobilized in this way [313, 317]. Bacteria that naturally form biofilms are simple to study as biofilms are perfect samples for AFM, although some washing may be required. See Section 4.1 for more sample preparation (p.166) details. Although higher resolution is usually obtained in air, bacteria imaged in liquid are closer to the native state, and dried bacteria usually have a small fraction of their hydrated height [6, 315, 621].

One of the most important areas in studies of bacteria is the study of the method of action of antibiotics and other antibacterial agents, due to the ongoing increase in antibiotic resistance in bacteria [627]. Several studies have imaged bacteria treated with antimicrobial agents, including the morphological changes to E. coli caused by the antibiotic cefodizime [628] and also E. coli and P. aeruginosa response to antibacterial peptides [629, 630], S. aureus response to antibiotics [631, 632] and others [624, 633, 634]. The response to the natural antimicrobial polymer chitosan, of E. coli, S. aureus, B. cereus and B. cereus spores has been measured by both AFM imaging and nanoindentation measurements [169, 178]. The changes that can be seen include morphological alterations such as appearance of holes, shrinking, cell shape changes and cell lysis, and also mechanical changes. An example showing the response of S. aureus to antibiotic treatment by both topographic changes and changes in cell elasticity is shown in Figure 7.21.
Fig. 7.21. An example of the use of AFM to measure bacterial response to antibiotic treatment. Top: topographical images showing cell wall roughening in *S. aureus* after increasing times of treatment by the antibiotic lysostaphin. The images are deflection images measured in contact mode. Below: the effect on cell wall stiffness. The drug appears to severely degrade the cell wall within 80 minutes. This data was collected in buffer solution, on a cell trapped in a membrane pore. Reproduced with permission from [632].

(p.167) It's also useful to make measurements of bacteria by non-imaging modes of AFM, because the high positioning resolution of AFM allows such measurements to directly address individual bacterial cells, which is difficult by other techniques [611]. For example, nanomechanical measurements (e.g. nanoindentation) of bacteria have been shown to be sensitive to treatment with antimicrobial agents [169, 629, 632], bacterial species and strain [155, 635, 636], physiological state of the organisms and the environment in which the measurements are made [155]. With the AFM it's relatively simple to perform nanoindentation experiments on individual micro-organisms, and even to differentiate one part of a cell from another by stiffness measurements [171]. For this sort of experiment, it's important to remember that the response of the probe will be different when the cell surface is perpendicular to the probe motion, than when it's at an angle, however [637]. Thus, all measurements should normally be carried out only on the upper portion of the cell which is relatively flat [382].

Other non-imaging experiments which may be carried out on bacteria using AFM include force spectroscopy in order to measure the distribution of specific adhesion factors on cell surfaces [156], cell hydrophobicity/hydrophilicity [475, 638], or the distribution of other molecules across the cell surface [611, 637, 639].

Bacterial colonization of surfaces is an important process, and reducing the process requires knowledge of individual bacteria–surface interactions. Bacteria–surface adhesion studies can be carried out using a number of experimental methodologies, the most commonly applied ones being direct force spectroscopy with bacteria immobilized on the AFM probe and lateral force microscopy measurements of the force required for removal of cells [637, 640–644]. AFM allows the combination of studies of cell-surface
adhesion, with measurements of the surface itself, which can help to understand how factors such as roughness, hydrophobicity, etc. can affect colonization by bacteria [645].

7.3.3 Lipid membrane imaging

Plasma membranes are ubiquitous in animal cells, forming a barrier between the intracellular components and the extracellular environment. The membrane’s purpose is to selectively allow molecules in and out of the cell, while blocking unwanted material. In addition the membranes form a scaffold for a large number of cell surface molecules, mainly proteins, which regulate activities such as cell adhesion, recognition, signalling, etc. By AFM it is possible to study the cell membrane in its native environment, i.e. as part of a cell (see next section), but for increased stability and higher resolution, it’s useful to use a model system. The major component of the plasma membrane is a bilayer of phospholipids, with their hydrophilic heads pointing out into solution, and the hydrophobic tails on the inside of the bilayer, so that they are shielded from the aqueous environment. Due to their importance in biology such lipid bilayers have been widely studied by a number of techniques. While they are simple to study by other techniques in solution (they form spherical vesicles), for AFM they can be deposited easily on a flat surface. This creates a flat, stable model for the plasma membrane, which is an ideal sample for AFM studies.

Formation of lipid bilayers for AFM studies has been carried out using a number of different methodologies. The two most common of these, however, are Langmuir–Blodgett film deposition, and fusion of vesicles from solution directly onto the substrate surface. (p.168) Lipid membranes form extremely flat surfaces, and membrane proteins or lipid domains often are visible in AFM as sub-nanometre height features, so it is generally desired that the substrate be atomically flat. In order to form a bilayer the substrate should be hydrophilic, so freshly cleaved mica is typically the best substrate to use, although silicon [646] or template-stripped gold [647] may be used, particularly when a specific surface chemistry is required. Langmuir–Blodgett (LB) film deposition is carried out by first forming a layer of the lipids on a water surface, and passing the substrate through the air–water interface [648]. This has some advantages in terms of control of the pressure of the bilayer, and the ability to form half-bilayers or mixed bilayers. On the other hand, vesicle fusion is an extremely simple process. A solution of vesicles is pipetted onto a mica surface and typically left for 20–40 minutes for fusion to take place, before rinsing with buffer solution. The vesicles collapse on the surface, leaving a well-organized bilayer, with typically some remnant vesicles which are washed away [646, 649]. This preparation process is quick and simple to carry out, although it lacks some of the control of the LB film technique. An advantage for imaging under liquid is that the sample need never dry out during the preparation process. Imaging of bilayers can be carried out in contact or oscillating modes, (either non-contact AFM or IC-AFM) [649], [125] and in air or, more commonly, in liquid [650].

In either case, when bilayers are prepared, the sample is often referred to as a supported lipid bilayer (SLB). This is to make explicit the fact that these bilayers are not in vesicle form, but on a surface; they will therefore have some interaction with the
substrate surface. In fact it’s known that the interaction with the surface changes somewhat the properties of the bilayer compared to a vesicular bilayer. An example showing height differences in the bottom bilayer of a multi-bilayer stack due to this surface interaction is shown in Figure 7.22. Studies of bilayer structure can include measurement of phase separation in mixed lipid systems, which is an important process due to the involvement of lipid rafts in many biological processes [129, 651]. Phase separation can be studied by a number of AFM techniques. Due to the incredibly high height resolution of AFM, discrimination of phases with a few Å height difference can be carried out directly [129]. In addition, friction contrast (measured by LFM) [652–654], or phase imaging [471, 655] can help to differentiate phases of very similar heights. Examples of this are shown in Figure 7.22. If a nanoindentation-type experiment is carried out on lipid bilayer with a flexible probe, then at a certain threshold force, a ‘breakthrough’ can be observed in the force–distance curve [129, 656]. This can be used as a measure of the coherence of the lipid film, and its thickness may be measured from the curve. Practically, this measurement can be used to prove the existence of the films, as lipid bilayers on mica can be uniform to the point of being featureless [308].

AFM is also ideal to study the interaction of peptides or proteins with membranes [657]. Some peptides are known to disrupt and damage lipid membranes, whilst other are drug candidates that need to be able to cross membranes. The changes in lipid membranes are typically disruptions with dimensions on the order of a few nanometres, so the action of these materials is usually studied indirectly. With AFM their action can be observed directly, typically manifesting as appearance of small ‘holes’ or other morphological changes in the SLBs [658–661].

Probably the most important application of studies of SLBs is the study of protein incorporation in membranes. By AFM this is rather simple, and can be performed by...
Fig. 7.22. Examples of bilayer structural studies. Top: demonstration of the influence of the substrate surface on bilayer height. The four layers are identical in composition, but the one next to the substrate appears considerably thinner. Below: differentiating phases in phospholipid mixtures by LFM and phase imaging. Left: topography (A,C) and LFM (B,D) of a mixture of DSPE and DOPE. The monolayers were imaged in air (A,B) and under liquid (C,D). Right: phase imaging of E. coli total extract in liquid. Note that there are several different lipids in this mixture, and some are discriminated by phase imaging, while others are not. Reproduced with permission from [129] (top), and [652] (left).

simply fusing proteoliposomes instead of liposomes onto a mica surface [308]. The protrusion of the membrane proteins above the flat lipid surface can then be imaged directly, and can give important information about protein insertion, see for instance the example on the left of Figure 7.23. Lipid membranes have also been used as a scaffold to study the fine structure of membrane protein oligomers in their native state. In AFM, membrane proteins have been widely studied in 2-D crystals, which are a highly stable configuration, allowing high-resolution imaging [320]. However such systems do not very closely represent the native conditions for membrane proteins. On the other hand, incorporation of protein complexes into membranes allows their imaging in conditions much closer to the native ones, and can demonstrate different structures to those found in the (p.170)
Fig. 7.23. Examples of proteins and protein oligomers inserted in lipid membranes. Left: low-resolution imaging of single outer membrane proteins (OmpF) inserted into DMPC supported lipid bilayer. The inset histogram shows two peaks in the histogram of protein heights suggesting two insertion mechanisms. Right: high-resolution images of light-harvesting complex incorporated into a DOPC/DPPC SLB. The inset shows an averaged image of the complexes, showing the discrimination of the 16 sub-units. Reproduced with permission from [662] (right).

crystallized complexes, which are presumably closer to the native structures. AFM is the only technique to allow high-resolution imaging of protein complexes in near-native conditions [662]. An example showing high-resolution imaging of protein complexes inserted into an SLB is given in Figure 7.24.

7.3.4 Mammalian cell imaging

Due to their importance in biological and particularly biomedical sciences, animal cells have been widely studied by AFM. As a high-resolution microscopy technique able to image samples under physiological conditions (in buffer or growth medium, at controlled temperature, with controlled ionic strength), AFM has some unique advantages for cell biology. In addition, the ability to make mechanical/chemical measurements using the AFM probe enables further possibilities. AFM is also particularly easy to combine with optical microscopy, so overlaying florescence images with AFM data is possible, allowing the combination of the unambiguous identification of features by florescence labelling and the high-resolution imaging of AFM. However, it is also true that imaging living cells presents the AFM operator with some unique challenges. Notably, very high resolution, such as may be obtained regularly with other samples can be extremely
challenging to achieve when imaging living animal cells [103]. This is likely to be due to a combination of the living cell’s high sensitivity and flexibility. Living cell surfaces are able to move spontaneously in solution [663], thus establishing with certainty their location with a mechanical technique is difficult.

However, while requiring great care, high-resolution imaging is possible, and even the comparatively low-resolution images that may be attained more routinely have resolution many times greater than optical microscopy [101]. In imaging mammal cells, the first decision to be made is whether to image the cells live, in solution, or dried and fixed. This will typically depend on the particular application, and the information required. While imaging dried and fixed cells can give useful information [664], live cells in liquid will be less prone to fixation artefacts, and closer to native conditions, and can also enable imaging of dynamic processes [665]. The second decision is whether to image the cells in contact or oscillating modes.

Despite the mechanical softness of living cells in solution, contact-mode imaging can give surprisingly good results [101], and is probably used more commonly for live cell imaging than oscillating modes [103]. Typically contact-mode imaging is carried out with very small applied forces and very soft cantilevers (spring constant, $K < 0.1 \text{ N/m}$) [101, 666]. For oscillating modes in liquid, slightly stiffer levers are usually used to overcome probe–cell adhesion. Unlike most other applications, for live cell imaging, it may be preferable to use unsharpened silicon nitride probes, which have relatively large tip radii (ca. 20 nm). These may be less likely to penetrate the cell membrane, leading to higher achievable resolution than with sharper probes [667].

Because of the flexibility of the cell membrane, different imaging conditions can lead to different images. Typically with contact-mode imaging (especially at high applied force), sub-membrane features (such as the cytoskeleton, actin fibres, etc. [668]) are visible, while for oscillating modes, the membrane itself is shown and the cytoskeleton is not seen [103, 669]. In addition, changing the applied force can affect the visibility of sub-membrane features. While increasing the applied force to make more features visible, can cause greater apparent resolution, it can also result in sample damage [101, 666, 670]. A few examples of contact and IC-AFM imaging of live cells, illustrating the differences commonly seen, are shown in Figure 7.24. One important aspect of cell imaging for AFM can be seen in the figure: animal cells are very large samples for AFM. While high-resolution imaging of the cell membranes can be very useful, it is usually convenient to also obtain overview images showing whole cells such as seen in Figure 7.25. This requires a large scanner, with a wide X-Y range (>50 μm, preferably 100 μm), and some cell types necessitate a long Z axis travel (ca. 10 μm) as well – this is particularly the case for measuring cell–cell or cell–substrate adhesion [671, 672].

Some of the more common applications of cellular imaging include morphological studies, which includes the morphological changes in cells upon interaction with drugs or other biological molecules [668, 673, 674], observation of dynamic cellular processes [675, 676], and observation of morphological changes in diseased cells [665, 677].
Fig. 7.25. Using AFM to diagnose cancer – nanomechanical measurements of live cells. Left: optical view of an AFM cantilever probing mixed healthy and tumourous cells. Middle: Young’s modulus of healthy cells. Right: Young’s modulus of tumour cells. The cells were differentiated by immunostaining. The AFM-based nanoindentation results show that the tumour cells are much less stiff, enabling cell motility in the body. Adapted with permission from [680].

the high-resolution imaging, and also the ability to image not only the cell, but it’s surroundings, studies of the interactions between cells and their substrate are a strong point of AFM-based cell studies [175, 678]. For example via AFM it’s possible to observe cell responses to nanostructured surfaces, and even to observe the cell-surface interactions in real time with living cells [664].

Mechanical properties of animal cells are very important for their functions, and thus it is useful that AFM can measure cellular mechanical properties at the single cell, or sub-cellular level [668, 679–681]. It is possible to determine differences in stiffness between different parts of the same cell, typically showing the presence of sub-membrane cell components. One example of the utility of this is in probing the mechanical differences between diseased and healthy cells. Recently it was realized that an important factor leading to tumour invasion and metastasis is a decrease in cell stiffness, leading to increased ability to spread throughout the body [682]. AFM has been used to prove that tumour cells collected from patients are considerably less stiff than healthy cells from the same patients. Further, it appeared that various types of cancer cell, all from different patients exhibited very similar stiffness values [681]. The AFM-based measurement of stiffness was proposed as a diagnostic method for forms of cancer that are difficult to diagnose by traditional methods [680, 681]. In addition to nanoindentation experiments, force spectroscopy experiments have been widely applied to living mammalian cells, which are covered in the next section.

7.3.5 Biological force spectroscopy
Intermolecular interactions are the basis of life, and an extremely important part of biological research, so an enormous range of techniques have been applied to their study. For the interactions of biological molecules, AFM has some unique advantages. It is very sensitive, allowing the interactions between single pairs of molecules to be studied due to force resolution in the 10 pN range [683]. Moreover, it can be very selective. The control over the x, y and z position of the probe with immobilized molecules means that unlike most techniques, by AFM-based force spectroscopy, it’s possible to control exactly (p.173) which molecules interact, and where they are doing it. This is fundamentally different from bulk techniques, where the molecules of interest are placed in a relatively
large volume, and some signal change observed. In addition, because the molecules are brought together and pulled apart under the control of the experimentalist, factors such as force of interaction and the rate of separation can be finely controlled. Combining all this with the possibility to carry out such reactions in physiological conditions means that AFM-based biological force measurements represent one of the most powerful techniques available to make molecular interaction measurements in biology [684].

There are two major ways in which force spectroscopy can be carried out: in one or in three dimensions. One-dimensional (1-D) force spectroscopy refers to experiments where the factor of interest is the intermolecular force to be measured, rather than the spatial distribution of the measured forces. In order to carry out this sort of experiment, the AFM probe and a flat surface will be modified to bind the two molecules of interest. The flatness of the substrate will reduce artefacts related to increased adhesion at the edges of features [159]. For self-interaction, the same molecule could be bound to both the surfaces [144, 685]. There are a number of issues related to the binding strategy used, which were discussed in Section 3.2.1, but essentially, the molecules must have a resistant yet flexible linker, and not have their recognition sites blocked [686, 687]. When suitably modified probes and samples have been generated, experiments are carried out as described in Section 3.2.1, and any pairs of interacting molecules may be studied. A common way to prove the nature of the interaction being probed is to add a ‘blocking’ molecule to the solution [163]. For example, having established that with molecule A on the tip and molecule B on the surface, a measurable interaction force is recorded, molecule B is added in excess to the medium. These excess ‘B’ molecules bind to ‘A’ on the tip, and the force spectroscopy experiments are repeated. If the interaction being probed is really of type ‘A-B’, the measured force will be changed (often it will disappear), with the blocking molecules in solution. The main parameter which is studied is the force or range of forces, at which detachment occurs (hence force spectroscopy). However, for molecules bound by a flexible linker, or for macromolecules, the distance of unfolding is also important (see next section). Typically the rate of pulling (i.e. the speed at which the probe is moved) can be varied, and this can also allow measurements of the kinetics of the dissociation process [688–690].

Some examples of interactions that have been studied include biotin–avidin binding [163, 691, 692] (which has become a ‘standard measurement’ in AFM force spectroscopy, due to its very high strength and specificity [690, 693]), other antibody–antigen interactions [689, 694, 695], carbohydrate–carbohydrate binding [144, 584], and fibrinogen binding [685, 688, 696], see the examples in Figure 7.26. AFM can also be used to measure cell–cell adhesion, or virus–cell adhesion by attachment of a cell or virus to the probe [697, 698]. This is only a small selection of the interactions that could be studied; for further details see the reviews [142, 684, 699].

Three-dimensional force spectroscopy or force-mapping is the second major methodology of force spectroscopy by AFM. In this sort of experiment, the aim is to use the specific force of interaction between two molecules to determine the location on the sample surface of one of the molecules. For instance, this can be used to determine the location
of receptor molecules on a cell surface. Probably the most commonly used way to do this is by measuring force–distance curves in a grid pattern over the sample surface. This is sometimes referred to as force volume imaging [700]. In this mode, the (p.174)

![Image](https://example.com/image.png)

**Fig. 7.26.** Examples of biological force spectroscopy. The two examples show different methods of studying interactions with human blood cells – at the top, one-dimensional force spectroscopy, and below, three-dimensional force mapping. Top: force spectroscopy on human platelets. The top force–distance curve was made with an unmodified tip, and the bottom two with a tip modified with peptide sequences from fibrinogen, showing the results of single (middle curve) and multiple (lower curve) adhesion events. On the right is a ‘force spectrum’, showing the presence of peaks at multiples of ca. 93 pN. Below: force mapping on red blood cells with a lectin-modified probe. Image A is the total adhesion force and image B is the topography of a mixed layer of group A and O cells. The topography shows no difference between the cells, while the adhesion image clearly distinguishes ‘A’ from ‘O’. Adapted from [688] and [710].

AFM instrument approaches the probe to contact the surface, and then pulls the probe away, before moving a small amount while out of contact, approaching again, etc. This is done in a grid pattern defined by the user. This method of measuring interactions has great advantages in that the individual force curves are as well-defined and controllable as via normal (1-D) force spectroscopy, and are carried out normal to the sample surface. However, it is also a rather slow technique, and normally the maps are produced with reduced resolution (e.g. 64×64 points [142]), in order to make the experiments reasonably short. In addition, the data processing can be complicated and time-consuming.

**(p.175)** Force mapping in this way has two major advantages over more commonly used approaches to determine molecular distributions in biology, which usually involve labelling the receptor. Firstly, no labelling, which could affect the results, and requires prior knowledge of receptor chemistry, is required, and secondly, the resolution is
Applications of AFM

higher than optical techniques. It can be used for a very broad range of applications, principally the mapping of the locations of various molecules on the outer membranes of live cultured mammalian cells [672, 701, 702], yeasts [703], bacteria [156, 704, 705], etc.

In addition to force-mapping via force–distance curves, it is possible to carry out similar experiments using dynamic AFM modes, with the main aim of increasing the speed and resolution of force mapping. This is sometimes known as dynamic recognition imaging or affinity imaging [706]. This can be done in a type of intermittent-contact AFM, using a probe modified as for force–distance curve acquisition [707]. The interaction between the modified probe and the targets on the sample surface should alter the probe's response in IC-AFM, and the signal corresponding to this change must be extracted from the data measured while the probe scans over the surface. One way to do this is by oscillating the probe (with an amplitude lower than the length of the linker between the probe molecules and tip), and electronically extracting two signals from the measured probe oscillation – that from the upper part of the oscillation (the oscillation maxima) and that from the lower part (the oscillation minima). The lower oscillation signal is for feedback. This is the part of the oscillation most affected by the mechanical damping of the probe by the sample surface. The upper part of the oscillation signal is used for the recognition image. This part is sensitive to the interaction between the probe-absorbed molecules and those on the sample surface [708]. The chief advantage of this technique over force–distance curve mapping is that molecular recognition can be recorded simultaneously with topographical imaging – at the same speed as normal AFM imaging. The trade-off for this speed is a reduction in the amount of information available at each point (for example the pull-off force, chain extension length, etc. which can be measured directly when using force-mapping). Such dynamic techniques may be applied to molecules absorbed to flat surfaces [709], or even to receptors on cell surfaces [685].

In addition to making measurements of intermolecular binding, force spectroscopy can measure the strength of intramolecular bonds, for example the force required to separate the two strands of dsDNA [711], to unfold the secondary structure of ssRNA [712], or to unfold proteins, which is covered in the next section.

7.3.5.1 Protein unfolding

Measuring protein unfolding with AFM is an advanced application of force spectroscopy. However, because protein unfolding is a huge area in biophysics and biochemistry, the adaptation of AFM to measuring protein unfolding created a whole new field of experiments [713, 714, 715, 716], and it is becoming an increasingly common application that has led to improvements in experimental technique in force spectroscopy and in instrumental capabilities and force resolution [586]. One reason that protein unfolding by AFM is so interesting is that in the classical techniques, protein unfolding is induced by either chemical or thermal denaturing. While these are important pathways, the ability to induce unfolding via completely different mechanisms allows researchers to probe the process in a very different way, revealing aspects of the unfolding process previously inaccessible [717, 718]. Furthermore, certain proteins require tensile strength for their physiological (p.176)
function, and thus mechanical resistance is an important part of their design [719]. While there are other techniques which may be used to study mechanical unfolding of proteins (such as optical tweezers), the accessibility and simplicity of protein unfolding by AFM has meant it is the most popular technique with which to study the phenomenon today [717]. The folded structures of proteins are typically held together by forces such as hydrogen bonding, hydrophobic, ionic, and van de Waals interactions, which are all weak interactions, but are collectively strong enough to hold the structure together. Therefore, the fine details of unfolding pathways require high force resolution. The way in which traditional force spectroscopy works is not ideal for protein unfolding studies, because normal force spectroscopy is carried out at a constant velocity. This means that in protein unfolding, large changes in applied force will occur during the process. A method that was developed to overcome this limitation is force clamp spectroscopy, in which an additional force feedback loop is added to the instrument, to maintain a constant force during unfolding [720, 721]. A further derivative of this technique is force ramp spectroscopy, where the feedback loop is used to maintain a constant increase in force during unfolding. At the time of writing, nearly all force-clamp spectroscopy has been carried out with modified instruments [722], but commercial instruments with such capabilities have also begun to appear. Typically such experiments are carried out with synthesized polyproteins, large molecules with multiple copies of a single protein domain [716, 718]. Pulling this type of molecules should give rise to a characteristic ‘fingerprint’ force curve (i.e. the sawtooth- or staircase-shaped profiles shown in Figure 7.27), which helps to reduce the ambiguities in force spectroscopy data discussed above. A new generation of commercial instruments have been produced recently, which are designed to optimize the ultimate force resolution by reducing noise in the z axis, largely spurred by the requirements of protein unfolding experiments. It has been estimated that using standard commercial cantilevers, thermal noise limits the resolution to approximately 6 pN [723]. These novel experimental methodologies and instrumental improvements have led to new insights into how protein structures control their physiological functions [717].
7.4 Industrial AFM applications

Presently the majority of AFM applications are in basic and applied research at universities, government and large company laboratories. However, there is a growing trend in the use of AFMs for commercial applications. The growth depends on the following requirements:

(a) Images and data from the AFM must be reproducible.
(b) The instruments must be operable by technicians and users that are not experts in AFM.
(c) There must be an awareness of AFM techniques at all levels in a company from bench level to senior managers.

For the purposes of this book AFM applications are divided into two categories: applications associated with commodity products, and applications for high-technology products. One of the limitations in presenting commercial applications is that companies often do not permit publication of their images and applications, because the information is considered confidential. It is not until many years after the application is identified by a company, that it is made public. Due to this, there are relatively few references for industrial work.

The following sections provide some insight into how atomic force microscopes are used in an industrial setting. It is by no means a complete list, as many applications are being carried out in complete secrecy, and cannot be presented publicly.

7.4.1 Commodity product applications

Commodity products are made in extremely high volumes with processes that are for the most part very well understood. Typically the products have very low profit margins per unit but can be very profitable because they are made in such large volumes. Because of the nature of this type of business, expenses for product development and research account for a very small percentage of a company's operating budget, and AFM has been used less often in this sort of industry than in the high-technology sector.

It is interesting to note that many commodity products have historically had critical components that were nanometre sized. Without knowing it, highly controlled processes were developed to manufacture of products having these nanometre sized components. Car tyres are an example. A car tyre is constructed mainly from rubber and carbon black. The carbon black is a nanoparticle. The quality of a tyre is related to the size of the carbon black particles and the dispersion of the carbon black in the rubber.

The motivation for companies to use atomic force microscopes when they are selling commodity product is typically to improve existing products. The goal is to make the products more cheaply, better, faster, stronger, etc. There are many consumer products now having claims of improved performance because of nanosized components. Examples include tennis rackets, golf balls, hair products, clothes and washing machines.

(p.178)
Fig. 7.28. Examples of commercial products with applications in AFM. Left: 4μm×4μm image of a razor blade. Middle: image of clay-containing coating on paper, showing hexagonal kaolin particles. Right: 35μm×35μm image of human hair.

Another motivation for companies that make commodity products to use the AFM is to solve production problems. As an example, a company that makes photocopy machines was having problems with the machines breaking because the particles used in the reproduction process were not working correctly. By using an AFM the company was able to find that the particles purchased from another company did not meet their specifications. Before it was solved the problem was costing the company approximately 1 million dollars per month.

In addition, AFM analysis can be used to support advertising claims, or to resolve industrial disputes. As an example, a toothbrush manufacturer was claiming that their product caused less damage to teeth than their competitors. The competitor challenged this claim with a law suit. An AFM was then used to compare the wear on teeth from the two companies’ products. In fact, it was demonstrated that the claims were true.

Figure 7.28 shows a few examples of commercial samples scanned with an AFM. The razor blade image is an example of using AFM to characterize defects caused by faulty processes. Razor blades are sharpened to atomic dimensions in extremely high volumes. The AFM was used to profile the sharpened surface and view imperfections at the very edge of the blade. Mounting the razor blade in the AFM so that the scans could be made without causing further damage was a substantial challenge.

Secondly, a paper coating is shown. The quality of paper coatings greatly affects their performance, and many papers are complex composite materials, containing different mineral particles, elastic binders, and fibres in the surface structure. For centuries paper products have been made from nanoparticles. A common component which controls the quality of the paper is clay. Often, the higher the clay content, the higher quality the paper. An AFM can readily be used to visualize the clay nanoparticles in paper. The image in Figure 7.28 shows an AFM image of Astr-Plus/Carbinal 95 coating. This ‘engineered’ coating is comprised of kaolin in a narrow particle size distribution and an ultra-fine ground calcium carbonate with a latex binder. AFM is a highly suitable tool for imaging and quantitative analysis of paper coatings [724, 725].

The final example of a commercial sample for AFM is hair treatments. Hair care products can cause substantial changes to the overall geometry of human hair. The changes can affect the hair’s optical and frictional properties which are very important to consumers. AFM is the only method that is able to measure the three-dimensional
topography of human hair, and in addition can make quantitative measurements of hair surface roughness and frictional properties [726, 727]. AFM can be applied to image the effects of many different cosmetic products on hair, for instance, the effect on topography and friction of hair bleaching [728], or the effects of shampooing and conditioning with different products can be studied [729].

7.4.2 High-technology applications

High-technology companies produce high profit-margin products that usually use advanced manufacturing technologies. These types of companies commonly adopt new techniques such as AFM to facilitate their research efforts, and less often, may use AFM to support production when necessary. The digital data storage industry and the semiconductor industries rely on AFM for solving some of their most difficult problems. Other products that have used AFM for product development include flat panel displays, optics, microelectromechanical systems (MEMS), and biosensors.

High-technology industries require novel materials, devices and processing capabilities. In a commodity-based company product development is 5% of revenue whereas high-technology companies spend 10–15% of their revenue on product development. Thus high-technology companies can afford to invest in atomic force microscopes.

Such industries often require high dimensional tolerance. Further, high-technology companies need to have an understanding of fundamental material behaviour in order to improve performance, manage quality control, and to improve product yields. An atomic force microscope is one of several tools available to high-tech companies which are capable of making such measurements. It should be noted though, that the AFM is a relatively new tool and typically other microscopes, such as electron microscopes, are used before an AFM for applications in technology industries.

Another characteristic of technology companies is that they often have to improve and change their processes in a very short time period when compared to traditional commodity-based companies. Thus, a company may need to use an AFM to support a manufacturing process for only a few years, after which the company may not need the microscope.

The limiting issues for broad acceptance of atomic force microscopes in the high-technology industries are probably probe quality and image capture rates. Variations in AFM probe geometries result in unwanted variations in quantitative measurements. Additionally, if a probe is damaged while scanning a surface, it is difficult for an operator to know, and erroneous data may be collected. When compared to optical and electron beam techniques, the AFM is sometimes considered to be slow. Thus, the AFM is used only for product development and off-line production applications.

Specialized AFM products are often required for high-technology industries. For example, in the semiconductor industry, the AFM must be particulate free so that it does not contaminate product wafers. Also, sample sizes can be very large in the high-technology industries. In order to design an AFM that can handle large sheets of glass
for the LCD industry, the final instrument must be very large and heavy. It is not uncommon for these AFM instruments to weigh several thousand pounds. For many applications the AFM must be able to automatically measure several images on the same sample.

(p.180) 7.4.2.1 Semiconductor industry
The use of AFM in the semiconductor industry started in the mid-1990s. Since that time several hundred atomic force microscopes have been employed by semiconductor companies. The majority of the applications were never made public. The applications presented here are some of those that were made public. Most of these applications were associated with developing new processes, processes that most likely have a lifespan of 5–10 years.

Front-end (wafer fabrication) applications include measurement of surface texture and dimensional metrology. The polishing of silicon wafers in the planarization step of semiconductor manufacture can be studied by AFM. The tolerance in these manufacturing processes is very low, and the signal-to-noise ratio of the measurements must be very high, which is why for such applications only AFM is suitable. The noise floor of the AFM must be below 0.5 Å for adequate characterization. The planarization method of chemical–mechanical polishing (CMP) was developed with the aid of AFMs for quality control. The second major application in semiconductors is in metrology. The measurement of feature and trench dimensions is very important, and due to its high resolution and three-dimensional information, AFM is a powerful tool for this [730]. Specialized probes such as those in the shape of a pole, which reduce ambiguity at feature edges, or even with flared ends that can measure line edge roughness have been developed for these applications [731]. Such measurements do not require measuring an entire image but only a line profile over the trench. The time required for the measurement is greatly reduced when only a line profile is required. Atomic force microscopes give excellent contrast on extremely flat surfaces, and so are very useful for thin film characterization. Because the AFM creates a 3-D map of the surface, software algorithms for measuring grain sizes are very reliable. In the case of insulating films, there is a great advantage of AFM over SEM because the sample does not need to be coated with a conductive film.

Back-end applications (product assembly, packaging and testing) in the semiconductor industry include solving problems associated with packaging, thermal management, adhesion of contacts and bonding. The thermal measurement capabilities of the AFM have been used to help solve heat transfer problems in several generations of microprocessors. Defects associated with problems in production processes can be investigated in detail with AFM. The defects can sometimes be seen with an optical microscope, but the AFM image can give greater insight into the source of the defect. Direct electrical measurements with the AFM can also help in such cases. Examples of these semiconductor applications are given in Figure 7.29.

7.4.2.2 Data storage
Advances in the data storage industry occur at a staggering pace. Data densities of all types of media increase dramatically on an annual basis. Atomic force microscopes were first used by the data storage industry to study the magnetic domains on hard disk drives in the early 1990s. At the same time, atomic force microscopes were used to improve and study optical disk drives. This has continued until today, and AFMs played a critical role in the development of CDs, DVDs and their successors. Custom stages are available to hold disk masters, stampers and replicas. With automated software it is possible to make statistical measurements of bit dimension distributions. Common measurements are the bit width, (p.181)

Fig. 7.29. Examples of high-technology applications in the semiconductor industry. Top left: using AFM to measure the quality of polishing by CMP. The CMP processes can achieve extraordinary flatness, as may be seen from the very small z-scale of this height image. The rms roughness ($R_q$) of this image is less than 1 nm. Top right: metrological measurement over a trench on a patterned wafer. Bottom left: thin film characterization by grain analysis on a polysilicon film. Bottom right: example of a defect imaged in a cross-sectioned device (circled).

length, and the angles of the bits sides. Although the AFM is helpful in developing these products, the AFM is typically not used in the mass production of optical media. A very common application for the AFM in the data storage industry is the study of pole tip recession on hard disk drive read/write heads [732]. The manufacture of hard disk heads is another example of a process that has extremely low error tolerances, due to the small head–platter distance in hard drives. AFM is used to accurately measure the recession (trench) that is used to protect the poles in operation [733]. Of course, MFM is also capable of measuring the magnetic domains with high resolution, which becomes increasingly important as data density increases [734]. Another application in the hard disk industry is measurement of laser bumps. These bumps on the surface of hard drives are used to (p.182)
facilitate the landing of the drive head when not in use. They are created in the landing zone of the disk surface with a short laser pulse. The bumps are not very deep and cover a small area of the disks surface. The AFM helped with the development of the process to create these pits; the height of the pit’s lip as well as the depth of the pit can be directly measured with an AFM. Some examples of these applications from the data storage industry are shown in Figure 7.30.

7.4.2.3 MEMS devices

One of the first commercial applications for MEMS devices was the cantilevers/probes used in the atomic force microscope. Applications for the AFM in MEMs devices include measuring the surface roughness of reflective surfaces, measuring the forces required to move MEMS fabricated devices, and metrology measurements on MEMS devices. Figure 7.31 illustrates a device imaged by both SEM and AFM, showing how the true

(p.183)
Applications of AFM

7.4.2.4 Optical products

Optical components can be somewhat less demanding than semiconductors, but still require very low manufacturing tolerances, and AFM can be useful to characterize several high-technology optical products. LCD screens are created by patterning a glass surface with several layers of thin films. The AFM is an excellent tool for measuring the thickness of the thin films on the surface of the glass. In the example in Figure 7.32, an image of an LCD screen is shown, with a critical measurement on a thin film step. The step height at the left is 30 nm, and the step at the right is 73 nm.

Optical components are required for many high-technology products such as optical storage device lenses, digital cameras, and fibre optics required for telecommunications. The AFM is an excellent method for imaging optical components. These components are often quite large, but measurement of these components is not possible with an optical profiler because the devices are transparent. A mechanical profiler cannot be used because it would potentially scratch their surfaces. The example in Figure 7.32 shows a micro-lens array comprising a series of microscopic lenses fabricated onto a surface. Each lens is approximately 7 microns across. The AFM is capable of precisely measuring the dimensions of each of the lenses, as well as the surface roughness of the lenses. Any anisotropy in the lenses will cause unwanted distortion in the devices that the lenses are used in.

![Image of optical products](image-url)