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New technologies in scanning probe microscopy for studying molecular interactions in cells

Petri P. Lehenkari, Guillaume T. Charras, Stephen A. Nesbitt and Mike A. Horton

Atomic force microscopy (AFM) is a specialised form of scanning probe microscopy, which was invented by Binnig and colleagues in 1986. Since then, AFM has been increasingly used to study biomedical problems. Because of its high resolution, AFM has been used to examine the topography or shape of surfaces, such as during the molecular imaging of proteins. This, combined with the ability to operate under known force regimes, makes AFM technology particularly useful for measuring intermolecular bond forces and assessing the mechanical properties of biological materials. Many of the constraints (e.g. complex instrumentation, slow acquisition speeds and poor vertical range) that previously limited the use of AFM in cell biology are now beginning to be resolved. Technological advances will enable AFM to challenge both confocal laser scanning microscopy and scanning electron microscopy as a method for carrying out three-dimensional imaging. Its use as both a precise micro-manipulator and a measurement tool will probably result in many novel and exciting applications in the future. In this article, we have reviewed some of the current biological applications of AFM, and illustrated these applications using studies of the cell biology of bone and integrin-mediated adhesion.

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Biochemical assays such as enzyme-linked immunosorbent assays and surface plasmon resonance analysis are extremely efficient and widely used to measure many of the chemical interactions upon which physiological phenomena in living organisms are based. This is especially so for those reactions that are not appreciably influenced by isolation from their normal surroundings. However, to function optimally, many biomolecules must be embedded in their natural surroundings, for example within a matrix or cell membrane. Such biomolecules must be analysed using even more specialised techniques that operate at the level of individual cells and target specific receptor proteins. Here, these receptors often need to be activated, either by their anchoring to the cytoskeleton or associated membrane proteins, or through interactions with intracellular signalling molecules. An example that typifies these requirements is the integrin family of cell-adhesion receptors (Ref. 1). Thus, integrin receptors are coupled 'mechanically' to the cytoskeleton and are functionally dependent upon associated signal-transduction processes, which are considered in more detail later in this review.

Previously, molecular interactions at the level of the cell membrane have been investigated using various micro-manipulative techniques, such as the surface-force apparatus (Ref. 2), pipette suction (Ref. 3), laser tweezers (Ref. 4), magnetic beads or hydrodynamic drag (Ref. 5). Several methods have also been developed to measure more specifically the affinity between receptors and their ligands. However, these have been limited either to the analysis of protein–protein interactions analysed with radioactively labelled probes and by surface plasmon resonance (Ref. 6), or to the characterisation of average 'affinities' in bulk cell-binding assays. All of the micro-manipulative techniques are based on the use of a physical probe that is 'functionalised' with a molecule that acts as a ligand, which binds to a cell-surface receptor. A classical example of such a micro-manipulative approach is the use of laser tweezers to study the molecular basis of muscle contraction, during which myosin molecules move along actin filaments (Refs 7, 8); the size and direction of the forces imparted by this 'molecular motor' have been characterised in detail.

'May the force be with you!'

Atomic force microscopy (AFM) is a specialised form of scanning probe microscopy that enables the acquisition of high-resolution topographical images of intact cells; furthermore, it allows the measurement of single molecular events under physiological conditions (Ref. 9). AFM was devised by Binnig and colleagues in 1986 (Ref. 10) for the examination of material surfaces, and was subsequently applied to the life sciences by Marti and colleagues (Ref. 11). Today, AFM is used widely in biology, and a summary of the range of the applications of AFM in cell biology is provided in Table 1. Intriguingly, AFM is increasingly being used as an ultra-precise micro-manipulator rather than an imaging tool, because it can apply precisely regulated, small forces. Indeed, as regards its imaging capabilities for cell-biological work, the rate at which it captures images is rather

Table 1. Examples of cell-biology subjects that have been studied using atomic force microscopy (tab001mhu)

Subject studied	Refs
Cell-surface morphology; cell volume	17, 63, 64, 65, 66, 67, 68, 69
Cytoskeleton and cytoskeletal elements	17, 19, 20, 23, 70, 71, 72
Cell migration and locomotion	50, 71, 73, 74, 75
Organelles and organelle movement	17, 23, 73, 76
Secretory structures	21, 23
Viral budding in infected cells	77, 78
Cell–matrix and cell–cell binding forces	28, 33, 42, 51
Receptor–ligand binding forces on cells	42

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disappointing (usually requiring several minutes per image); thus, AFM faces 'competition' from confocal laser scanning microscopy and scanning electron microscopy, both of which can accumulate data faster. However, owing to the method by which surface topography is determined, AFM has many other uses, including: (1) the measurement of the binding force between a molecule and its ligand, (2) the determination of the elastic properties of cells, (3) the evaluation of the friction coefficient of materials and (4) the high-resolution imaging of isolated proteins and structures. Importantly, AFM can be performed on living samples such as cells and tissues.

In this review, our aim was to outline some of the different uses of AFM in the life sciences, specifically focusing on its uses as a nanoscale micro-manipulator and a detector of molecular interactions. As an example, we have also described current views of the mechanics involved in integrin–ligand recognition, and considered the potential applications of AFM in bone cell biology (Ref. 12).

Principles of AFM

The measurement of the topography of a sample using AFM involves a micro-fabricated cantilever (usually 100–200 μm long) with a very small tip (which has a contact area of only a few square nanometers) being raster scanned above the surface of the sample (i.e. the microscope tip is moved progressively backwards and forwards across the surface; see Fig. 1). A piezo-electric crystal is used to raise or lower the cantilever, to maintain a constant bending of the cantilever. A laser beam is reflected from the top of the cantilever towards a photo-detector, which detects any bending of the cantilever, thus enabling the actual position of the cantilever to be back-calculated. As a result, the atomic force microscope records images of surface topography under a constant applied force (in the low nN range), which is optimised to produce maximal resolution without damaging the surface of the sample.

Many different imaging modes have been developed for AFM, and these have been reviewed by Hansma and colleagues (Ref. 13). Contact-mode imaging involves the whole surface being scanned with the tip of the cantilever in constant contact with the surface. Using the contact mode, it is also possible to measure the torque applied on the cantilever as it is scanned sideways over the surface. Differences in torque equate to differences in the friction of the surface and variation in material properties (Refs 14, 15). An alternative non-contact, or 'tapping', mode involves the cantilever being rapidly oscillated while it is slowly lowered towards the surface. When the cantilever comes close to the surface, the amplitude of the oscillations is dampened and the surface can be detected. This mode is much less damaging to the samples (Ref. 16). During a third mode of operation, 'force–distance measurement', the cantilever is slowly lowered towards the surface and its deflection is constantly recorded. This mode yields a curve showing the bending of the cantilever as a function of the distance travelled (see Fig. 2). Using the spring constant of the cantilever, it is then possible to calculate the force needed to create that deflection. This final mode is the basis for the measurement of the material properties of cells and the binding forces between ligands and receptors.

Applications of AFM

Topography measurements

AFM was originally designed for the study of surface topography. Initially, the technique yields a height map, which is generated from height measurements taken at multiple positions along the cell surface. From this map, it is possible to construct a three-dimensional profile of the cell surface (Fig. 1b). The resolution is proportional to the deformation caused by the force used for imaging. When an atomic force microscope is used in contact mode on surfaces that are harder than the probe, the lateral resolution of the microscope is at the atomic level. However, for surfaces that are 'softer' than the tip of the atomic force microscope, the resolution obtained is lower than the 'atomic scale', owing to indentation of the tip along the probed surface. Indeed, on materials as soft as living cells, the resolution can be as low as in the order of 500 nm. In comparison, a resolution of 350 nm is achievable using confocal laser scanning microscopy, which is, in turn, much lower than that achievable using scanning electron microscopy (i.e. ~1 nm). The resolution achievable using AFM can be enhanced by operating the atomic force microscope in non-contact mode. The estimated resolution in tapping mode is in the order of a few tens of

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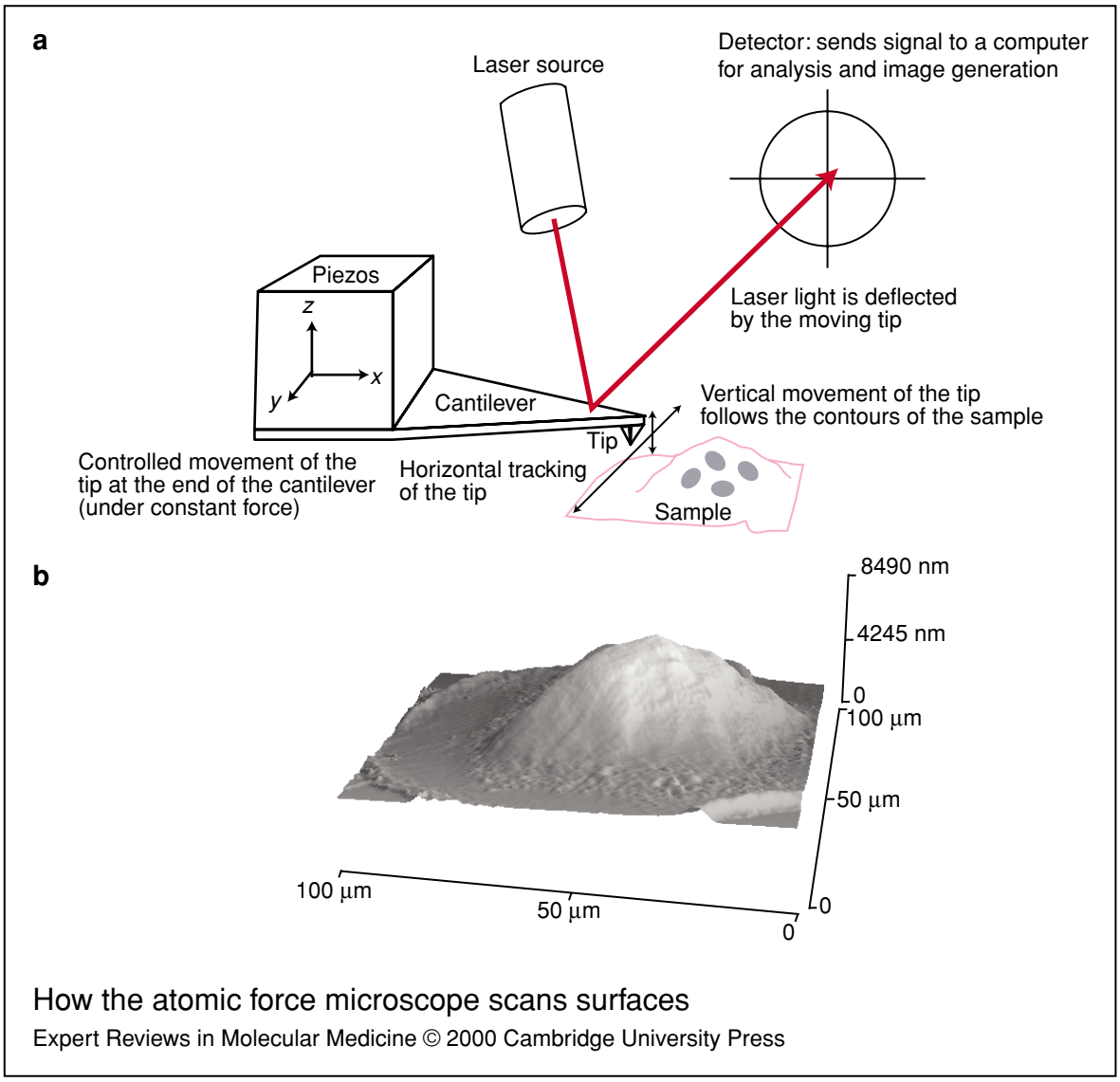

How the atomic force microscope scans surfaces

Figure 1. How the atomic force microscope scans surfaces. (a) A schematic illustration of the method of operation of an atomic force microscope. In this example, the sample being analysed is a multi-nucleated osteoclast derived from bone (osteoclasts, which resorb bone matrix, are large cells, with dimensions up to 100 µm x 100 µm x 8 µm). The position of the cantilever of the atomic force microscope is controlled by three piezo-ceramic controllers, which place the tip of the atomic force microscope in the *x* and *y* directions and additionally, under a controlled and known downward force, in the *z* or vertical direction. A laser beam is directed at the reflective, upper surface of the cantilever, and the deflected light detected by a four-quadrant photo-detector. Both the size and position of the current created in the detector are linked via a computer to a feedback circuit, which maintains the cantilever position at a defined location on the surface that is being analysed. Meanwhile the cantilever is scanned backwards and forwards across the surface (raster scanned) to produce an image (of surface topography, using the simplest form of atomic force microscopy) that reflects the change in position of the cantilever tip. Other forms of imaging and measurement using atomic force microscopy can be generated by analysing the different types of information produced in the feedback circuits and also by employing different modes of controlling the movement of the cantilever (see text, for further details). (b) An example of a three-dimensional image of the surface topography of an osteoclast that was cultured on a glass coverslip. This atomic force microscopy image was produced using the simplest mode of operation (the so-called contact mode, whereby the tip of the microscope is kept on the surface of the sample that is being analysed) **(fig001mhu)**.

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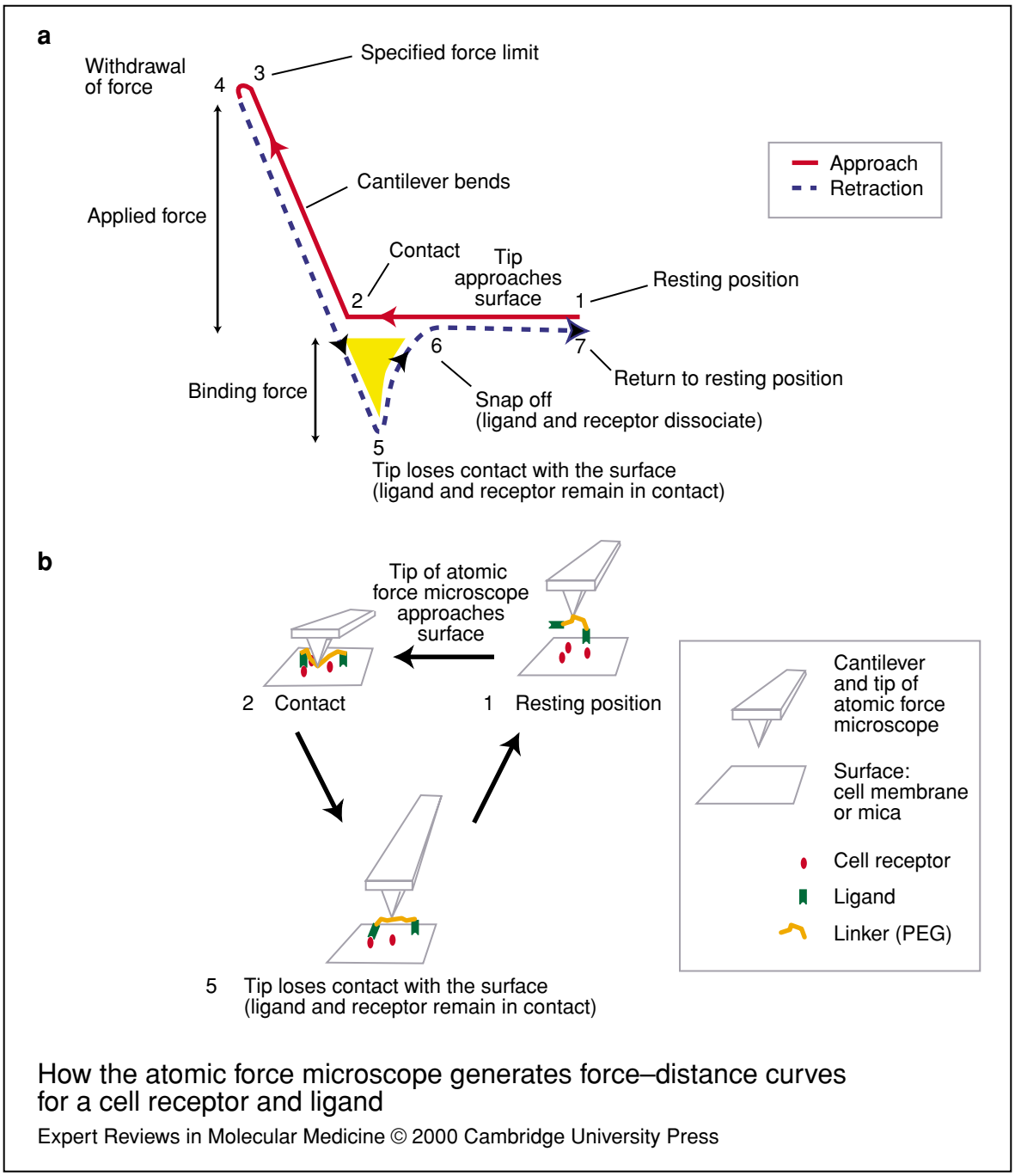

How the atomic force microscope generates force–distance curves for a cell receptor and ligand

Figure 2. How the atomic force microscope generates force–distance curves for a cell receptor and ligand (*see next page for legend*) **(fig002mhu)**.

nanometers, which is better than that of confocal laser scanning microscopy (Ref. 16). However, non-contact imaging is difficult to operate in liquids and particularly so in the case of living cells in an aqueous phase; this is due to the confounding effects of oscillations of the object being imaged and the surrounding fluid that are induced by movement of the cantilever.

Topography measurements yield the three-dimensional aspect of the examined surface with a vertical resolution at the nanometer level (Fig. 1b). Samples can be either fixed (i.e. using conventional histological fixatives) or unfixed.

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Figure 2. How the atomic force microscope generates force–distance curves for a cell receptor and ligand. (a) A representative force–distance curve between a ligand, which is bound to the tip of an atomic force microscope, and a receptor molecule, which is bound to a solid surface. (b) A schematic illustration of the molecular interactions observed. In both parts, from positions '1' to '2', the tip is approaching the surface, and at position '2' contact is made. From positions '2' to '3', the cantilever bends until it reaches the specified force limit that is to be applied; it is then withdrawn during positions '4' and '5'. At position '5', the tip relinquishes contact with the surface that is being analysed; however, the ligand, which is coupled to the tip, remains bound to its receptor molecule on the surface of the sample and both molecules are extended. Following the further application of the retraction force, the molecule–ligand complex dissociates (at position '6', which is referred to as 'snap off'). Between positions '6' and '7', the cantilever returns to its resting position (i.e. position '1') and is ready for another measurement. The maximum difference between the approach curve (i.e. the upper, solid line) and the retraction curves (i.e. the lower, dotted line), and the shape of the curve between positions '2', '5' and '6' yield information on the interaction 'binding' force between the ligand on the tip of the microscope and the receptor molecule on the surface, and their physical properties. Abbreviations used: PEG = polyethylene glycol, a polymer that is used as a linker molecule between the tip of the atomic force microscope and the ligand that is used to probe the cellular receptor **(fig002mhu)**.

However, as shown in Figure 3, fixed cells present striking morphological differences when compared with live cells, as has been reported by several investigators (Refs 17, 18). In contact mode, because the softer parts of the cytoskeleton inside the cell are indented more than the harder parts of the cell by the tip of the atomic force microscope, this mode of imaging can also reveal components of the underlying cytoskeleton, thereby providing structural information about

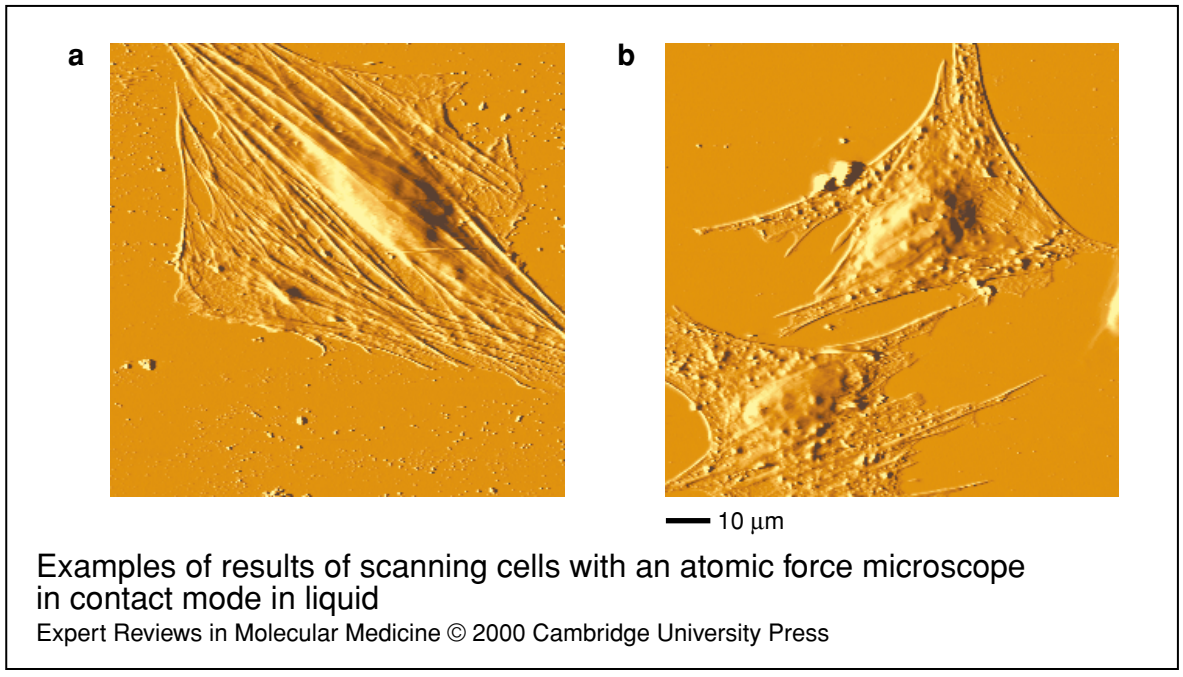

Figure 3. Examples of results of scanning cells with an atomic force microscope in contact mode in liquid. (a) An atomic force microscopy image of a live osteoblast (i.e. a bone cell that synthesises bone matrix), which has been scanned in contact mode using a constant force (1 nN). Prominent cytoskeletal fibres can be seen within the cell; these are evident because the downward force on the tip of the atomic force microscope allowed the relatively rigid cytoskeleton to be 'seen' through the compliant cell membrane. (b) An atomic force microscopy image of similar osteoblasts to that shown in part (a), but after pre-treatment with a commonly used histological aldehyde fixative. Note the change in morphology compared with that shown in (a); the underlying cytoskeletal elements are less visible in the fixed cells, because they are 'stiffer' (i.e. have a higher elastic modulus). Additional granular structures are evident in the fixed cells; these are fixation artifacts. These images were produced using a modified TopoMetrix Explorer™ atomic force microscope (Ref. 9) **(fig003mhu)**.

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Table 2. Examples of the interactive forces between specific molecules (tab002mhu)

Ligand–receptor or cell-structure interactions	Force (pN)	Refs
Proteins		
Meromyosin and actin	15–25	7
Avidin and biotin	160–200	32, 34, 38, 58
Streptavidin and biotin	200–257	32, 79
Cell-adhesion proteoglycans	40–125	36
Antibiotin antibody and biotin	60	35
Anti-HAS and HAS; other antibodies and antigen	49–244	30, 80
P-selectin and glycoprotein ligand-1	165	38
Cells		
RGD and cell receptor	35–120	42
Trophoblast and uterine epithelium	1000–16 000	28
Cell and uncoated surface	19 000–100 000	51
Cell and coated surface	100 000–220 000	51

Abbreviations used: HAS = human serum albumin; RGD = arginine–glycine–asparagine peptide motif

the interior of the cell. Thus, sub-surface biological processes such as vesicle trafficking, mitosis or cytoskeletal rearrangement can be evaluated under ambient conditions (Refs 19, 20), and the imaging of these processes enhanced in live cells because they are 'softer' than the 'more rigid' fixed cells. Furthermore, AFM is beginning to be used to measure other physiological responses. For example, Schneider and colleagues (Refs 21, 22) have used AFM to measure the aldosterone-induced volume increase in live endothelial cells, whereas Spudich and Braunstain used it to follow the process of basophil de-granulation (Ref. 23).

Even though AFM can be used to acquire images of surface topography, similar images can be obtained by using confocal laser scanning microscopy. This can be achieved by labelling cells with fluorescent markers (e.g. tagged antibodies or lectins) that bind to structures that are found solely on the cell surface, such as the αvβ3 integrin (i.e. the vitronectin receptor) on osteoclasts (Refs 24, 25, 26). Similar confocal techniques that utilise membrane-excluded dyes (e.g. fluorescein-linked dextrans, which have a high molecular weight) have been developed to reveal surface topography (Ref. 27). Using confocal laser scanning microscopy, a series of confocal cross-sectional images (separated by a minimum thickness of 350 nm) are collected vertically through a cell. The stack of images is then post-processed (using a computer and commercially available software) to recreate a three-dimensional rendered image (Fig. 4). The resolution and surface detail obtained using AFM and optical (confocal) techniques are comparable.

Measurements of binding forces

Since its invention, AFM has proven of value for quantifying the binding forces of interactions between proteins, between cells, between receptors and ligands (either on cells or plated onto mica), or between cells and their substrates (Table 2). Receptor–ligand binding forces in the range of 15–250 pN have been reported for protein interactions, and up to 220 nN for total cellular binding forces (Refs 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39). Using the atomic force microscope, it is possible to evaluate directly the binding force between a single receptor molecule and its ligand (Refs 40, 41). To achieve

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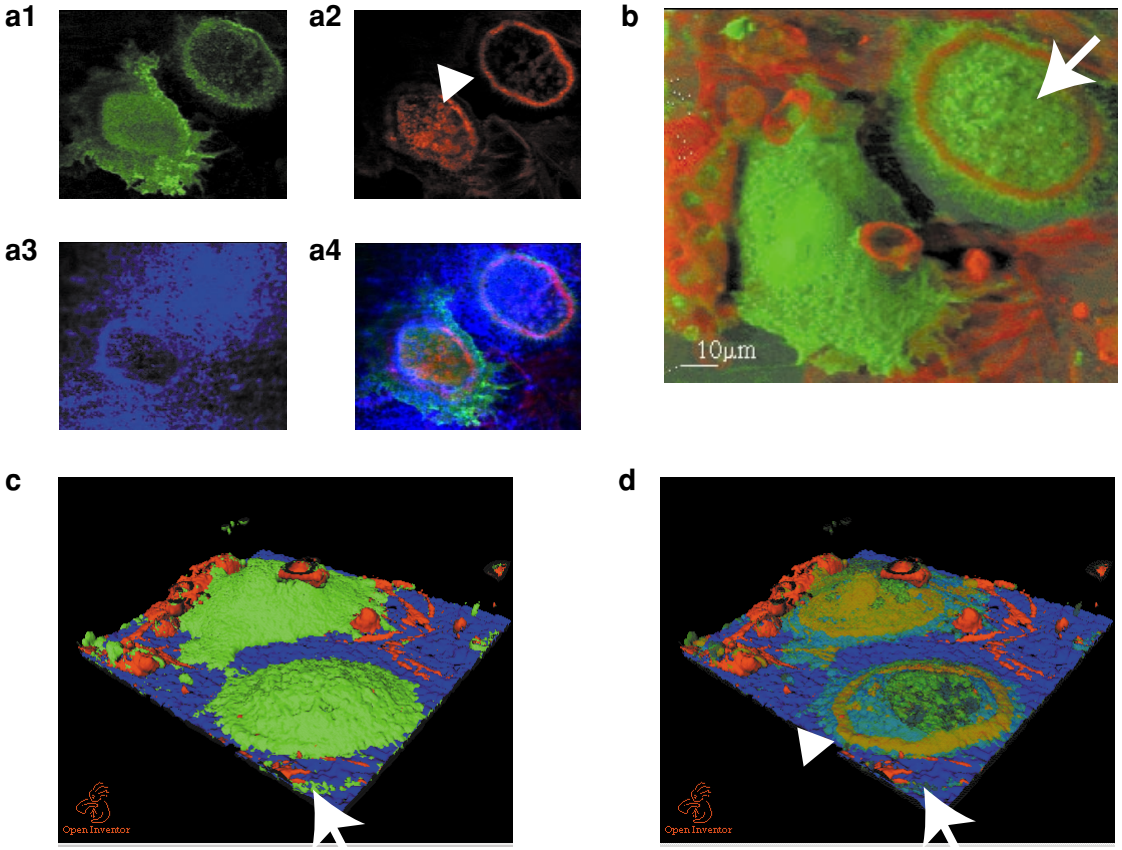

Confocal laser scanning microscopy of fixed and stained cells

Figure 4. Confocal laser scanning microscopy of fixed and stained cells. A computer-produced image reconstruction is shown of fixed and immuno-stained osteoclasts (bone-resorbing cells) that were cultured on dentine. Three-dimensional and volumetric data have been derived from a series of confocal laser scanning microscope images that were produced using a Leica TCS-NT system. The surface features shown in this figure can be compared with those of the atomic force microscope topographical image of a living osteoclast [see Fig. 1b]. Osteoclasts resorb mineralised tissues following a series of cellular polarisation events. Cytoskeletal rearrangement in osteoclasts creates a structure that is rich in F-actin, which forms a tight seal that encloses a specialised secretory membrane called the ruffled border. Protons and proteases cross the ruffled border and degrade bone matrix by extracellular demineralisation and proteolytic activity. Subsequently, a resorption pit is formed beneath the osteoclast, as released bone matrix is transported through the cell before being expelled into the extracellular space (Refs 61, 62). (a1–a4) An image, taken in a single confocal plane, of two osteoclasts, one in an early resorption state (top right), the other merely adhering to the dentine surface. (a1) The vitronectin receptors of the osteoclasts have been stained with fluorescein-labelled antibodies (and appear green); (a2) F-actin has been stained using rhodamine-phalloidin (and appears red); (a3) the dentine surface has been biotinylated and stained with Cy5-labelled streptavidin (and appears blue). The images were taken at the surface of the dentine, where the F-actin 'ring' structure (a tight seal at the resorption area) is maximal [as shown by an arrowhead on image (a2)]. (a4) This image has been produced by merging the previous three images. (b) A pseudo three-dimensional projection of a stack of single confocal slices viewed from above; the arrow indicates the view direction for the images shown in parts (c) and (d). (c) This image shows an isosurface reconstruction of the data-set for all three colours [i.e. from (a1–a3)], which has been tilted 30° downwards and rotated through 90° clockwise relative to the image shown in (b). (d) This shows the same view as that shown in (c), but the intensity of the 'green signal' (i.e. of the vitronectin receptor) has been reduced to visualise the internal structure of the osteoclasts (particularly the F-actin ring, which is indicated by the arrowhead) **(fig004mhu)**.

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such a measurement, the tip of the atomic force microscope is 'functionalised' by adding a specific interactive molecule, such as an antibody or protein ligand. Two basic techniques have been developed to achieve this. One relies on the passive chemi-sorption of a solution of the 'coating' molecule onto the tip of the microscope, often using polyethylene glycol as a linker or adsorbent (Ref. 42). The other technique uses covalent coupling (using various chemistries) to link the 'coating' molecule directly onto the tip (Ref. 39). Force–distance curves are then recorded in different locations until an adhesion event occurs, which is easily recognised by the shape of the force–distance curve. The shape and interpretation of a typical force–distance measurement are shown in Figure 2.

The measurement of the binding forces of cellular components depends on the ligand being either adsorbed onto atomically smooth, cleaved mica, or present as a natural component of the cell membrane. However, it is inherently difficult to carry out measurements on living cells for three reasons: (1) the target molecule is easily lost from the cell membrane, (2) the target molecule may be present at too low a density to make a successful interaction likely or (3) the tip of the atomic force microscope may become contaminated by cellular debris during the analysis (Ref. 43). Recently, our group has reported the first measurements of binding forces between an arginine–glycine–asparagine (arg–gly–asp or RGD) peptide and integrin, its cognate receptor, on living cells (Ref. 42). Details of this application of AFM are described later in this review.

Measuring the material properties of a cell

The intracellular cytoskeleton gives a cell its physical integrity but the cytoskeleton also adapts to the external environment and the activity levels of the cell. However, the exact role of each element of the cytoskeleton is still unclear. By measuring the material properties of a cell (e.g. stiffness, plasticity and visco-elasticity) and determining the effects of induced cytoskeletal changes on them, it is possible to gain insight into the particular role of each cytoskeletal element. Moreover, by analysing the slopes of force–distance curves that are recorded during indentation with the probe of an atomic force microscope (Fig. 5a and 5b), the elastic modulus (i.e. the stiffness) of a cell can be determined at a particular location, using the theory of indentation (Ref. 44). Thus, AFM can determine the cellular elasticity of tissues (as reviewed by Radmacher in Ref. 45). Applying these principles (Fig. 5), such information has been accumulated during raster scanning across a cell, and a three-dimensional map of information about elastic properties and height of structures generated (Fig. 5c, 5d and 5e).

Common materials, such as steel or bone, have elastic moduli of 200 000 kPa and 10 000 kPa, respectively, whereas living cells are more compliant, with stiffness values in the range of 1–150 kPa. A summary of the reported elastic moduli of different cell types and cell structures is presented in Table 3. As expected, different constituents and regions of a cell have very different stiffness values. The effects of cytoskeleton-disrupting drugs on cell stiffness have been studied extensively (Ref. 46; Table 4). In particular, it was found that disrupting the F-actin network with cytochalasin D reduced cell stiffness. In contrast, glutaraldehyde (a common histological fixative) increased cell stiffness by up to threefold, by increasing the degree of crosslinking between the F-actin fibres and the rest of the cytoskeletal network. Together, these studies show that F-actin filaments participate in the maintenance of cellular elasticity. In focal-adhesion plaques, the F-actin cytoskeleton is linked to integrin receptors on the cell surface by vinculin. Recently, Goldmann and colleagues (Ref. 47) reported that the elastic modulus of vinculin-deficient cells was lower than that of wild-type cells; when vinculin expression was reinstated by gene transfer of the gene encoding vinculin, an elastic modulus close to that of the wild-type cells was attained. These results point to a role for vinculin in stabilising focal adhesions and transferring mechanical stresses to the cytoskeletal network. Banes and colleagues have extensively reviewed the field of transduction of mechanical signals through the cytoskeleton (Ref. 48). Other material properties such as the visco-elasticity and the plasticity of cells have also been measured. Visco-elasticity measurements are determined by indenting (deforming) a cell, and following its recovery over a period of time (Ref. 46). Plasticity measurements are, in contrast, determined by measuring the permanent deformations that are inflicted on a cell as a result of a similar indention (for a review of the basic biomechanical measurements see Ref. 49).

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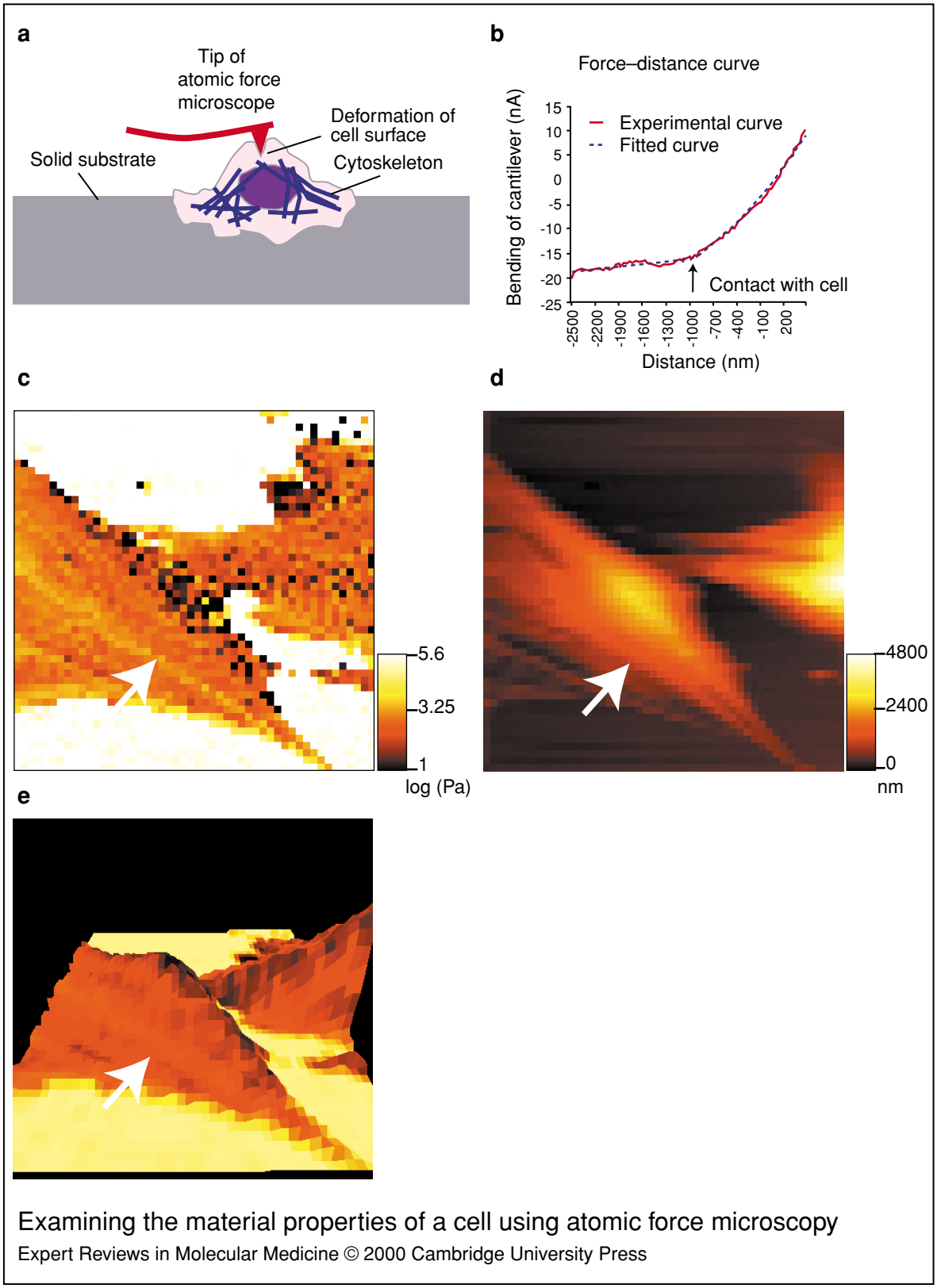

Examining the material properties of a cell using atomic force microscopy

Figure 5. Examining the material properties of a cell using atomic force microscopy (*see next page for legend*) **(fig005mhu)**.

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Figure 5. Examining the material properties of a cell using atomic force microscopy. (a) Because the tip of the cantilever is much stiffer (i.e. has a higher elastic modulus) than the cell, it deforms the cell membrane. (b) Using the theory of indentation, experimental force–distance curves can be compared with, and fitted to, theoretical curves and thus used to quantify cell stiffness. Raster scanning (i.e. moving the tip of the atomic force microscope progressively backwards and forwards across the surface) yields a map of the distribution of derived elasticity (c), and topographical information (d). (c) In the elasticity map, 'hard' areas appear as yellow, and compliant 'softer' areas as black. (e) The two sets of data can be merged to produce a three-dimensional representation of the topography; different colours represent different degrees of stiffness (elastic modulus) in a particular location. In (c), (d) and (e), cytoskeletal elements, which are seen as linear structures and have different material properties compared with those of the surrounding cellular elements, are marked with an arrow **(fig005mhu)**.

Using the atomic force microscope as a micro-manipulator

Recently, there have been several reports of experiments that fall under neither of the previous categories in that they are not aimed at surface measurement, but instead these studies use the atomic force microscope as either a micro-manipulator or a micro-detector. Domke and colleagues used AFM to map the mechanical 'pulse' (i.e. contraction and relaxation) of cardiomyocytes in culture (Ref. 50). Thie and colleagues, using similar technology, examined the adhesive forces between a trophoblast (i.e. an implanting embryo) and the uterine epithelium using whole trophoblast cells, instead of isolated molecules, bound to the tips of atomic force microscopes (Ref. 28). Adhesion forces of ~3 nN were recorded between the cells,

Table 3. Elastic properties of different cell types and cell structures (tab003mhu)

Sample type	Elastic modulus (kPa)	Refs
Cell		
Living cells	1–100	45
Rat liver macrophage	1–10	81
Lung carcinoma cell	13–150	82
Human platelet	1–50	83
Embryonic carcinoma cell	3.8	47
Vinculin-deficient F9 embryonic carcinoma cell	2.5	47
Chicken cardiocyte	5–200	84
Endothelial cell	2 (at cell centre)	85
Fibroblast-like cell	2.23 (at cell centre)	46
Organelle		
Nuclear region of rat atrial mycocytes	500–670	72
Secretory granules from rat mast cells	37–4300	86
Stress fibres	200	84
'Stable' edge of a fibroblast	12	75
Leading edge of a fibroblast	3–5	75
Pseudo-nucleus of a platelet	4	83
Cortex of a platelet	50	83

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Table 4. Effects of cytoskeleton-disrupting drugs on the material properties of cells (tab004mhu)

Cytoskeletal-disrupting drug	Effect on cytoskeleton	Effect on elastic modulus	Refs
Cytochalasin D	Actin filament in actin cytoskeleton disrupted	Decreased by up to threefold	46, 81, 84
Nocodazole, colcemid	Microtubular network disrupted	Increased	46
Glutaraldehyde	Cytoskeletal elements crosslinked	Increased from 1 kPa for living cells to >100 kPa on fixation	85, 46
Protein crosslinking agent (DTSSP)	Receptors on cell surface crosslinked	Increased (no effect, after reduction of disulphide bonds)	46
Concanavalin A	Bound to cell-surface glycoproteins	Increased twofold	46
Pore-forming protein	Pores formed in cell membrane	Decreased	87

which is an order of magnitude higher than reported adhesion forces between receptors and ligands. Recently, Sagvolden and colleagues developed a new use for AFM; they used it to quantify the adhesion forces of cells to a substrate (Ref. 51). This new application is of interest in the field of bioengineering, particularly in orthopaedic and vascular surgery. For example, such data might be useful in the selection of suitable biomaterials for tissue reconstruction. In the future, we are likely to see an increase in the number of similar studies, and their findings used in the development of new therapies.

Integrin–RGD recognition: an example of intermolecular-force measurement in cells using AFM

Members of the integrin family of cell-adhesion receptors play key roles in cell–matrix recognition (Ref. 52). They are found in virtually all types of cells (Ref. 52) as transmembrane receptors, consisting of two non-covalently linked subunits that require the presence of divalent cations for optimal function (Refs 53, 54). In cells that adhere to extracellular-matrix substrates, integrins are concentrated at the underside of the cells in focal-adhesion plaques that are associated with intracellular linker proteins, such as vinculin with both the F-actin cytoskeleton and signalling molecules (Ref. 55). Many integrins recognise peptides that contain the RGD consensus sequence. This sequence is found in several ligands including cell-surface and extracellular-matrix proteins; these proteins include non-collagenous proteins that are found in the bone matrix, such as osteopontin and vitronectin (Ref. 56). The affinity of the integrin receptor for a given ligand depends not only on the integrin type, but also on the conformational changes that follow receptor activation. Such conformational changes are triggered by alterations in the extracellular microenvironment of the integrin receptor, and also by interactions between: (1) the cytoplasmic part of the receptor, (2) the intracellular signalling molecules and (3) the cytoskeleton (Ref. 57). Knowledge of the biological properties of integrins suggests that the reliable evaluation of their affinity for ligands depends on the properties of the intact cells that are being used (Ref. 42). It is likely that this is also true for many other systems of receptors on the cell-surface membrane.

RGD–integrin binding forces, measured using AFM in live osteoclasts and osteoblasts (bone cells), appear to depend not only on the integrin type and its activation status, but also on the spatial conformation of the sequence of the peptide or protein containing the RGD sequence (Ref. 42). Thus, the forces observed for a linear RGD peptide are lower than those for echistatin, a snake-venom protein that contains RGD in a different conformation and is a potent antagonist of integrins. This dependence on conformation is most likely because the more favourable orientation between the ligand and the integrin

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receptor results in shorter interaction ranges for the molecules, and thus higher interaction forces between RGD and the binding site. Surprisingly, the dynamic range of RGD-binding forces is small compared with affinity measurements that are based on the inhibition of receptor–ligand interactions (see Table 2). For example, the binding affinity of a linear RGD peptide and echistatin differs by a factor of ~1 x 10^5, whereas the binding forces that are measured using AFM vary by only approximately twofold (Ref. 42). The relationship between these two values is unclear, and it is probable that fundamental differences exist between the parameters measured. AFM can be used to determine binding force and binding probability separately, whereas affinity measurements combine both (Ref. 30).

AFM also provides further insights into the nature of intermolecular interactions. During the evaluation of intermolecular interactions, the 'pulling velocity' (i.e. the rate at which the tip of the atomic force microscope is removed from the object under analysis during a force–distance measurement) is critical, as shown by Fritz and colleagues, using isolated proteins (Ref. 38), and by our group, using intact cells (Ref. 42). Thus, the measured binding force (e.g. that for the biotin–avidin interaction) varies with the pull-out speed (Ref. 58); higher speeds yield higher binding forces. There are several possible explanations for this phenomenon. The first explanation is based on the occurrence of different adhesive forces within the binding pocket of the receptor that rupture separately; thus, the observed force consists of a series of sub-binding forces, which are added together to yield a higher force at high pulling speeds. The separation into different forces can be explained in two different ways: (1) the bound molecules can undergo different orientations during the pull-out step from the receptor molecule, and the initial binding energy can then be absorbed by both of the molecules and finally transformed into conformational changes; or (2) a series of forces could be explained by the existence of physically separate binding and acceptor domains within the interacting molecules. A second, more simplified explanation is based on the gradual increase in distance and the proportional decrease in force during a slow pull-out. This model does not assume any conformational changes in the molecules, but rather is based on linear binding fields between the molecules, which get weaker the further they are separated. A third explanation considers the properties of the atomic force microscope itself, rather than intermolecular forces. Thus, different pull-out speeds change the recognition capabilities of the probe of the microscope, owing to changes in either the visco-elasticity of the 'vehicle' (i.e. the culture fluid) or the absorption of energy by the probe itself. Which of the three proposed explanations is true awaits confirmation. Meanwhile, the pull-out speeds and cantilever mechanics must be carefully considered when 'absolute' force values are cited.

Research in progress and outstanding research questions

Intriguingly, the use of AFM in biology in the future might be based not so much on its atomic-scale imaging capabilities, but rather on the opportunity it offers to apply and measure forces between biological entities, such as molecules, cells or biomaterials. As a consequence, the atomic force microscope is being used increasingly as an ultra-precise micro-manipulator rather than an imaging tool. This capability has already been used in some extremely interesting preliminary studies that have focused not only on intermolecular forces but also on intramolecular bonds, such as unfolding force measurements of the muscle protein titin or DNA (Refs 59, 60). As instrumentation for AFM improves and a wider range of equipment becomes commercially available, it is likely that cell biologists will routinely use AFM to measure molecular interaction forces.

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Further reading, resources and contacts

The following list of websites comprises our personal favourites relating to scanning probe microscopy and other microscopy techniques. It is not intended to be comprehensive but instead to provide a source of basic data on commercially available instruments; a list of academic research groups; and information on confocal laser scanning microscopy, image processing and other types of microscopy. Together they contain a plethora of basic technical information for the range of techniques described in this review, including links to other websites, downloadable images and teaching information.

Manufacturers of AFM instrumentation

The following list includes some of the AFM instrument manufacturers and suppliers with whom we have had contact. These websites contain useful introductions to the field in terms of instrumentation, links and basic information.

Burleigh Instruments Inc., New York, USA.
http://www.burleigh.com/Pages/surface.htm

Digital Instruments, California, USA.
http://www.di.com/

Molecular Imaging Corporation, Arizona, USA.
http://www.molec.com/index.html

OMICRON, GmbH, Germany.
http://www.omicron-instruments.com/

ThermoMicroscopes/Park Scientific Instruments, California, USA.
http://www.thermomicro.com/

TopoMetrix, California, USA.
http://www.topometrix.com/

WITec GmbH, Germany.
http://www.WITec.de/

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Academic research groups

Below is a fairly comprehensive listing of the websites of various academic research groups who are currently active in the field of AFM. Although such sites have a tendency to change with time, these are the most stable and informative.

Applied Optics Group in the Department of Applied Physics at the University of Twente, The Netherlands.
http://www.tn.utwente.nl/top/index_bft.html

Blaine's Scanning Probe Microscopy Home Page. This site provides a long list of useful links.
http://www.mcs.net/~wbstine/spm/spm.html

Hansma Research Group in the Department of Physics at the University of California Santa Barbara, USA.
http://www.physics.ucsb.edu/Research/BP.phtml

Heckl's Nano Science Research Group at the University of Munchen, Germany.
http://www.nano.geo.uni-muenchen.de/

Hermann Gaub's Group at the University of Munchen, Germany.
http://www.biophysik.physik.uni-muenchen.de/index_e.html

Institute for Biophysics at the University of Linz, Austria.
http://www.bphys.uni-linz.ac.at/

Jan Hoh's Laboratory in the Department of Physiology at the Johns Hopkins University School of Medicine in Baltimore, Maryland, USA.
http://www.hohlab.bs.jhmi.edu/

John Cross's website. This site provides a useful introduction to scanning probe microscopy (or 'imaging surfaces on a fine scale').
http://www.mobot.org/jwcross/spm/

Laboratory of Biophysics and Surface Analysis in the School of Pharmaceutical Sciences at the University of Nottingham, UK.
http://pharm6.pharm.nottingham.ac.uk/

Lindsay Laboratory at the Arizona State University, USA.
http://green.la.asu.edu/

Nottingham Nanoscience Group at the University of Nottingham, UK.
http://www.ccc.nottingham.ac.uk/~ppzstm/home.html

Phil Haydon's Research Group at the Laboratory of Cellular Signalling, Iowa State University, USA.
http://molebio.iastate.edu/~p_haydon/haydon1.pub

Scanning Probe Microscopy Group in the Department of Physics at the University of Bristol, UK.
http://polymer.physics.bristol.ac.uk/spm/

Scanning Probe Microscopy Group at the Institute of Food Research, Norwich, UK.
http://www.ifr.bbsrc.ac.uk/FQM/SPM

Scanning Probe Microscopy Laboratory at the North Carolina State University, USA.
http://spm.aif.ncsu.edu/

The Local Probes Group in the European Molecular Biology Laboratory, Heidelberg, Germany. This site is produced by Stephan Altmann and contains links through to many useful sites.
http://www.embl-heidelberg.de/~altmann/

Zhifeng Shao's Research Group at the Department of Molecular Physics and Biological Physics, University of Virginia, USA.
http://www.med.virginia.edu/medicine/basic-sci/physio/profiles/shao.html

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Nanotechnology
Center for Nanotechnology at the University of Washington, USA. This site includes information and links on the broader subject of 'nanotechnology' rather than microscopy on the nanoscale.
http://www.nano.washington.edu/

General microscopy
The following sites provide links to information related to general (optical) microscopy, imaging and computation, and confocal laser scanning microscopy, etc.

Confocal laser scanning microscopy
3-D Laser Scanning Confocal Microscopy home page, at the Department of Physiology, University of British Columbia, Canada.
http://www.cs.ubc.ca/spider/ladic/confocal.html

Leica Microsystems Heidelberg GmbH, Germany. This site is dedicated to confocal laser scanning microscopy.
http://www.llt.de/

Microscopy index sites
Microscopy & Imaging Resources on the WWW. This collection of on-line resources on microscopy and imaging is produced by the Center for Toxicology, University of Arizona College of Pharmacy, USA.
http://www.pharm.arizona.edu/centers/tox_center/swehsc/exp_path/m-i_onw3.html

Microscopy links. This site is compiled by Bio-Rad Laboratories, Inc., and aims to provide 'a definitive links page for microscopy-related issues'.
http://microscopy.bio-rad.com/Technical_Info/links/index.html

Molecular Expressions Microscopy Primer. This site was produced by Michael W. Davidson (of Mortimer Abramowitz, Olympus America Inc., and the Florida State University).
http://micro.magnet.fsu.edu/primer/index.html

Computation
Bitplane AG, Zurich, Switzerland. This company 'provides scientific solutions', including confocal software.
http://www.bitplane.ch/

NIH Image. This site provides information on NIH *Image*, a public domain image-processing and analysis program for the Macintosh, which was developed at the Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), part of the National Institutes of Health (NIH).
http://rsb.info.nih.gov/nih-image/

The BioImage Homepage. This site describes a European initiative for a new database of multidimensional biological images.
http://www.bioimage.org/

Features associated with this article

Figures
Figure 1. How the atomic force microscope scans surfaces (fig001mhu).
Figure 2. How the atomic force microscope generates force–distance curves for a cell receptor and ligand (fig002mhu).
Figure 3. Examples of results of scanning cells with an atomic force microscope in contact mode in liquid (fig003mhu).
Figure 4. Confocal laser scanning microscopy of fixed and stained cells (fig004mhu).
Figure 5. Examining the material properties of a cell using atomic force microscopy (fig005mhu).

Tables
Table 1. Examples of cell-biology subjects that have been studied using atomic force microscopy (tab001mhu).
Table 2. Examples of the interactive forces between specific molecules (tab002mhu).
Table 3. Elastic properties of different cell types and cell structures (tab003mhu).
Table 4. Effects of cytoskeleton-disrupting drugs on the material properties of cells (tab004mhu).

Movie
Movie 1. An osteoclast in three dimensions (swf001mhu).

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