USING NANOTECHNIQUES TO EXPLORE MICROBIAL SURFACES

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Our current understanding of microbial surfaces owes much to the development of electron microscopy techniques. Yet, a crucial limitation of electron microscopy is that it cannot be used to examine biological structures directly in aqueous solutions. In recent years, however, atomic force microscopy (AFM) has provided a range of new opportunities for viewing and manipulating microbial surfaces in their native environments. Examples of AFM-based analyses include visualizing conformational changes in single membrane proteins, the real-time observation of cell-surface dynamics, analysing the unfolding of cell-surface proteins and detecting individual cell-surface receptors. These analyses have contributed to our understanding of the structure–function relationships of cell surfaces and will hopefully allow new applications to be developed for AFM in medicine and biotechnology.

CRYO-ELECTRON MICROSCOPY
An electron microscopy technique in which the sample is frozen to protect it during imaging.

ATOMIC FORCE MICROSCOPY (AFM). A relatively new form of microscopy in which a sharp tip is scanned over the surface of a sample, while sensing the interaction force between the tip and the sample. Because AFM does not rely on an incident beam, as in electron or light microscopy, the specimen can be directly observed at high resolution in aqueous solution.

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Why study microbial surfaces? Most microorganisms have a well-defined cell wall that has presumably evolved during the course of evolution by selection in response to environmental and ecological pressures[1,2]. As microbial surfaces form a boundary between the external environment and the cell, they have several important functions, including determining cell shape, growth and division, enabling cells to resist turgor pressure, acting as molecular sieves, and mediating molecular recognition and cellular interactions. Therefore, studying the structure, properties and functions of microbial surfaces is an exciting and continuously expanding field of microbiology.

Our current view of the organization of microbial surfaces owes much to the tremendous developments in electron microscopy techniques over the past 20–30 years. In particular, CRYO-ELECTRON MICROSCOPY methods have allowed researchers to obtain high-resolution images of cell-surface structures in conditions close to their native state[3,4]. Yet, these sophisticated methods are labour- and equipment-intensive, and they are not suitable for examining specimens in aqueous solutions. As well as surface structure, physical properties such as elasticity and surface charge are also important determinants of the functions of cell surfaces. These properties have traditionally been difficult to study in microorganisms owing to their small size. Valuable information can be obtained using electron microscopy and surface-analysis techniques[1,5]; however, these assays require cell manipulation (for example, extraction, drying and labelling) before examination and they generally provide averaged information obtained from many cells. Similarly, the forces that determine cellular interactions and molecular-recognition events remain largely unknown owing to the lack of appropriate probing techniques. Consequently, there was an urgent need to develop new high-resolution tools to probe the structure, properties and interactions of cell surfaces in their native environments.

The invention of ATOMIC FORCE MICROSCOPY (AFM) in the mid-1980s[6], followed by continuous progress in instrumentation, sample preparation and recording conditions, has revolutionized the way in which microscopists explore biological structures. This surface-imaging technique involves scanning a sharp tip over the surface of a sample, while sensing the interaction force between the tip and the sample (FIG. 1). The images are therefore created not by using an incident beam, as in other classical microscopy methods, but by measuring 'near-field' physical interactions between the instrument tip and the surface of interest. The main advantage of an atomic force microscope is that it provides direct images

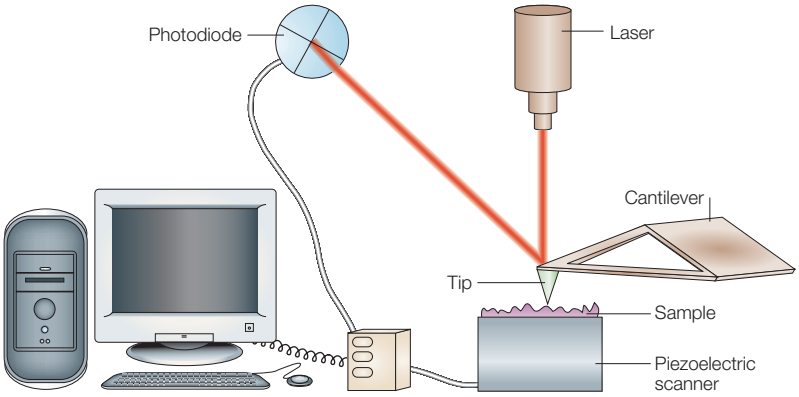

Figure 1 | **The principles of atomic force microscopy.** Atomic force microscopy (AFM) is a member of a family of new microscopic techniques that are referred to as scanning-probe microscopies (SPMs). The concept on which all SPMs are based is the generation of images of surfaces by measuring the physical interaction between a sharp tip and the sample rather than by using an incident beam (light or electrons) as in classical microscopy. The main parts of an atomic force microscope are the sample stage, the CANTILEVER and the optical detection system, which comprises a laser diode and a photodetector. The sample is moved relative to the cantilever in three dimensions using PIEZOELECTRIC CERAMICS. The force interacting between the tip and the sample is monitored with piconewton (10^{-12} N) sensitivity, by attaching the tip to a soft cantilever, which acts as a spring, and measuring the bending (or deflection) of the cantilever. The cantilever deflection is usually detected by a laser beam focused on the free end of the cantilever and is reflected into a photodiode. AFM cantilevers and tips are usually made of silicon or silicon nitride using MICROFABRICATION techniques.

of biological specimens at sub-nanometre resolution under physiological conditions (BOX 1). In fact, an atomic force microscope is more than just a microscope as it can also measure minute forces within or between biological molecules — a method known as FORCE SPECTROSCOPY — in a way that was not previously possible (BOX 1).

Since the late 1980s, many biological specimens have been examined by AFM, ranging from biological molecules such as DNA, to lipid membranes and cells (for reviews on the biological applications of AFM, see REFS 7–16). In recent years, rapid advances have been made in applying AFM techniques to issues of microbiological importance, which indicates that AFM is becoming an established technique amongst microbiological researchers. Some of the exciting questions that have been addressed using AFM are listed in TABLE 1 and include: is the surface ultrastructure of a living cell similar to that observed by electron microscopy?; how does the surface morphology of bacterial cells change as they grow?; do physical properties such as cell-wall elasticity and surface charge vary across cell surfaces?; how flexible are cell-surface polysaccharides?; and what forces are required to unfold a membrane protein?

Visualizing single membrane proteins

S-LAYERS — two-dimensional (2-D) arrays of protein or glycoprotein subunits — are one of the most common cell-surface structures in bacteria[1,2]. As many isolated S-layer subunits can reassemble into stable 2-D crystalline arrays on solid surfaces, they have proved to be particularly well suited for high-resolution AFM analysis. After pioneering studies in the mid-1990s[17,18], continuous improvements in AFM instrumentation and methodology have allowed many different bacterial protein crystals to be studied at sub-nanometre resolution, including not only S-layers in *Bacillus* spp.[19] and *Deinococcus radiodurans*[20] (where the S-layer is also referred to as the hexagonally packed intermediate (HPI) layer), but also the purple membrane from the archaeon *Halobacterium salinarium*[21] and PORINS from *Escherichia coli*[22,23]. As the specimens are probed directly under physiological conditions, the information provided by AFM studies is complementary to that obtained by electron and X-ray crystallography.

An elegant example of the analysis of a membrane protein by AFM is shown in FIG. 2, in which the atomic model and the AFM images obtained for the surface of the OmpF porin are compared[24]. The OmpF porin is a channel-forming protein found in the *E. coli* outer membrane that consists of 16 antiparallel β-strands lining a transmembrane pore. Individual protrusions of single OmpF porin trimers were clearly resolved at sub-nanometre resolution using AFM. The antiparallel β-strands are connected by short turns at the periplasmic side of the membrane (FIG. 2a) and by long loops at the extracellular surface — these loops form a domain that protrudes up to 1.3 nm above the membrane (FIG. 2b).

FORCE SPECTROSCOPY
A form of AFM in which the force acting on the tip is measured with piconewton (10^{-12} N) sensitivity as the tip is pushed towards the sample then retracted from it.

S-LAYERS
Two-dimensional arrays of protein or glycoprotein subunits with a molecular mass between 40,000 and 200,000 Daltons that are common constituents of bacterial cell walls.

HPI
The hexagonally packed intermediate (HPI) layer from *Deinococcus radiodurans* is an S-layer and was amongst the first S-layer systems to be viewed by AFM at submolecular resolution.

PORIN
A membrane protein that allows the passage of small molecules such as glucose through the membrane.

CANTILEVER
AFM tips are mounted on cantilever beams or triangles, which are typically made of silicon or silicon nitride, that behave like springs. Using Hooke's law, the magnitude of the tip–sample force is proportional to the deflection of the cantilever.

Box 1 | **The different operating modes of atomic force microscopy**

Atomic force microscopes can be operated in various modes[7,8]. In the constant-force imaging mode, images are created by bringing the tip and sample into contact and scanning the tip across the surface while the sample height is adjusted using a feedback loop to keep the bending — or DEFLECTION — of the atomic force microscope cantilever constant. This yields a topographic image that gives calibrated height information about the sample. In many cases, small cantilever deflections occur because the feedback loop is not perfect, and the resulting error signal can be used to generate a deflection image, which is useful for revealing surface details of corrugated samples.

In tapping mode atomic force microscopy (TMAFM), an oscillating tip is scanned over the surface and the amplitude and phase of the cantilever are monitored near its resonance frequency. As the tip touches the sample surface only at the very end of its downward movement, lateral forces during imaging are greatly reduced, which is advantageous for imaging 'soft' biological samples.

In addition to being used as a microscope, an atomic force microscope can measure biomolecular forces with piconewton (10^{-12} N) sensitivity[7–10]. In this mode — known as force spectroscopy — the cantilever deflection is recorded as the tip is pushed towards the sample and retracted from it. Using appropriate corrections, a force versus separation distance curve is obtained. Such a curve can be exploited to gain insights into a variety of surface properties and molecular interactions and to manipulate single molecules. Importantly, force–distance curves can be recorded at multiple locations of the (x, y) plane to yield spatially resolved maps of properties and interactions.

PIEZOELECTRIC CERAMICS
Materials that expand or contract when subjected to a potential difference.

MICROFABRICATION
A range of techniques that are derived from the techniques used in microelectronics to make integrated circuits and which are used to make AFM tips and cantilevers.

DEFLECTION
The vertical bending of the AFM cantilever resulting from the tip–sample interaction force.

BACTERIORHODOPSIN
A light-driven proton pump that is packed into a two-dimensional crystal lattice — known as the purple membrane — and integrated into the plasma membrane of *Halobacterium salinarium*.

Table 1 | **Applications of AFM in microbiology**

Property investigated*	Selected references
Structural properties	
Structure of native membrane proteins at sub-nanometre resolution	17–23
Function-related conformational changes in single proteins	20,24,25
Surface ultrastructure of living cells	26–28,32,36,61,62
Cell-surface dynamics	26,32,36,61
Morphology of biofilms	57,58
Physical properties and biomolecular interactions	
Stiffness of cell walls	33,37–40
Local surface charge and hydrophobicity	41,42
Elasticity and conformational properties of single molecules	36,44–48
Mechanical stability of supramolecular assemblies	49,51
Unfolding pathways of membrane proteins	50
Molecular forces determining cell adhesion and cell aggregation	52,53,69–71

*This article focuses on microbial surfaces. Studies on biological molecules such as DNA are beyond the scope of this paper (for general reviews on biological AFM, see REFS 7,8,11–14).

Structure–function relationships are a key feature of the biological sciences. AFM offers new opportunities to probe function-related conformational changes in membrane proteins. FIGURE 3 illustrates how the technique can be used to resolve conformational changes such as, in this example, the extracellular surface of the OmpF porin[24]. After a critical transmembrane potential is applied, the large extracellular loops that normally protrude into the aqueous solution reversibly collapse onto the channel entrance. Interestingly, the same effect is obtained by decreasing the pH. The significance of this finding is that channel closure might be a mechanism that *E. coli* cells have evolved as a protective device against marked changes in the environment. This led to the proposal that *E. coli* might survive longer in the acidic environment of the stomach by closing its outer-membrane pores[24].

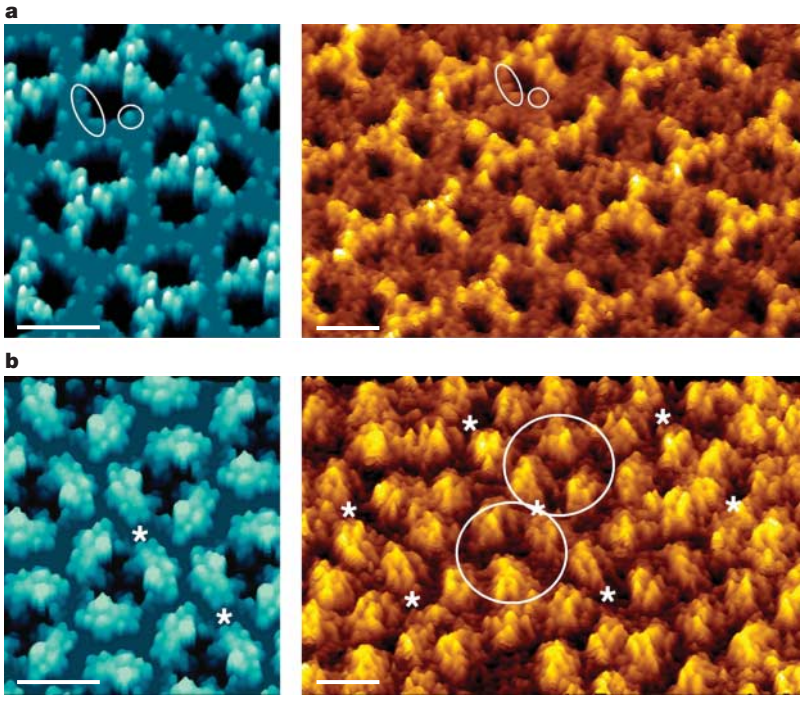

Figure 2 | **Observing native membrane proteins at sub-nanometre resolution.** Comparison of high-resolution atomic force microscopy (AFM) images recorded in buffer solution of the outer-membrane porin OmpF from *Escherichia coli* (brown-yellow) and the atomic model derived from X-ray crystallographic data (blue). **a** | The periplasmic surface. Short β-strands comprising only a few amino acids can sometimes be seen in the AFM image (circled). The surface features correlate directly with the atomic model rendered at 3 Å. **b** | The extracellular surface. Comparison between the atomic model and the AFM image indicates that the loops protrude away from the membrane and are flexible, leading to a disordered appearance. The asterisks mark the two-fold axis of symmetry of rectangular unit cells, whereas the circles indicate two porin trimers with their triangular vestibules. The scale bars represent 50 Å. Reproduced with permission from REF. 24 © (1999) Elsevier.

Conformational changes have been detected in other membrane proteins. In the HPI layer in *D. radiodurans*, individual pores were observed to switch from an open to a closed conformation, which indicates that the HPI layer could function as a molecular sieve, thereby supporting its putative protective function[20]. In BACTERIORHODOPSIN from *H. salinarium* purple membranes[25], the force applied by the AFM instrument tip was used to induce conformational changes in the protein subunits and to estimate the local flexibilities of the protein, indicating that, as well as probing conformations, AFM can also characterize dynamic aspects of protein structures.

Tracking cell surfaces in their native environment

AFM allows researchers to visualize the surface of microbial cells in aqueous environments, thereby providing information that is complementary to that obtained using other structure-determination techniques. An important requirement for successful and reliable cell imaging is that the samples be firmly attached to a support while avoiding denaturation (BOX 2). This can be achieved by immobilizing the cells mechanically within porous membranes. FIGURE 4a illustrates how this strategy can be used to generate three-dimensional (3-D) topographic images (that is, images showing the surface features) of native, living cells, in this case for the yeast *Saccharomyces cerevisiae*.

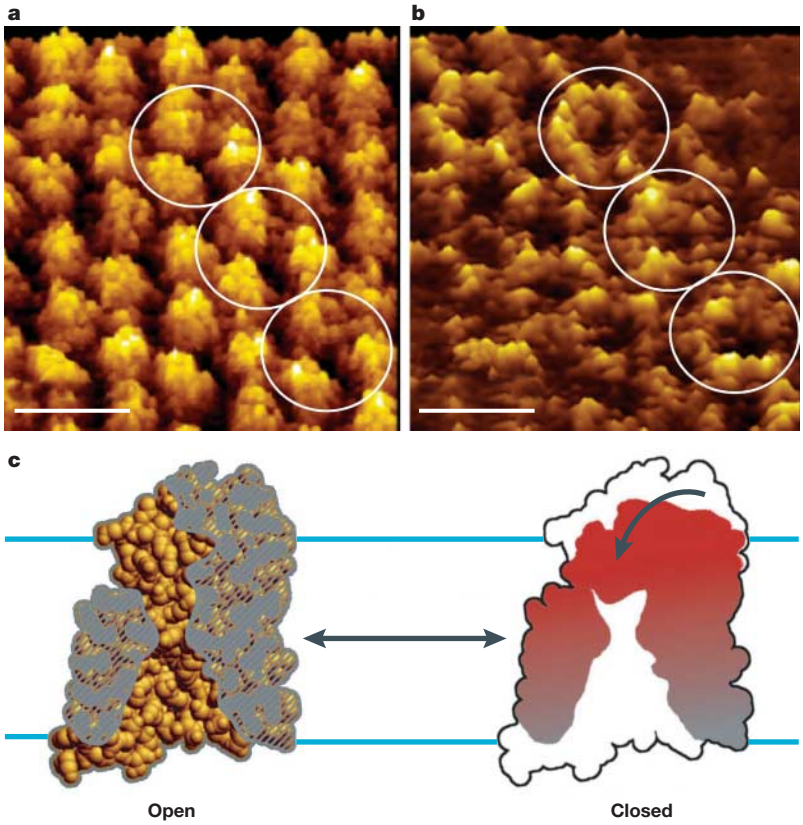

Figure 3 | **Visualizing conformational changes in membrane proteins.** Voltage-induced channel closure of the extracellular surface of the *Escherichia coli* porin OmpF. **a** | Trigonally packed porin trimers (circled) have 13-Å-long protrusions surrounding a triangular vestibule. The scale bar represents 50 Å. **b** | After a membrane potential is applied, a marked conformational change occurs: the extracellular domains form ring-shaped structures about 6 Å in height (circles). The scale bar represents 50 Å. **c** | Cross-sections of the monomer illustrating the open (left panel) and closed (right panel) conformations: the conformational change leading to channel closure can be modelled as a rotation of the extracellular domain about a hinge at the rim of the β-barrel. Reproduced with permission from REF. 24 © (1999) Elsevier.

Using this approach, high-resolution (for example, 250 nm × 250 nm) images can be obtained from different locations on the cell surface, allowing subtle structural variations to be discerned. For *S. cerevisiae*, images of the surface were obtained to a lateral resolution of 2 nm, the highest resolution yet reported for a living microbial cell[26]. The images revealed a smooth surface, which is consistent with earlier electron microscopy data and the presence of a glucan/mannoprotein outer layer. By contrast, it can be seen in FIG. 4b that the surface of spores of the fungus *Phanerochaete chrysosporium* is covered by a crystalline layer of regularly arranged rodlets that has a periodicity of 10 nm and is up to several hundred nanometres in length[27]. These protein structures are remarkably stable during repeated scanning, even when strong forces are applied, confirming their protective function. In recent years, images from a variety of other microorganisms in aqueous solutions have been obtained, including the freshwater diatom *Pinnularia viridis*[28], lactic acid bacteria[29], spores of *Bacillus* species[30], *Staphylococcus aureus*[31,32], *E. coli*[31], *Enterococcus hirae*[33], *Pseudomonas aeruginosa*[33], HIV[34] and vaccinia virus[35]. So far, high-resolution imaging of 'soft' specimens such as Gram-negative bacteria, which lack the thick peptidoglycan layer that is found in Gram-positive bacteria, is often limited by the strong interaction between the instrument tip and the surface being analysed.

A unique feature of AFM is the ability to visualize — in real-time — the surfaces of cells as they interact with external agents or change during cell growth and division. An example of such a dynamic study is the direct monitoring of the enzymatic digestion of cell walls, as was recently shown with *S. cerevisiae*[26]. Successive images of the cell surface that were recorded after the addition of protease revealed the progressive formation of large depressions, about 500 nm in diameter and 50 nm in depth, which reflect the erosion of the mannoprotein outer layer.

Touhami *et al.* recently combined AFM with thin-section transmission electron microscopy (TEM) to investigate the changes in the cell wall of *S. aureus* cells as they grow and divide[32]. Nanoscale holes were seen around the SEPTAL ANNULUS at the onset of division and were attributed to the presence of cell wall structures with high autolytic activity. After cell separation, concentric rings were observed on the surface of the new cell wall that were proposed to reflect newly formed peptidoglycan. Marked changes in the surface ultrastructure of germinating fungal spores have also been observed[36]: the crystalline rodlet layer changed into a gel layer of polysaccharide material that interacted strongly with the instrument tip.

Box 2 | **Advanced methodologies for sample preparation**

To observe biological structures in their native state by atomic force microscopy (AFM), samples must be firmly attached to a solid support to resist the lateral forces exerted by the scanning tip. Several immobilization strategies have been established for microbial specimens. For isolated membrane proteins, the most frequently used procedure is based on physical adsorption on a flat support, such as mica, glass or silicon oxide, in the presence of the appropriate electrolytes[60]. For whole cells, however, immobilization by means of simple adsorption procedures is generally inappropriate because the contact area between the cells and the support is very small, often leading to the cell being detached by the scanning tip. To solve this problem, air-drying and chemical fixation can be used to promote cell attachment, but these treatments can cause significant denaturation of the specimen. Alternatively, more gentle immobilization procedures have been developed in which the cells are mechanically trapped either in an agar gel[61] or in a porous membrane[27,62]. In the latter method, a concentrated cell suspension is gently sucked through an isopore polycarbonate membrane with a pore size that is slightly smaller than that of the cell. This simple approach can be used to image single, spherical, bacterial, yeast and fungal cells under aqueous conditions, while minimizing denaturation of the surface molecules (FIG. 4).

SEPTAL ANNULUS
A structure formed during cell division that corresponds to the growth of wall material into the cytoplasm.

Mapping cell-surface properties

Although AFM was initially developed as an imaging tool, it has rapidly evolved into a quantitative probe of biomolecular forces and physical properties. As is discussed in the following sections, a mode known as force spectroscopy (BOX 1), which combines high force sensitivity with positional precision, has several novel applications in microbiology — for example, the nanoscale probing of cell-surface properties, the controlled manipulation of individual biological molecules, the dissection of supramolecular assemblies and the detection of molecular-recognition events. Such precise and sensitive analyses are important as there are significant variations in the individual properties and interactions of different cell surfaces.

An interesting example of such measurements is the characterization of cell-wall elasticity. By pushing the AFM tip onto microbial cell walls, their elastic properties (expressed as elastic or YOUNG'S MODULUS) can be measured quantitatively. These studies, which have been pioneered by the research groups of Jericho and Beveridge, are important for understanding the mechanisms underlying cell shape and cell growth. In this way, the elasticity of the sheath of the archaeon *Methanospirillum hungatei* was determined, indicating that this single-layered structure of unusual strength can withstand an internal pressure of 400 atm[37]. The elastic properties of the MUREIN SACCULI of Gram-negative bacteria were also assessed and the results obtained by AFM were found to be in agreement with theoretical calculations[38].

Nanoscale mechanical measurements can also be carried out on whole cells, allowing, for example, the elasticity and turgor pressure of bacteria in liquid medium to be assessed[33,39]. Importantly, spatially resolved force maps — maps that are obtained by recording force curves at many locations on the surface being analysed (BOX 1) — allow us to discern local variations of elasticity on single cells, as was recently demonstrated for the yeast *S. cerevisiae*[40]. Force maps that were recorded in parallel with topographic images of the surface of individual budding cells yielded Young's modulus values of 6.1 MPa and 0.6 MPa for the BUD SCAR and surrounding cell surface, respectively. This provided the first demonstration that, in yeast, the bud scar is ten times stiffer than the surrounding cell wall, a finding that is consistent with the accumulation of chitin in this area. So, force spectroscopy is a complementary approach to AFM imaging to investigate cell growth and cell division.

Surface charge and surface hydrophobicity both have essential roles in governing cellular interactions. Modifying the surface of AFM tips with specific chemical groups allows researchers to map local variations of such properties across single cells (BOX 3). For example, AFM tips modified with ionizable carboxyl groups have been used to probe the local ISOELECTRIC POINT of *S. cerevisiae*[41]. In other work, hydrophilic and hydrophobic tips (modified with hydroxyl and methyl groups, respectively) were used to map the surface hydrophobicity of *P. chrysosporium* spores[42]. As chemically modified tips provide information with nanoscale lateral resolution, they are a valuable complement to the more traditional approaches[1,5] used to assess the properties of cell surfaces. The future of this kind of experiment lies in the detailed understanding of interfacial interactions, such as cell–cell support and cell–cell, cell–drug and cell–ion interactions.

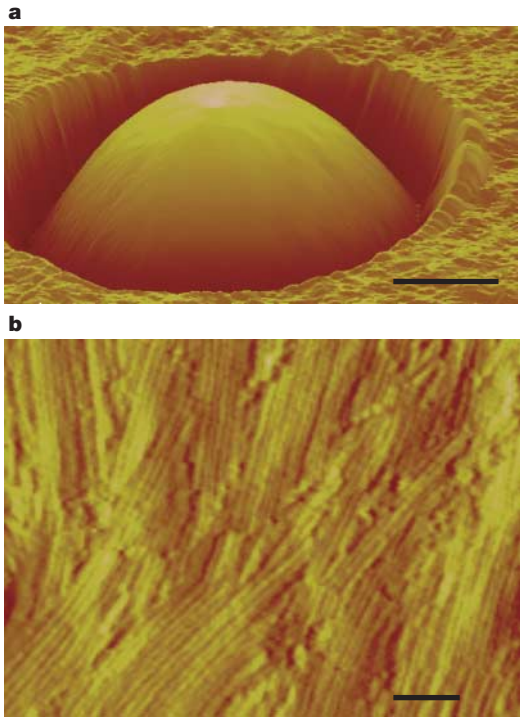

Figure 4 | **Tracking cell surfaces in their native state.** **a** | Three-dimensional atomic force microscopy (AFM) height image of a *Saccharomyces cerevisiae* yeast cell immobilized in a porous membrane. This simple preparation method allows single bacterial, yeast and fungal cells to be viewed in solution, while minimizing denaturation of the specimen (BOX 2). The scale bar represents 1 μm. **b** | High-resolution deflection image of the surface of *Phanerochaete chrysosporium* fungal spores obtained using the same preparation procedure; as opposed to the very smooth yeast surface, the spore surface is covered with nanoscale rodlets. The scale bar represents 100 nm.

Manipulating single molecules

AFM allows researchers to manipulate and dissect individual molecules and molecular assemblies. In doing so, the mechanical and conformational properties of DNA, proteins and polysaccharides can be investigated at the single-molecule level (for reviews, see REFS 13,15,43,44). An example of a pioneering single-molecule experiment is shown in FIG. 5a (REF. 45). The AFM tip was used to 'fish' for and then stretch individual filaments from the microbial polysaccharide dextran. On pulling the tip away from the surface, the so-called force-extension curves show attractive elongation forces that reflect the stretching of the flexible filaments. Models from statistical mechanics can be used to understand the elasticity of the stretched molecules. Interestingly, for dextran and other related polysaccharides the molecules undergo a reversible conformational change at high forces, which

YOUNG'S MODULUS
Young's modulus, or the tensile elastic modulus, is a parameter that reflects the resistance of a material to elongation. The higher the Young's modulus, the larger the force needed to deform the material.

MUREIN SACCULI
Murein sacculus is the term used to refer to the net-like peptidoglycan layer that is found in the cell wall of bacteria.

BUD SCAR
The process by which *S. cerevisiae* proliferates is known as budding. A ring of chitin is formed between the mother cell and the daughter cell (or bud) and once the bud has been pinched off, a mark is left on the surface of the mother cell that is known as the bud scar. Chitin is the main constituent of the bud scar.

ISOELECTRIC POINT
The isoelectric point (or pI) of a protein is the pH at which the protein has an equal number of positive and negative charges.

Box 3 | **Making tips with chemical and biological specificity**

Most commercial atomic force microscope tips have a poorly defined surface chemistry, and might not be suited for quantitative force measurements. Therefore, methods have been introduced to attach well-defined chemical groups (for example, hydroxyl, methyl, carboxyl or amino groups) to their surface. The most common and versatile approach is based on the formation of self-assembled monolayers of alkanethiols on gold surfaces[63]. The procedure involves coating, by thermal evaporation, microfabricated cantilevers with a thin adhesive layer of chromium or titanium, followed by a thin layer of gold, immersing the coated cantilevers in dilute ethanol solutions of the selected alkanethiol, rinsing with ethanol and drying. Such chemically modified nanotips are very sensitive to specific chemical groups[63] and can be used to probe the nanoscale surface properties of cell surfaces[41,42].

Understanding biomolecular forces such as those involved in molecular recognition requires modification of the tip with biological molecules. Here, several important issues must be considered[64]. First, the binding of the biological molecules should be stronger than the intermolecular force being studied. Second, the attached biological molecules should have enough mobility so that they can freely interact with complementary molecules. Third, the contribution of nonspecific adhesion to the measured forces should be minimized. To fulfil these requirements, several flexible spacer molecules have been introduced, including polyethylene glycol[64] and carboxymethyl-amylose[65]. The biological molecules are covalently bound and able to move and orient freely, while nonspecific adsorption is inhibited. Using these 'biotips', a variety of intermolecular forces have been measured, including the forces between biotin–avidin[66], antibody–antigen[64], complementary strands of DNA[67] and lectin–carbohydrate[65,68].

Importantly, several techniques have also been developed to attach cells directly onto AFM cantilevers, thereby allowing researchers to probe cell–solid support and cell–cell interactions[53,69–71]. Current strategies involve the use of specific receptor–ligand interactions[53], electrostatic interactions[69], glue[70] or chemical fixation[71]. An important issue when applying these approaches is to ensure that the surface of the attached cells is still representative of the native surface.

might have an important role in accommodating mechanical stresses and modulating ligand binding in biological systems[46].

Single-molecule-stretching experiments can also be carried out on whole cells. In FIG. 5b, the force-extension curves that were recorded for germinating spores of *Aspergillus oryzae* show the presence of attractive elongation forces, which have been attributed to the stretching of long cell-surface polysaccharide chains[36]. This interpretation is supported by the underlying theory: that is, modelling the force-extension curves yields mechanical parameters that are similar to those reported for pure amylose and dextran molecules[45,46]. The sticky and flexible nature of the surface polysaccharides on *A. oryzae* spores could have a biological significance in that it might promote the spore aggregation that is observed when the spores germinate. In other studies, experiments on the biopolymer chains found on the cell surface of *Pseudomonas putida* showed that these chains were less extended in the presence of salt[47] and that heterogeneity in their properties, both on an individual bacterium and within a population of bacterial cells, could be much greater than previously believed and should be incorporated into models of bacterial adhesion[48].

The functions of membrane proteins are determined by the folding of the peptide chains and their interactions with the cell membrane. Single-molecule force spectroscopy and high-resolution imaging can be combined to gain an insight into the folding and assembly of membrane proteins. In the first such study, the force-extension curves that were recorded for the HPI layer of *D. radiodurans* showed multiple adhesion peaks that were attributed to the sequential 'unzipping' of the HPI-layer pores, which are formed by six protomers[49]. After recording the force-extension curve, the resulting molecular defect could be localized using high-resolution imaging. In another remarkable study, individual bacteriorhodopsin molecules were extracted from the native purple membranes of *H. salinarium*[50]. This well-characterized membrane protein comprises seven transmembrane helices. Once the AFM tip was retracted, the helices unfolded and the force spectra revealed the pattern of the unfolding of the individual molecules. Here again, high-resolution images of the manipulated surface could be obtained, revealing molecular vacancies in the membrane. Similar nanodissection has recently been performed on the S-layer of *Corynebacterium glutamicum*, providing new information on its stability[51]. Accordingly, the combination of AFM imaging and single-molecule force spectroscopy allows us to subject membrane proteins to controlled manipulation, thereby providing details of their unfolding pathways and of the forces that anchor them into the membrane. In the future, it is hoped that this technology will be applicable to living cells, enabling us to learn more about the organization and stability of their surface constituents.

Detecting molecular-recognition events

Molecular recognition is an important event in immunological reactions, microbial infection and symbiosis. It is also of great relevance for developing new bioanalytical (detection) and medical (diagnostic) techniques. Researchers can now measure the minute forces between complementary biological molecules using biologically modified AFM tips (BOX 3).

FIGURE 6 illustrates how this form of nanobiotechnology can be used in microbiology to gain an insight into the molecular bases of cell adhesion and cell aggregation. This example focuses on the lectin–carbohydrate interactions that mediate yeast FLOCCULATION, an aggregation event that occurs in brewing and wine-making. The force curves that are recorded between

FLOCCULATION
A process involving the aggregation of microbial cells. In beer-brewing, yeast flocculation occurs spontaneously near the end of fermentation, thereby providing an easy method to dispose of the cells.

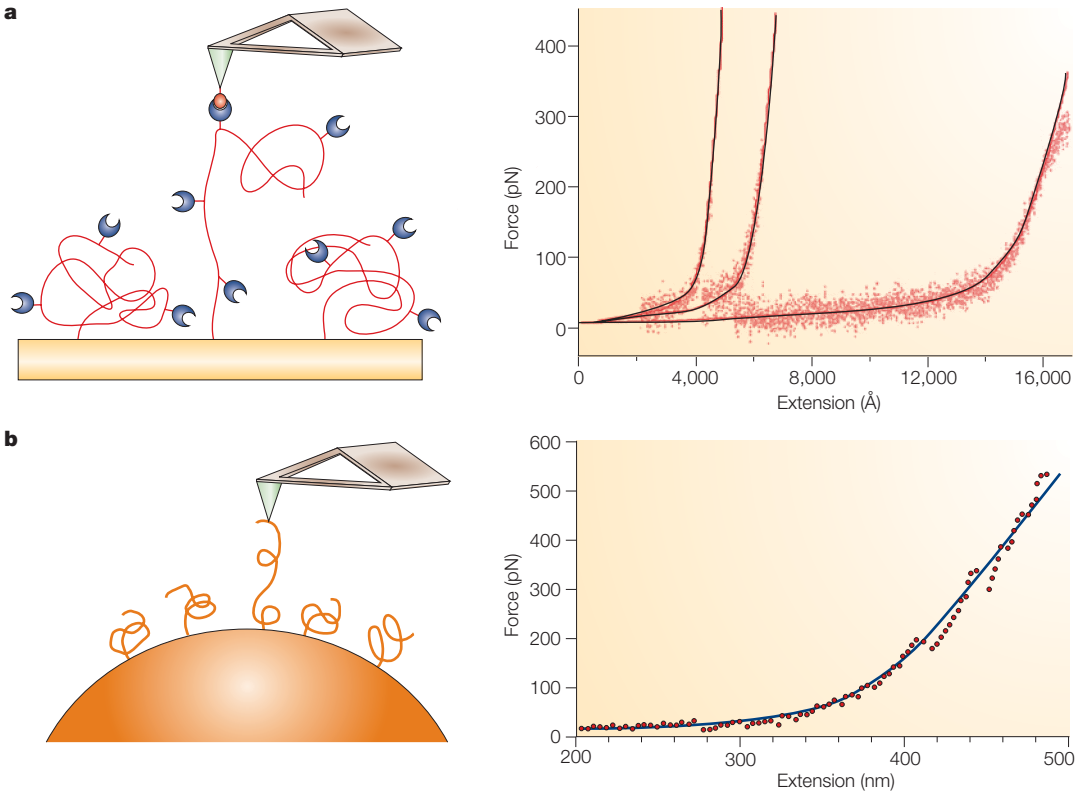

Figure 5 | **Manipulation of single molecules. a** | Stretching of dextran molecules. As shown in the schematic (left panel), dextran filaments linked to a gold support are picked up by an atomic force microscopy (AFM) tip through a biotin–streptavidin bond. The measured force-extension curves (right panel) show elongation events reflecting the deformation of individual filaments. The data (shown as dots) can be modelled by entropy springs with segment elasticity (solid line). Reproduced with permission from REF. 45 © (1997) American Association for the Advancement of Science. **b** | Stretching of polysaccharides on cell surfaces. A typical force-extension curve recorded between a silicon nitride tip and the surface of *Aspergillus oryzae*. The elongation force (shown as dots) can be described using the same model as in part **a** (shown as a solid line), supporting the notion that individual polysaccharide filaments are stretched. Modified with permission from REF. 36 © (2001) American Chemical Society.

carbohydrate-coated AFM tips and *Saccharomyces carlsbergensis* under flocculating conditions show typical adhesion forces, which reflect the specific interactions between individual cell-surface lectins and the glucose residues on the tip. In the presence of mannose, however, the adhesion is strongly reduced, indicating that mannose has blocked the lectin receptor sites, thereby confirming the specificity of the measured adhesion force. Specific adhesion forces were correlated with the ability of the cells to flocculate, indicating their involvement in the flocculation process[52].

Another striking example of cellular aggregation is found in the eukaryote *Dictyostelium discoideum*. In this organism, an adhesion glycoprotein is expressed in aggregating cells that are engaged in the development of a multicellular organism. By attaching individual cells to an AFM tip (BOX 3), Benoit and co-workers were able to measure an adhesion force of 23 pN between two adjacent cells[53]. Using genetic manipulation, this force could be ascribed to a discrete interaction between two adhesion glycoproteins.

Molecular recognition is a crucial step in the infection of mammalian cells by pathogenic bacteria and viruses. Although the mechanisms that are involved in the recognition of host cell-surface receptors by microbial adhesins are well known, little is known about the molecular forces that control these interactions. In collaboration with Menozzi's group[54], we are currently addressing this challenge by combining AFM force spectroscopy with biochemical and genetic approaches. The project focuses on *Mycobacterium tuberculosis*, which is known to interact specifically with epithelial cells through a so-called heparin-binding haemagglutinin adhesin, and will hopefully shed new light on the molecular mechanisms of *M. tuberculosis* pathogenicity.

Implications for basic and applied research

Although microbiological AFM is still in its infancy, it is believed that both imaging and force spectroscopy analyses will have an important impact on many areas of microbiology (BOX 4).

As the instrumentation and methodologies are further developed (for example, faster imaging and weaker interaction forces, see BOX 5 for more details), AFM imaging will be able to help address many previously inaccessible questions. For example, in S-layer research, and possibly also in cellular microbiology, AFM imaging will allow an increasing number of conformational changes and other

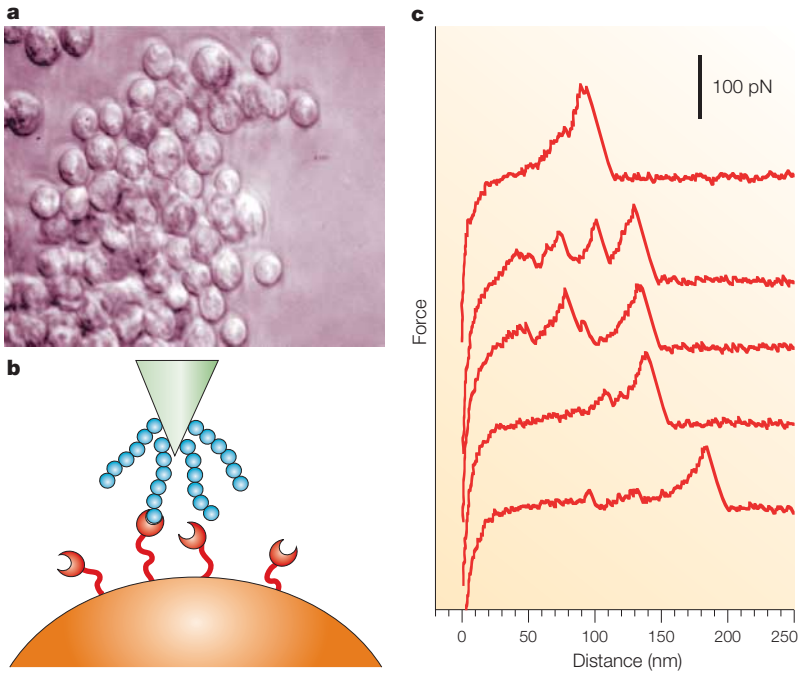

Figure 6 | **Detection of molecular-recognition events at cell surfaces. a** | Flocculation of *Saccharomyces carlsbergensis* cells is mediated by the interaction between complementary cell-surface lectins and carbohydrates. **b** | To probe this interaction at the level of single molecules, force–distance curves are recorded between the cell surface and an atomic force microscopy (AFM) tip functionalized with carbohydrates. **c** | Set of typical force–distance curves showing single or multiple adhesion peaks of 121 ± 53 pN magnitude attributed to the rupture of single lectin–carbohydrate complexes. Modified with permission from REF. 52 © (2003) The Society for General Microbiology.

dynamic events to be understood. In antimicrobial therapy, it is anticipated that real-time imaging of cells in liquid environments will make it possible to visualize small surface alterations that are induced by antibiotics and other drugs, thereby shedding new light on their mechanisms of action. In fact, morphological changes in bacteria that have been subject to antibiotic therapy have already been observed using AFM in air[55], indicating that monitoring drug-induced surface alterations in hydrated cells might soon become possible.

In pathogenesis research, nanoscale imaging provides the opportunity for visualizing animal and plant cell cultures that are infected with viruses or bacteria. In this context, high-resolution images of fibroblasts infected with mouse leukaemia virus were recently obtained after cell fixation[56], which indicates that AFM has the potential to become an important tool to investigate cell–virus interactions.

Environmental and industrial microbiology are two other fields that should benefit from AFM imaging. Here, the technology will be useful to investigate the formation of biofilms on solid surfaces[57]. Such studies could have an impact on geomicrobiology by providing an insight into the microbial–mineral interface[58], as well as in industrial situations, where the association of microorganisms with solid surfaces in the form of contamination or corrosion can cause large economic losses.

What about force spectroscopy analyses? As standard protocols for attaching biological molecules and cells to AFM cantilevers become available, biologically modified tips will increasingly be used to investigate cellular interactions and to detect bioanalytes. Such 'biotips' have particular promise for clinical microbiology and pathogenesis as they could be used to investigate the interactions between microbial pathogens and host cells and to localize cell-surface receptors, which might help to develop new therapeutic approaches. Promising applications are also expected in environmental microbiology, where biotips will contribute to the elucidation of, and perhaps also modulate, microbial–plant interactions and microbial–mineral interactions at the molecular level. In diagnosis, highly parallel cantilever arrays[59] combined with automatic analyses will provide a basis for a new generation of biochips and binding assays for

Box 4 | **Ten questions that AFM could help to answer in the next decade**

Molecular and cellular microbiology
- How does the conformation of cell-surface molecules change in response to environmental alterations?
- What are the forces driving the folding and assembly of membrane proteins?
- What are the nanomechanical properties of cell-wall constituents and how do they change during growth?

Clinical microbiology and pathogenesis
- How does the surface architecture of microorganisms change as they interact with antibiotics?
- What are the molecular forces driving host–pathogen interactions and how can they be modulated for therapy?
- How does a virus or a bacterium penetrate a plant or animal host cell?

Diagnostics
- What are the concentrations of toxins or pathogens in a medical sample?

Environmental microbiology
- What are the molecular forces involved in microbial–plant and microbial–mineral interactions?
- How do biofilms form on solid surfaces and what do they look like in the hydrated state?

Industrial microbiology
- What are the concentrations of microbial metabolites, such as enzymes, in a liquid culture and how do they vary during fermentation?

Box 5 | **Technological challenges that remain to be addressed**

Although significant progress has been made in applying atomic force microscopy (AFM) to biological problems, there are still many limitations and technological issues that must be solved before the full potential of this technique can be exploited. As already discussed (BOX 2), a key prerequisite for successful AFM imaging is sample preparation. Although excellent immobilization strategies are available for membrane protein layers, improved procedures are still needed for many cell types, such as rod-shaped bacteria.

An important problem in cell imaging is the alteration of the specimen that can be caused by the scanning tip. In fact, images of 'soft' samples, such as bacteria coated with gel layers and appendages, are generally of poor resolution due to strong interaction with the tip. The recent development of an active resonance control in tapping mode AFM (TMAFM) is an important step towards solving this problem[72]. With this new technology, tip–specimen interactions are much weaker, resulting in images at higher resolution or images that would otherwise be unattainable[16].

Time resolution is a crucial factor that currently limits dynamic imaging studies. For two-dimensional protein crystals, acquiring a high-resolution image takes about 30 seconds, whereas for whole cells this can take several minutes owing to the highly corrugated character of the surface. This is much greater than the timescale at which dynamic processes usually occur in biology. Interestingly, remarkable advances have been made in developing scanning-probe instruments with increased imaging rates, giving access to unprecedented timescales (to millisecond resolution)[73–75]. These very fast techniques will provide fascinating new prospects in microbiology to explore molecular and cellular dynamics.

Another exciting challenge will be the imaging of intracellular structures in three dimensions. This might become possible using a new class of instruments, referred to as photonic force microscopes, in which the AFM cantilever is replaced by the three-dimensional trapping potential of a laser focus[16].

In force spectroscopy, current procedures for attaching chemical groups, biological molecules and cells to AFM cantilevers are labour-intensive and require specific expertise that is not usually found in microbiology laboratories. In the future, defining simple standard protocols or developing kits for modifying tips and making them readily available to the microbiological community will contribute to increase the use of force spectroscopy in the field. The use of nanotube tips terminated with single active biological molecules[76] should make it possible to map cell-surface receptors with a resolution that would be difficult to achieve with conventional tips. In applied research, automating force spectroscopy analyses will provide a basis for the development of ultrasensitive biosensors for diagnosis and screening purposes.

the rapid, highly sensitive detection of microbial antigens and toxins, and for the screening of cell-surface markers. AFM-based biochips could also have valuable applications in industrial microbiology for identifying microbial metabolites (for example, enzymes and antibiotics) during fermentation.

Conclusion

AFM has become a powerful addition to the range of techniques that are available to investigate the structure, properties and functions of microbial surfaces. When used as a microscope, AFM can visualize membrane proteins at sub-nanometre resolution, monitor conformational changes in single molecules, observe the surface of untreated living cells with unprecedented resolution and follow structural changes as the cells grow or interact with their environment. Importantly, atomic force microscopes have matured from surface-imaging tools to quantitative probes of biomolecular interactions. With force spectroscopy, microbiologists can apply force to cell-wall constituents to understand their elasticity, map the surface charge and hydrophobicity across single cells, manipulate single molecules to gain insights into the molecular bases of protein folding and protein–protein assemblies, and detect molecular-recognition sites. In conclusion, I am confident that as AFM further develops and becomes a more routine technique, it will engage the interest of microbiologists from all disciplines, whether fundamental, environmental, clinical or industrial.

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Competing interests statement

The author declares that he has no competing financial interests.

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