Towards nanomicrobiology using atomic force microscopy

Yves F. Dufrêne

Abstract | At the cross-roads of nanoscience and microbiology, the nanoscale analysis of microbial cells using atomic force microscopy (AFM) is an exciting, rapidly evolving research field. Over the past decade, there has been tremendous progress in our use of AFM to observe membrane proteins and live cells at high resolution. Remarkable advances have also been made in applying force spectroscopy to manipulate single membrane proteins, to map surface properties and receptor sites on cells and to measure cellular interactions at the single-cell and single-molecule levels. In addition, recent developments in cantilever nanosensors have opened up new avenues for the label-free detection of microorganisms and bioanalytes.

Atomic force microscopy
A sharp tip is scanned over the surface of a sample, which allows the interaction force between the tip and the sample to be measured and three-dimensional images to be generated.

Force spectroscopy
Uses atomic force microscopy in the force spectroscopy mode. The force that acts on the tip is measured as the sample is pushed towards the tip and retracted. In single-molecule force spectroscopy, single molecules are manipulated and their interaction forces are measured.

Unité de chimie des interfaces, Université catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium.
e-mail: Yves.Dufrene@uclouvain.be
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Microbial cell walls fulfil several important functions, such as determining and maintaining cell shape, protecting cells against unfavourable environments, mediating host interactions and biofilm formation, and regulating immune responses[1–5]. Although the structures and biochemical compositions of microbial cell-wall constituents are generally well-characterized, little is known about the spatial organization, assembly, conformational properties and interactions of the individual components. This is largely due to the fact that traditional methods in microbiology focus on large ensembles of cells or molecules, rather than on single cells and single molecules. In fact, it is well-established that individual microbial cells can differ widely from each other in terms of their genetic composition, physiology, biochemistry and behaviour, and that this variability — or heterogeneity — can have an important impact on various processes, such as antibiotic resistance, fermentation productivity, the efficacy of food preservatives and the potential of pathogens to cause disease[6]. There is also growing evidence that single cells can show heterogeneous, polar organization[7]. For example, some bacteria display polar organelles, such as flagella and pili, or lack capsular material at the new poles following cell division.

Recent advances in nanotechnology, particularly in atomic force microscopy (AFM)[8,9] (BOX 1), have provided new opportunities for studying single cells and single molecules in microbiology[9–15]. AFM can image membrane proteins and live cells at nanometre resolution directly in buffer solution, which is a key advantage over electron-microscopy techniques. In particular, real-time imaging of single cells provides novel insight into dynamic structural events, such as structural changes that are caused by growth or drug interactions. As well as structural imaging, the force spectroscopy mode (BOX 1) is increasingly being used to measure the nanoscale chemical and physical properties of cells. Chemical force microscopy (CFM), in which AFM tips are modified with specific functional groups, provides a way to map chemical groups on cell surfaces and measure their interactions. Single-molecule force spectroscopy (SMFS) is now routinely used to probe the unfolding pathways of membrane proteins, the conformational properties of cell-surface polymers and the localization of surface receptors.

Here, I discuss recent progress that has been made in the use of AFM to address microbiological questions. Although the primary focus of this article is on single cells, selected examples that deal with membrane proteins are also presented. Specific highlights include the high-resolution imaging of native photosynthetic membranes, the real-time observation of wall remodelling on growing cells, the mapping of cell-surface elasticity, hydrophobicity and receptor sites, the quantitative measurement of cell–cell and cell–solid interactions and the label-free detection of bioanalytes and cells.

Submolecular imaging of membranes

Membrane proteins are nanomachineries that fulfil various crucial functions in the cell membrane. AFM is increasingly used to visualize the nanoscale organization of bacterial membrane proteins. The atomic structure of membrane proteins has traditionally been determined using X-ray crystallography[16]. Although electron

Box 1 | **How does atomic force microscopy work?**

In atomic force microscopy (AFM), a sharp tip is scanned over the surface of a sample, which allows so-called near-field physical interactions between the tip and the sample to be sensed and three-dimensional images to be generated. The sample is mounted on a piezoelectric scanner, which ensures three-dimensional positioning with high accuracy. While the tip (or sample) is being scanned in both the x and y directions, the force that interacts between the tip and the specimen is monitored with piconewton sensitivity. This force is measured by deflection of a soft cantilever, which is detected by a laser beam that is focused on the free end of the cantilever and reflected into a photodiode.

Different AFM-imaging modes differ mainly in the way that the tip moves over the sample. The most widely used imaging mode is the contact mode, in which sample topography can be measured in two ways: constant height or constant force. In the constant-height mode, cantilever deflection is recorded while the sample is scanned at constant height. However, it is often necessary to minimize large deflections and therefore maintain the applied force to small values to prevent sample damage. This is achieved in the constant-force mode, in which the sample height is adjusted to keep the deflection of the cantilever, and thus the force that is applied to the tip, constant using a feedback loop. In the dynamic or intermittent mode, an oscillating tip is scanned over the surface and the amplitude and phase of the cantilever are monitored near its resonance frequency.

In force-spectroscopy modes, such as chemical force microscopy (CFM) and single-molecule force spectroscopy (SMFS), cantilever deflection is recorded as a function of the vertical displacement of the scanner — that is, the sample is pushed towards the tip and retracted. This results in a cantilever-deflection versus scanner-displacement curve, which can be transformed into a force–distance curve using appropriate corrections. The characteristic adhesion (or unbinding) force between the tip and the sample that is observed during retraction can then be used to map chemical groups (in CFM) and receptor sites (in SMFS). Force curves can be recorded at multiple locations of the x–y plane to yield spatially resolved information on surface properties and receptor sites. In CFM, the tips must be modified with well-defined functional groups to measure interaction forces and chemical properties. Ligands are attached to the tips to detect specific receptors using SMFS.

crystallography and AFM cannot compete with X-ray techniques in terms of resolution, they do allow membrane proteins to be studied in a lipid bilayer and therefore in their native environment. Whereas electron crystallography can resolve the three-dimensional, atomic-scale structure of membrane proteins, AFM can image the surfaces of membrane proteins at subnanometre resolution directly in aqueous solutions. AFM is therefore a complementary tool to X-ray and electron-crystallography techniques in structural biology[9,10,16].

Following pioneering studies by Engel and Müller[10,13], Scheuring and colleagues[12] pushed the limits of submolecular imaging on native bacterial membranes by providing new insight into the supramolecular architecture of photosynthetic membranes from *Rhodopseudomonas* species[17,18], *Rhodospirillum photometricum*[19,20] and *Rhodobacter blasticus*[21]. Remarkably, AFM data could explain how the organization of the *R. photometricum* photosynthetic membrane is modulated in response to light[20]. High-resolution images revealed two different types of membrane protein assemblies in confined areas, with distinct architectures dividing functional tasks and therefore ensuring optimized photosynthetic activity under high-light and low-light conditions.

In addition to imaging, the AFM tip can be used to manipulate and dissect individual membrane proteins. By applying additional loading forces to the imaging tip, stacked S-layer proteins from *Corynebacterium glutamicum* and *Deinococcus radiodurans* were removed to allow access to underlying membranes, and individual protein loops from bacteriorhodopsin and *Escherichia coli* aquaporins were manipulated[22]. Notably, SMFS can be used to manipulate individual membrane proteins, thereby providing details of their unfolding pathways and anchoring forces[23,24]. In early studies, force curves that were recorded for *D. radiodurans* S layers revealed saw-tooth patterns, with six force peaks that were attributed to the sequential pulling out of multiple protomers of the S layer. After recording the force curve, a molecular defect in the form of a hexameric complex was visualized by high-resolution imaging[23]. This approach was also used to unzip the S layer of *C. glutamicum*, thereby providing new insight into the stability of this protective coat[25]. Similarly, SMFS was used to examine the folding and assembly of the S layer protein CbsA from *Lactobacillus crispatus*[26].

Despite the remarkable capabilities of AFM, its widespread use in membrane research has been limited by the need to firmly attach specimens onto solid supports. To solve this problem, Goncalves *et al.*[27] developed a novel two-chamber AFM set-up which allows membrane proteins that separate two aqueous compartments to be investigated. This set-up was used to image the inner and outer surfaces of non-supported *C. glutamicum* membranes at a resolution of ~15Å and to determine the elastic properties and energy of the interaction of the membrane proteins. Using this device, microbiologists will be able to couple structural and functional AFM analyses of non-supported bacterial membranes and cell-wall layers. For example, they will be able to observe how ion, pH and solute gradients affect the conformation of channels, pumps and receptors.

Live-cell imaging

Microscopes have always been essential tools in microbiology. Light microscopy is useful for counting and identifying cells, as well as for determining their general shape, but the resolution is limited to the wavelength of light. Electron-microscopy techniques can provide high-resolution images of cells[28,29]. Although powerful, these methods are demanding and require vacuum conditions, which means that live cells cannot be investigated in aqueous solutions.

Over the past few years, rapid progress has been made in applying AFM imaging to single cells and thereby complementing data obtained with electron microscopy. Microorganisms that have recently been imaged include *E. coli*[30], *Bacillus* spp.[31,32], *Clostridium*[33] spp., *Mycobacterium bovis*[34], *Myxococcus xanthus*[35] and several fungal[36–38] and diatom species[39,40]. Several studies have demonstrated that AFM can be combined with electron microscopy to gain a comprehensive view of cell surfaces[33,36,41,42].

A recent trend that is receiving increasing attention and that holds promise for the study of cell functions is the use of AFM imaging for assessing the phenotypic characteristics of mutant strains with altered cell-wall constituents[30,31,35,36,43,44]. For example, the surfaces of

Chemical force microscopy
An atomic-force-microscopy modality in which modification of the tip with specific functional groups enables researchers to map the spatial arrangement of chemical groups and their interactions.

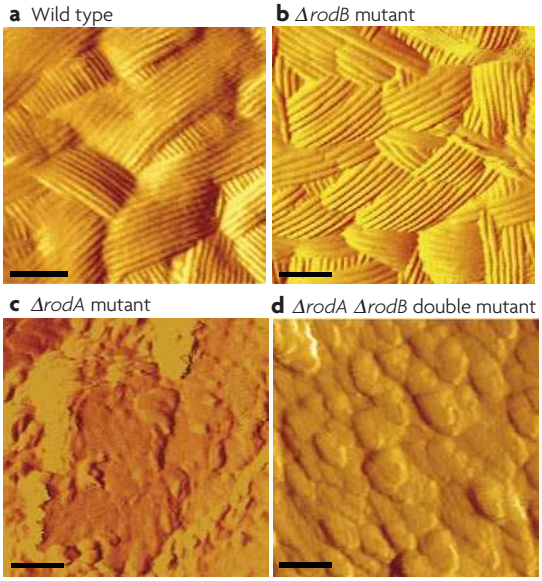

Figure 1 | **Live-cell imaging. a–d** | Atomic-force-microscopy topographic images in aqueous solution that reveal major structural differences at the surface of wild-type and mutant conidia of *Aspergillus fumigatus*. The scale bars represent 100 nm. Images reproduced, with permission, from REF. 44 © (2008) American Chemical Society.

wild-type and mutant conidia of the human fungal pathogen *Aspergillus fumigatus* were compared[44] (FIG. 1). AFM revealed 10-nm-wide rodlets on the wild-type and *ΔrodB* mutant, but these structures were absent on the *ΔrodA* and *ΔrodA ΔrodB* mutants, which had granular surfaces. This provided direct evidence that only *rodA* is required for rodlet formation. Consistent with AFM data, X-ray photoelectron spectroscopy and secondary ion mass spectrometry confirmed that the surface of wild-type conidia is protein rich, with large amounts of hydrophobic amino acids, whereas the surface of mutants without rodlets is richer in polysaccharides.

Real-time imaging offers new opportunities to probe dynamic events, such as the cell-wall remodelling that is associated with growth and division. A series of high-resolution images of the structural dynamics of single, germinating *Bacillus atrophaeus* spores provides an elegant example of this approach[32] (FIG. 2). AFM revealed previously unrecognized germination-induced alterations in spore-coat architecture, as well as the disassembly of outer-spore-coat rodlet structures. The nascent surface of emerging germ cells was revealed to be a porous network of peptidoglycan fibres, similar to the honeycomb structure of peptidoglycan oligomers that was detected by NMR. In a related study, the combined use of ultrathin-section transmission electron microscopy and AFM provided detailed information on the structure of dormant and germinating spores of *Clostridium novyi* NT[33]. The spores were shown to be surrounded by an amorphous layer that was intertwined with honeycomb parasporal layers. During germination and outgrowth, the honeycomb layers, as well as the underlying spore coat and undercoat layers, sequentially dissolved until the vegetative cell was released (FIG. 2).

AFM imaging is also well-suited to the study of the influence of drugs on microbial cell walls[34,45–47]. There is currently much interest in wall–drug interactions in *Mycobacterium tuberculosis*, as the biosynthesis of its cell wall is targeted by some of the most powerful anti-tuberculous drugs. In this context, *in situ* AFM was used to image the surface of mycobacteria before and after incubation with isoniazid, ethionamide, ethambutol or streptomycin[34]. After drug treatment, the overall integrity of the cells was generally maintained, but major structural alterations were observed in the form of layered structures, striations and porous morphologies. It was suggested that these modifications reflect the inhibition of the synthesis of three major cell-wall constituents: mycolic acids, arabinans and proteins. AFM is therefore a promising tool for investigating the organization and assembly of microbial cell walls and their dynamic changes after cell growth or interaction with drugs. In the future, these nanoscale analyses could have an important impact on drug development, as they may help elucidate the mode of action of novel antibiotics and identify new targets.

Functional imaging of live cells

Assessing the surface properties of microbial cells at the subcellular level has always been a challenge owing to their small size. However, AFM now provides new possibilities for mapping the elasticity, chemical properties and specific receptors of individual cells.

AFM can probe local cell-wall elasticity. Nanomechanical measurements of the yeast *Saccharomyces cerevisiae* revealed that cell-wall elasticity varies significantly across the cell, with the bud scar being much stiffer than the cell wall owing to the accumulation of chitin[48,49]. In addition, the cell wall exhibits local temperature-dependent nanomechanical motion[49]. Gaboriaud *et al.*[50] similarly quantified the nanomechanical properties of *Shewanella putrefaciens*, and observed that the cell surface swells when the pH is raised, an effect that they attributed to stronger repulsive forces between the cell-surface polymers.

CFM can provide insight into nanoscale chemical properties. CFM is a powerful, chemically sensitive imaging technique that can be used to probe chemical groups and their interactions on the nanoscale[51] and has recently been used to address microbiological questions[34,37,38,52]. For example, CFM was used to demonstrate that *A. fumigatus* conidia are strongly hydrophobic[37], a finding that is consistent with the presence of hydrophobic proteins (hydrophobins) in the rodlet layer and that provides direct indications as to the putative functions of these proteins as dispersion and adherence structures. Interestingly, surface hydrophobicity was lower after germination[38] and in a mutant without rodlets[37], which confirmed that the measured hydrophobic properties were associated with hydrophobins. In another study, treatment of mycobacteria with isoniazid or ethambutol

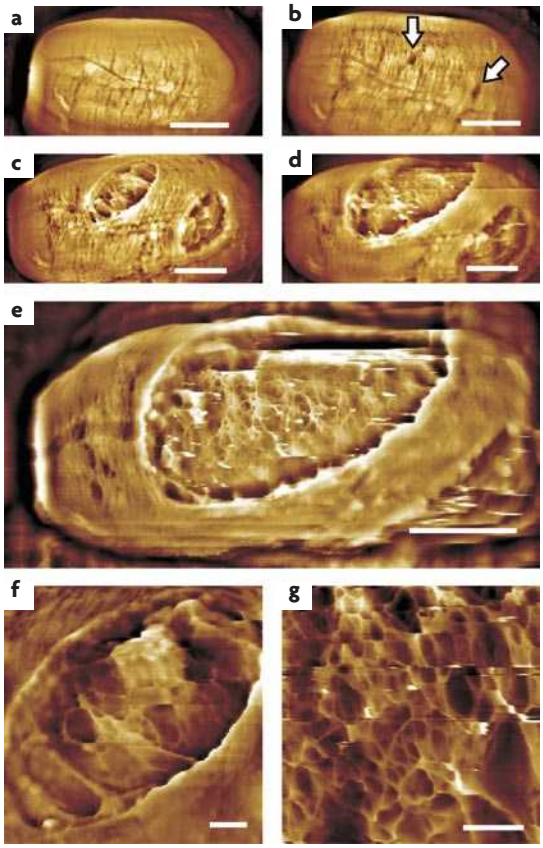

Figure 2 | **Real-time imaging of the structural dynamics of single *Bacillus atrophaeus* spores germinating under native conditions. a**–**g** | A series of atomic-force-microscopy images that show ~60-nm-deep apertures in the rodlet layer (indicated with arrows in **b**) that gradually enlarged (**c**,**d**) and subsequently eroded the entire spore coat. Germ cells then emerged from these apertures (**e**). Before germ-cell emergence from the spore coat, the peptidoglycan cell-wall structure was evident (**f**,**g**). At an early stage of emergence, the cell wall was still partly covered by spore remnants (**f**), whereas immediately before cell emergence, the cell wall was free of spore integument debris (**g**). The germ-cell surface contained nanofibres that formed a fibrous network which enclosed pores of 5–100 nm. Elapsed germination time in hours and minutes was as follows: 3 h and 40 min (**a**), 5 h and 45 min (**b**), 7 h and 5 min (**c**), 7 h and 30 min (**d**), 7 h and 45 min (**e**), 7 h and 15 min (**f**) and 7 h and 50 min (**g**). The scale bars represent 500 nm (**a**–**e**) and 100 nm (**f**,**g**). Images reproduced, with permission, from REF. 32 © (2007) National Academy of Sciences.

led to a dramatic decrease of cell-surface hydrophobicity, which was attributed to the removal of the mycolic acid layer[34]. CFM is therefore a valuable method for resolving the nanoscale chemical properties of microorganisms and observing the effects of cell growth and drug treatment.

SMFS can be used to map receptor distribution. SMFS with AFM tips that bear cognate ligands is well-suited for mapping the distribution of receptors, such as cell-adhesion proteins and antibiotic binding sites, and can provide novel insight into pathogen–host and pathogen–drug interactions[53,54]. This approach was used to map the distribution of single adhesins on mycobacteria with nanoscale resolution[53] (FIG. 3), revealing that the adhesin distribution was concentrated into nanodomains that could promote adhesion to target cells by inducing the recruitment of receptors within membrane rafts. Such single-molecule studies are therefore likely to help us understand the molecular basis of bacterial pathogenicity in the future.

In another study, vancomycin-bearing AFM tips were used to detect single D-alanine-D-alanine receptor sites on the septum of live *Lactococcus lactis*[54]. This new form of antibiotic probe offers exciting prospects for understanding the mode of action of antibiotics that target cell-wall constituents.

Measuring cellular interactions

Molecular interactions have essential roles in the mediation of cellular events, such as cell adhesion and biofilm formation[55]. There has been considerable recent progress in the use of force spectroscopy to quantify cell–cell and cell–solid interactions[14,55]. A common method for probing cellular interactions is to directly attach microbial cells to an AFM cantilever and measure the interaction forces towards other cells or solid surfaces. In pioneering work, Benoit *et al.*[56] measured the specific adhesion force between two adjacent cells of *Dictyostelium discoideum* at the single-molecule level. This interaction force could be ascribed to the discrete interaction between two cell-adhesion glycoproteins that were engaged in cell aggregation. In another early study, Lower *et al.*[57] measured the forces between living *Shewanella oneidensis* bacteria and mineral surfaces.

There is substantial interest in the application of cell probes to biomedical issues. For example, Emerson and Camesano[58] examined the initial adhesion forces of two infectious agents, *Candida parapsilosis* and *Pseudomonas aeruginosa*, to biomaterial and biofilm surfaces. More recently, Emerson *et al.*[59] explored the influence of the surface properties of solids on the adhesion strength of *Staphylococcus epidermidis*, a microorganism that is commonly isolated from medical-implant infections. Postollec *et al.*[60] measured the forces between co-aggregating and non-co-aggregating oral bacterial pairs, and the co-aggregating pair exhibited larger adhesive forces and energies than the non-co-aggregating pair.

Cell-surface polymers and appendages have long been recognized for their role in mediating cell adhesion. For the first time, SMFS measurements on microbial cells have enabled researchers to study the adhesion, elasticity and conformational properties of surface polysaccharides and proteins[14,61–66]. For example, SMFS was used to detect specific force signatures that were characteristic of the bond between a fluorescent chimeric protein on *E. coli* and a solid surface[64]. The chimera was composed of a portion of outer-membrane protein A fused to the cyan-fluorescent protein AmCyan. Atabek and Camesano[65] provided new insight into the roles of lipopolysaccharides and extracellular polymers on the

Cantilever
Atomic-force-microscopy tips are mounted on cantilever beams or triangles — that is, thin beams or triangles that behave as springs. The force that acts on the tip can thus be evaluated by measuring cantilever vertical bending (deflection) and by applying the classical Hooke's law for springs.

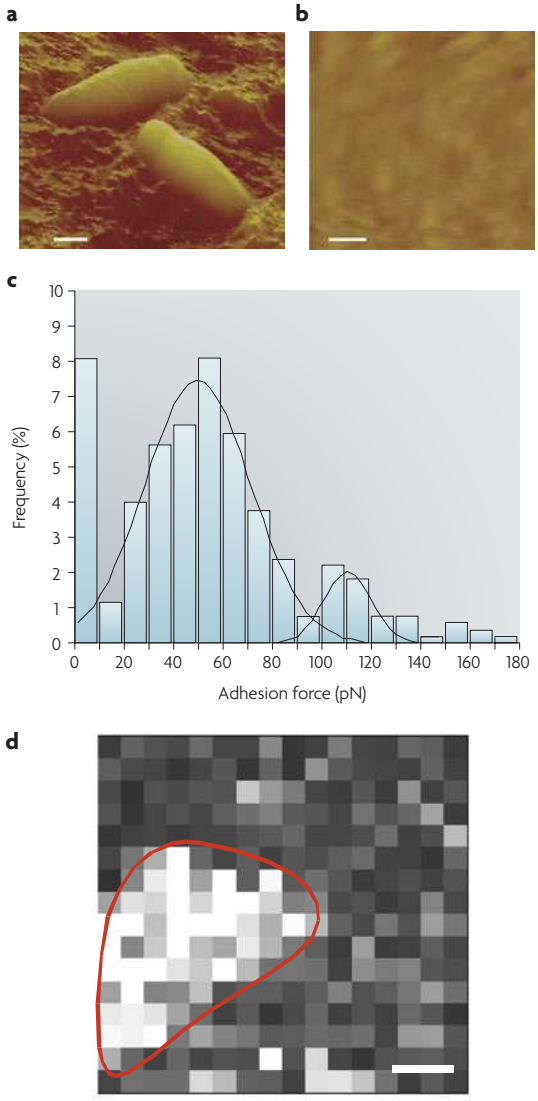

Figure 3 | **Mapping cell-surface receptors using single-molecule force spectroscopy. a** | Topographic image that shows two living *Mycobacterium bovis* cells on a polymer surface. **b** | A high-resolution image of the surface of an *M. bovis* cell reveals a smooth, homogeneous morphology. **c**,**d** | Histogram (**c**) and high-resolution map (**d**) (adhesion range of 100 pN) of adhesion forces that were recorded on a single cell with a heparin-terminated tip. The adhesion-force histogram reveals two maxima that reflect the detection of one or two cell-adhesion proteins (adhesins), whereas the map reveals that adhesins are concentrated in localized regions (clear pixels surrounded by a red line). The scale bars represent 1 μm (**a**) and 50 nm (**b**,**d**). Figure modified, with permission, from *Nature Methods* REF. 53

adhesion of *P. aeruginosa* strains. A few studies[67–69] have concentrated on pili, which play a key part in mediating the attachment of pathogens to host cells. In particular, Miller *et al.*[68] found that the rods of both P pili and type 1 pili from *E. coli* are highly extensible — that is, they can be extended by approximately twofold to fourfold their resting length. However, the force that was required to unravel each of these pili was unique, a finding that could represent a specific adaptation to the biological niche in which type 1 pili promote bacterial colonization.

Combining the power of SMFS with biologically modified AFM tips provides direct access to molecular recognition forces. In microbiology, this approach has been successfully used to measure the specific binding forces between *S. epidermidis* or *Staphylococcus aureus* and fibronectin[70,71]; mycobacteria and host receptors[53]; *L. lactis* and vancomycin[54]; and *Streptococcus mutans* and laminin[72] or salivary proteins[73]. In the future, we anticipate that single-cell and single-molecule force measurements, combined with macroscopic adhesion assays, will be widely used to shed light on the molecular mechanisms of microbial adhesion and biofilm formation.

AFM-based nanosensors

Over the past decade, arrays of cantilevers[74,75] have emerged as a powerful technique for biomedical and biotechnological sensing applications. An important advantage of such AFM-based sensors compared with other sensing methods is that they enable the rapid, ultra-sensitive detection of bioanalytes and cells without any need for labelling or external probes. This is achieved using cantilevers that are functionalized with receptor molecules (for example, antibodies) and incubated with cognate ligands. The resulting specific biomolecular recognition events are detected by monitoring either cantilever bending or the resonance frequency shift.

In the cantilever-bending mode, bending of the cantilever is induced upon binding of the ligands, which allows the measurement of picomolar concentrations. Cantilever bending has been successfully applied to the detection of DNA hybridization[76], prostate-specific antigens[77], cardiac biomarker proteins[78] and mRNA markers for cancer progression[79]. In the resonance-frequency-shift mode, the cantilever is oscillated at its resonance frequency and mass changes on the functionalized cantilever surface are derived from shifts in resonance frequency. Consequently, mass changes can be detected with subpicogram resolution. Nugaeva *et al.*[80,81] demonstrated the power of this sensing mode for microbiology: they used cantilevers that were coated with polyclonal antibodies to detect single *Aspergillus niger* spores with a sensitivity of 10^3 colony forming units per ml and could distinguish germinating spores from dormant spores within a few hours. In another study, *Bacillus anthracis* spores could be specifically detected at low concentrations in the presence of large numbers of *Bacillus thuringiensis* and *Bacillus cereus* spores[82].

A novel type of nanosensor has recently been reported in which fluid-filled microcantilevers are used to weigh single bacterial cells and sub-monolayers of adsorbed proteins in water with subfemtogram (fg) resolution[83] (FIG. 4). The sensor can measure the mass of individual live *E. coli* (110 fg) and *Bacillus subtilis* (150 fg) bacteria. In microbiology, the future of these AFM-based nanosensors lies in the development of

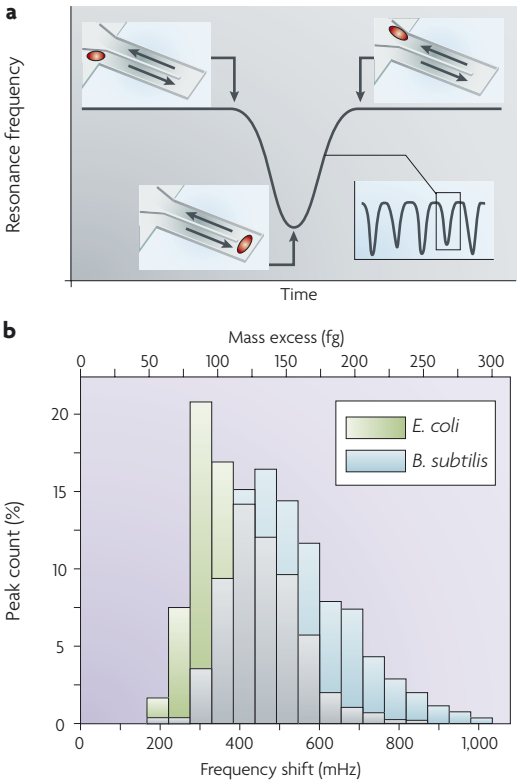

Figure 4 | **Use of cantilever nanosensors in microbiology.** **a** | Fluid-filled microcantilevers are used as suspended microchannel resonators for weighing single bacterial cells with subfemtogram resolution. Cells flow through the cantilever and the observed signal depends on the position of the cells along the channel. The exact mass excess of a cell can be quantified by the peak frequency shift that is induced at the apex. **b** | Histograms of peak frequency shifts that were caused by bacteria flowing through a cantilever. The masses of *Escherichia coli* and *Bacillus subtilis* were measured by passing the bacteria through the cantilever and collecting peak-height histograms. Figure reproduced, with permission, from *Nature* REF. 83 © (2007) Macmillan Publishers Ltd. All rights reserved.

novel, label-free bioassays for the rapid detection of pathogens and toxins.

Conclusions

The studies reviewed here demonstrate how the use of AFM in microbiology has established a strong, new field — nanomicrobiology — that offers fascinating new opportunities in basic and applied research. Compared with other types of microscopy, AFM offers two unique features: the ability to work directly at nanometre (and subnanometre) resolution in aqueous solutions, and the possibility of probing various properties and interactions at the single-molecule level. In the imaging mode, AFM can visualize the architecture of hydrated membrane proteins at subnanometre resolution, thereby complementing X-ray and electron-crystallography techniques. Real-time imaging can be used to track the surface of single live cells while they grow or interact with drugs, which opens up new possibilities for studying the assembly and remodelling of cell walls, and for understanding the action mode of antibiotics. Force spectroscopy provides direct, quantitative information on cell-surface elasticity, chemical properties and receptor distributions, and cellular interactions, which has lead to new insights into the molecular bases of cell-adhesion events. Cantilever arrays can be used for the rapid, ultra-sensitive detection of bioanalytes and cells without any need for labelling or external probes, and therefore offers new avenues for medical diagnostics and environmental monitoring. These sensors could serve as a platform for the detection of pathogens and toxins.

This new form of single-cell and single-molecule microbiology will undoubtedly have an important impact on many fields, particularly in biomedical and environmental contexts for elucidating the molecular bases of pathogen–host and microorganism–mineral interactions. Technological challenges that remain to be addressed include the improvement of protocols for attaching biomolecules and cells to AFM cantilevers[15] and the development of high-speed AFM instruments[84].

1. Cabeen, M. T. & Jacobs-Wagner, C. Bacterial cell shape. *Nature Rev. Microbiol.* **3**, 601–610 (2005).
2. Branda, S. S., Vik, S., Friedman, L. & Kolter, R. Biofilms: the matrix revisited. *Trends Microbiol.* **13**, 20–26 (2005).
3. Mazmanian, S. K. & Kasper, D. L. The love–hate relationship between bacterial polysaccharides and the host immune system. *Nature Rev. Immunol.* **6**, 849–858 (2006).
4. Cloud-Hansen, K. A. *et al.* Breaching the great wall: peptidoglycan and microbial interactions. *Nature Rev. Microbiol.* **4**, 710–716 (2006).
5. Weidenmaier, C. & Peschel, A. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nature Rev. Microbiol.* **6**, 276–287 (2008).
6. Brehm-Stecher, B. F. & Johnson, E. A. Single-cell microbiology: tools, technologies, and applications. *Microbiol. Mol. Biol. Rev.* **68**, 538–559 (2004).
7. Maddock, J. R., Alley, M. R. K. & Shapiro, L. Polarized cells, polar actions. *J. Bacteriol.* **175**, 7125–7129 (1993).
8. Gerber, C. & Lang, H. P. How the doors to the nanoworld were opened. *Nature Nanotechnol.* **1**, 3–5 (2006).
9. Müller, D. J. & Dufrêne, Y. F. Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology. *Nature Nanotechnol.* **3**, 261–269 (2008).
10. Engel, A. & Müller, D. J. Observing single biomolecules at work with the atomic force microscope. *Nature Struct. Biol.* **7**, 715–718 (2000).
11. Dufrêne, Y. F. Using nanotechniques to explore microbial surfaces. *Nature Rev. Microbiol.* **2**, 451–460 (2004).
12. Scheuring, S., Levy, D. & Rigaud, J. L. Watching the components of photosynthetic bacterial membranes and their *in situ* organisation by atomic force microscopy. *Biochim. Biophys. Acta* **1712**, 109–127 (2005).
13. Müller, D. J. *et al.* Single-molecule studies of membrane proteins. *Curr. Opin. Struct. Biol.* **16**, 489–495 (2006).
14. Camesano, T. A., Liu, Y. T. & Datta, M. Measuring bacterial adhesion at environmental interfaces with single-cell and single-molecule techniques. *Adv. Wat. Res.* **30**, 1470–1491 (2007).
Timely review on the use of single-cell and single-molecule techniques for measuring bacterial adhesion.
15. Hinterdorfer, P. & Dufrêne, Y. F. Detection and localization of single molecular recognition events using atomic force microscopy. *Nature Methods* **3**, 347–355 (2006).
16. Werten, P. J. L. *et al.* Progress in the analysis of membrane protein structure and function. *FEBS Lett.* **529**, 65–72 (2002).
17. Scheuring, S. *et al.* Nanodissection and high-resolution imaging of the *Rhodopseudomonas viridis* photosynthetic core-complex in native membranes by AFM. *Proc. Natl Acad. Sci. USA* **100**, 1690–1693 (2003).
18. Scheuring, S., Goncalves, R. P., Prima, V. & Sturgis, J. N. The photosynthetic apparatus of *Rhodopseudomonas palustris*: structures and organization. *J. Mol. Biol.* **358**, 83–96 (2006).
19. Scheuring, S., Rigaud, J. L. & Sturgis, J. N. Variable LH2 stoichiometry and core clustering in native membranes of *Rhodospirillum photometricum*. *EMBO J.* **23**, 4127–4133 (2004).
20. Scheuring, S. & Sturgis, J. N. Chromatic adaptation of photosynthetic membranes. *Science* **309**, 484–487 (2005).
Outstanding high-resolution imaging study that demonstrated how the organization of native photosynthetic membranes changes in response to light.
21. Scheuring, S., Busselez, J. & Levy, D. Structure of the dimeric PufX-containing core complex of *Rhodobacter blasticus* by *in situ* AFM. *J. Biol. Chem.* **180**, 1426–1431 (2005).
22. Goncalves, R. P. & Scheuring, S. Manipulating and imaging individual membrane proteins by AFM. *Surf. Interf. Anal.* **38**, 1413–1418 (2006).
23. Müller, D. J., Baumeister, W. & Engel, A. Controlled unzipping of a bacterial surface layer with atomic force microscopy. *Proc. Natl Acad. Sci. USA* **96**, 13170–13174 (1999).

A combination of AFM imaging and SMFS allowed the controlled nanomanipulation of single-membrane proteins.
24. Oesterhelt, F. *et al.* Unfolding pathways of individual bacteriorhodopsins. *Science* **288**, 143–146 (2000).
25. Scheuring, S. *et al.* Charting and unzipping the surface layer of *Corynebacterium glutamicum* with the atomic force microscope. *Mol. Microbiol.* **44**, 675–684 (2002).
26. Verbelen, C., Antikainen, J., Korhonen, T. K. & Dufrêne, Y. F. Exploring the molecular forces within and between CbsA S-layer proteins using single molecule force spectroscopy. *Ultramicroscopy* **107**, 1004–1011 (2007).
27. Goncalves, R. P. *et al.* Two-chamber AFM: probing membrane proteins separating two aqueous compartments. *Nature Methods* **3**, 1007–1012 (2006).
Presents a novel AFM set-up for probing membrane proteins that separate two aqueous compartments over which membrane gradients can be established.
28. Beveridge, T. J. & Graham, L. L. Surface layers of bacteria. *Microbiol. Rev.* **55**, 684–705 (1991).
29. Matias, V. R. F. & Beveridge, T. J. Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. *Mol. Microbiol.* **56**, 240–251 (2005).
30. Beckmann, M. A. *et al.* Measuring cell surface elasticity on enteroaggregative *Escherichia coli* wild type and dispersin mutant by AFM. *Ultramicroscopy* **106**, 695–702 (2006).
31. Chada, V. G. R., Sanstad, E. A., Wang, R. & Driks, A. Morphogenesis of *Bacillus* spore surfaces. *J. Bacteriol.* **185**, 6255–6261 (2003).
AFM was used to image the surfaces of *B. subtilis* spores that have mutations in several coat-protein genes.
32. Plomp, M., Leighton, T. J., Wheeler, K. E., Hill, H. D. & Malkin, A. J. *In vitro* high-resolution structural dynamics of single germinating bacterial spores. *Proc. Natl Acad. Sci. USA* **104**, 9644–9649 (2007).
Demonstrated the power of live-cell imaging for visualizing cell-wall remodelling during germination.
33. Plomp, M. *et al.* Spore coat architecture of *Clostridium novyi* NT spores. *J. Bacteriol.* **189**, 6457–6468 (2007).
34. Alsteens, D. *et al.* Organization of the mycobacterial cell wall: a nanoscale view. *Pflugers Arch.* **456**, 117–125 (2008).
35. Pelling, A. E., Li, Y. N., Shi, W. Y. & Gimzewski, J. K. Nanoscale visualization and characterization of *Myxococcus xanthus* cells with atomic force microscopy. *Proc. Natl Acad. Sci. USA* **102**, 6484–6489 (2005).
36. Ma, H., Snook, L. A., Tian, C., Kaminskyj, S. G. W. & Dahms, T. E. S. Fungal surface remodelling visualized by atomic force microscopy. *Mycol. Res.* **110**, 879–886 (2006).
37. Dague, E. *et al.* Chemical force microscopy of single live cells. *Nano Lett.* **7**, 3026–3030 (2007).
The first time that CFM was applied to microbial cells to address their nanoscale chemical properties and interactions.
38. Dague, E., Alsteens, D., Latgé, J. P. & Dufrêne, Y. F. High-resolution cell surface dynamics of germinating *Aspergillus fumigatus* conidia. *Biophys. J.* **94**, 656–660 (2008).
39. Hildebrand, M., Doktycz, M. J. & Allison, D. P. Application of AFM in understanding biomineral formation in diatoms. *Pflugers Arch.* **456**, 127–137 (2008).
40. Francius, G., Tesson, B., Dague, E., Martin-Jézéquel, V. & Dufrêne, Y. F. Nanostructure and nanomechanics of live *Phaeodactylum tricornutum* morphotypes. *Environ. Microbiol.* **10**, 1344–1356 (2008).
41. Touhami, A., Jericho, M. & Beveridge, T. J. Atomic force microscopy of cell growth and division in *Staphylococcus aureus*. *J. Bacteriol.* **186**, 3286–3295 (2004).
42. Read, N., Connell, S. & Adams, D. G. Nanoscale visualization of a fibrillar array in the cell wall of filamentous cyanobacteria and its implications for gliding motility *J. Bacteriol.* **189**, 7361–7366 (2007).
43. Cross, S. E. *et al.* Atomic force microscopy study of the structure–function relationships of the biofilm-forming bacterium *Streptococcus mutans*. *Nanotechnology* **17**, S1–S7 (2006).
44. Dague, E., Delcorte, A., Latgé, J. P. & Dufrêne, Y. F. Combined use of atomic force microscopy, X-ray photoelectron spectroscopy, and secondary ion mass spectrometry for cell surface analysis. *Langmuir* **24**, 2955–2959 (2008).
45. Braga, P. C. & Ricci, D. Atomic force microscopy: application to investigation of *Escherichia coli* morphology before and after exposure to cefodizime. *Antimicrob. Agents Chemother.* **42**, 18–22 (1998).
46. Yang, L. *et al.* Atomic force microscopy study of different effects of natural and semisynthetic β-lactam on the cell envelope of *Escherichia coli*. *Anal. Chem.* **78**, 7341–7345 (2006).
47. Verbelen, C. *et al.* Ethambutol-induced alterations in *Mycobacterium bovis* BCG imaged by atomic force microscopy. *FEMS Microbiol. Lett.* **264**, 192–197 (2006).
48. Touhami, A., Nysten, B. & Dufrêne, Y. F. Nanoscale mapping of the elasticity of microbial cells by atomic force microscopy. *Langmuir* **19**, 4539–4543 (2003).
49. Pelling, A. E., Sehati, S., Gralla, E. B., Valentine, J. S. & Gimzewski, J. K. Local nanomechanical motion of the cell wall of *Saccharomyces cerevisiae*. *Science* **305**, 1147–1150 (2004).
50. Gaboriaud, F., Bailet, S., Dague, E. & Jorand, F. Surface structure and nanomechanical properties of *Shewanella putrefaciens* bacteria at two pH values (4 and 10) determined by atomic force microscopy. *J. Bacteriol.* **187**, 3864–3868 (2005).
51. Frisbie, C. D., Rozsnyai, L. F., Noy, A., Wrighton, M. S. & Lieber, C. M. Functional group imaging by chemical force microscopy. *Science* **265**, 2071–2074 (1994).
52. Alsteens, D., Dague, E., Rouxhet, P. G., Baulard, A. R. & Dufrêne, Y. F. Direct measurement of hydrophobic forces on cell surfaces using AFM. *Langmuir* **23**, 11977–11979 (2007).
53. Dupres, V. *et al.* Nanoscale mapping and functional analysis of individual adhesins on living bacteria. *Nature Methods* **2**, 515–520 (2005).
SMFS with tips that bear biologically active molecules was used to map the distribution of individual adhesins on living mycobacteria.
54. Gilbert, Y. *et al.* Single-molecule force spectroscopy and imaging of the Vancomycin/D-Ala-D-Ala interaction. *Nano Lett.* **7**, 796–801 (2007).
55. Busscher, H. J., Norde, W. & van der Mei, H. C. Specific molecular recognition and nonspecific contributions to bacterial interaction forces. *Appl. Environ. Microbiol.* **74**, 2559–2564 (2008).
56. Benoit, M., Gabriel, D., Gerisch, G. & Gaub, H. E. Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. *Nature Cell Biol.* **2**, 313–317 (2000).
Pioneering paper that showed how SMFS can be used to measure cell–cell interactions at the single-molecule level.
57. Lower, S. K., Hochella, M. F. & Beveridge, T. J. Bacterial recognition of mineral surfaces: nanoscale interactions between *Shewanella* and α-FeOOH. *Science* **292**, 1360–1363 (2001).
58. Emerson, R. J. & Camesano, T. A. Nanoscale investigation of pathogenic microbial adhesion to a biomaterial. *Appl. Environ. Microbiol.* **70**, 6012–6022 (2004).
59. Emerson, R. J. *et al.* Microscale correlation between surface chemistry, texture, and the adhesive strength of *Staphylococcus epidermidis*. *Langmuir* **22**, 11311–11321 (2006).
60. Postollec, F., Norde, W., de Vries, J., Busscher, H. J. & van der Mei, H. C. Interactive forces between co-aggregating and non-co-aggregating oral bacterial pairs. *J. Dent. Res.* **85**, 231–234 (2006).
61. van der Aa, B. C. *et al.* Stretching cell surface macromolecules by atomic force microscopy. *Langmuir* **17**, 3116–3119 (2001).
62. Abu-Lail, N. I. & Camesano, T. A. Elasticity of *Pseudomonas putida* KT2442 surface polymers probed with single-molecule force microscopy. *Langmuir* **18**, 4071–4081 (2002).
63. Camesano, T. A. & Abu-Lail, N. I. Heterogeneity in bacterial surface polysaccharides, probed on a single-molecule basis. *Biomacromolecules* **3**, 661–667 (2002).
64. Lower, B. H., Yongsunthon, R., Vellano, F. P. & Lower, S. K. Simultaneous force and fluorescence measurements of a protein that forms a bond between a living bacterium and a solid surface. *J. Bacteriol.* **187**, 2127–2137 (2005).
65. Atabek, A. & Camesano, T. A. Atomic force microscopy study of the effect of lipopolysaccharides and extracellular polymers on adhesion of *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**, 8503–8509 (2007).
66. Dugdale, T. M., Dagastine, R., Chiovitti, A., Mulvaney, P. & Wetherbee, R. Single adhesive nanofibers from a live diatom have the signature fingerprint of modular proteins. *Biophys. J.* **89**, 4252–4260 (2005).
67. Touhami, A., Jericho, M. H., Boyd, J. M. & Beveridge, T. J. Nanoscale characterization and determination of adhesion forces of *Pseudomonas aeruginosa* pili by using atomic force microscopy. *J. Bacteriol.* **188**, 370–377 (2006).
68. Miller, E., Garcia, T., Hultgren, S. & Oberhauser, A. F. The mechanical properties of *E. coli* type 1 pili measured by atomic force microscopy techniques. *Biophys. J.* **91**, 3848–3856 (2006).
69. Lugmaier, R. A., Schedin, S., Kuhner, F. & Benoit, M. Dynamic restacking of *Escherichia coli* P-pili. *Eur. Biophys. J. Biophys. Lett.* **37**, 111–120 (2008).
70. Bustanji, Y. *et al.* Dynamics of the interaction between a fibronectin molecule and a living bacterium under mechanical force. *Proc. Natl Acad. Sci. USA* **100**, 13292–13297 (2003).
71. Yongsunthon, R. *et al.* Correlation between fundamental binding forces and clinical prognosis of *Staphylococcus aureus* infections of medical implants. *Langmuir* **23**, 2289–2292 (2007).
72. Busscher, H. J. *et al.* Intermolecular forces and enthalpies in the adhesion of *Streptococcus mutans* and an antigen I/II-deficient mutant to laminin films. *J. Bacteriol.* **189**, 2988–2995 (2007).
73. Xu, C. P. *et al.* Interaction forces between salivary proteins and *Streptococcus mutans* with and without antigen I/II. *Langmuir* **23**, 9423–9428 (2007).
74. Lang, H. P., Hegner, M., Meyer, E. & Gerber, C. Nanomechanics from atomic resolution to molecular recognition based on atomic force microscopy technology. *Nanotechnology* **13**, R29–R36 (2002).
75. Goeders, K. M., Colton, J. S. & Bottomley, L. A. Microcantilevers: sensing chemical interactions via mechanical motion. *Chem. Rev.* **108**, 522–542 (2008).
76. Fritz, J. *et al.* Translating biomolecular recognition into nanomechanics. *Science* **288**, 316–318 (2000).
77. Wu, G. H. *et al.* Bioassay of prostate-specific antigen (PSA) using microcantilevers. *Nature Biotechnol.* **19**, 856–860 (2001).
78. Arntz, Y. *et al.* Label-free protein assay based on a nanomechanical cantilever array. *Nanotechnology* **14**, 86–90 (2003).
79. Zhang, J. *et al.* Rapid and label-free nanomechanical detection of biomarker transcripts in human RNA. *Nature Nanotechnol.* **1**, 214–220 (2006).
80. Nugaeva, N. *et al.* Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection. *Biosens. Bioelectron.* **21**, 849–856 (2005).
81. Nugaeva, N. *et al.* An antibody-sensitized microfabricated cantilever for the growth detection of *Aspergillus niger* spores. *Microsc. Microanal.* **13**, 13–17 (2007).
82. Campbell, G. A. & Mutharasan, R. Method of measuring *Bacillus anthracis* spores in the presence of copious amounts of *Bacillus thuringiensis* and *Bacillus cereus*. *Anal. Chem.* **79**, 1145–1152 (2007).
83. Burg, T. P. *et al.* Weighing of biomolecules, single cells and single nanoparticles in fluid. *Nature* **446**, 1066–1069 (2007).
Elegant application of nanosensors in microbiology, in which single bacterial cells were weighed using fluid-filled microcantilevers.
84. Ando, T. *et al.* High-speed AFM and nano-visualization of biomolecular processes. *Pflugers Arch.* **456**, 211–225 (2008).

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DATABASES

Entrez Genome project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj

Aspergillus fumigatus | *Aspergillus niger* | *Bacillus anthracis* | *Bacillus cereus* | *Bacillus subtilis* | *Bacillus thuringiensis* | *Clostridium novyi* NT | *Corynebacterium glutamicum* | *Deinococcus radiodurans* | *Dictyostelium discoideum* | *Escherichia coli* | *Lactococcus lactis* | *Mycobacterium bovis* | *Mycobacterium tuberculosis* | *Myxococcus xanthus* | *Pseudomonas aeruginosa* | *Saccharomyces cerevisiae* | *Shewanella oneidensis* | *Shewanella putrefaciens* | *Staphylococcus aureus* | *Staphylococcus epidermidis* | *Streptococcus mutans*

FURTHER INFORMATION

Yves F. Dufrêne's homepage: http://www.cifa.ucl.ac.be

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