Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology

With its ability to observe, manipulate and explore the functional components of the biological cell at subnanometre resolution, atomic force microscopy (AFM) has produced a wealth of new opportunities in nanobiotechnology. Evolving from an imaging technique to a multifunctional 'lab-on-a-tip', AFM-based force spectroscopy is increasingly used to study the mechanisms of molecular recognition and protein folding, and to probe the local elasticity, chemical groups and dynamics of receptor–ligand interactions in live cells. AFM cantilever arrays allow the detection of bioanalytes with picomolar sensitivity, opening new avenues for medical diagnostics and environmental monitoring. Here we review the fascinating opportunities offered by the rapid advances in AFM.

DANIEL J. MÜLLER[1] AND YVES F. DUFRÊNE[2]

[1]Biotechnology Center, Technische Universität Dresden, Tatzberg 47-51, D-01307 Dresden, Germany; [2]Unité de Chimie des Interfaces, Université Catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium

e-mail: daniel.mueller@biotec.tu-dresden.de; yves.dufrene@uclouvain.be

Nanobiotechnology — the characterization, design and application of biological systems at the nanometre scale — is a rapidly evolving area at the crossroads of nanoscience, biology and engineering[1,2]. One fascinating challenge in nanobiotechnology is the bottom-up design and operation of nanoscale machines and motors made up of supramolecular systems[3,4]. The molecular components of these systems are typically cellular machineries such as haemolysin[5], porin[6], myosin[7], kinesin[8], RNA polymerase[9] and ATP synthase[10]. These machines can be set in motion in a controlled manner to work as bio-inspired nanoscale valves, engines, motors and shuttles.

Progress in nanobiotechnology strongly relies on the development of scanning probe techniques, particularly atomic force microscopy (AFM)[11,12]. For the first time, AFM techniques are allowing us to observe and manipulate biomolecular machinery in its native environment (Fig. 1). The principle of AFM is to raster-scan a sharp tip over the sample surface and to probe interaction forces with piconewton sensitivity. AFM can image surfaces in buffer solution with outstanding signal-to-noise ratio, offering a means of observing single biomolecules without the need for fixation or staining. Cellular functions are governed ultimately by the dynamic character of their working parts, which makes them difficult to understand solely from static structural information. Time-lapse AFM imaging directly observes biological specimens at work, thus providing a unique tool to approach the relationship between their structure and function. Moreover, using the AFM probe as a 'lab-on-a-tip' enables us to probe simultaneously the structure and specific biological, chemical and physical parameters of the cell's machinery.

Molecular interactions make up the basic language of all biological processes. They determine how molecules assemble into complex functional structures and switch these cellular machineries 'on' or 'off'. In addition, molecular interactions control the way that cells communicate with each other, and form higher-ordered organisms. Remarkably, AFM can measure interactions between and within single biomolecules, giving us clues on how cells direct adhesion between themselves. Such information is important for understanding tissue development, tumour metastasis, bacterial infection and an almost uncountable number of medical and biotechnological questions. AFM can also be used to determine the energy landscape of molecular recognition events and protein folding pathways. AFM is clearly emerging as a powerful, multifunctional nanoscale tool, flourishing in modern biological and medical laboratories, and opening up exciting new possibilities for nanobiotechnologists (Fig. 1).

OBSERVING CELLULAR MACHINERIES AT WORK

With current progress in AFM instrumentation, sample preparation and imaging, researchers can now routinely obtain topographs of biological specimens in physiological conditions[13–15]. The ability to visualize native biomolecules without the need for staining or fixation, together with superior signal-to-noise ratio, makes AFM a complementary tool to optical microscopy and even to X-ray and electron crystallography.

So far, the best spatial resolution obtained using AFM on corrugated cell surfaces is of the order of 10 nm. By contrast, AFM allows us to observe isolated cell membranes adsorbed onto flat supports with subnanometre resolution (Fig. 2). Prominent examples include the voltage-dependent ion channels of the mitochondrial membrane[16], ion-driven rotors of F_oF_1-ATP synthases[17,18], communication channels (Fig. 2a)[19], and G-protein coupled receptors (GPCRs) (Fig. 2b)[20], all of which cover key functions of the human body and are the targets of a large number of therapeutic drugs. The example of GPCRs demonstrates impressively that their temporary assembly with other GPCRs adapts their functions to the needs of the biological cell[21]. This is the case for many of the other cell machineries, and it is of outstanding interest to characterize factors that determine how they assemble into larger functional units. High-resolution topographs of light-harvesting complexes[22] provide insights into the molecular

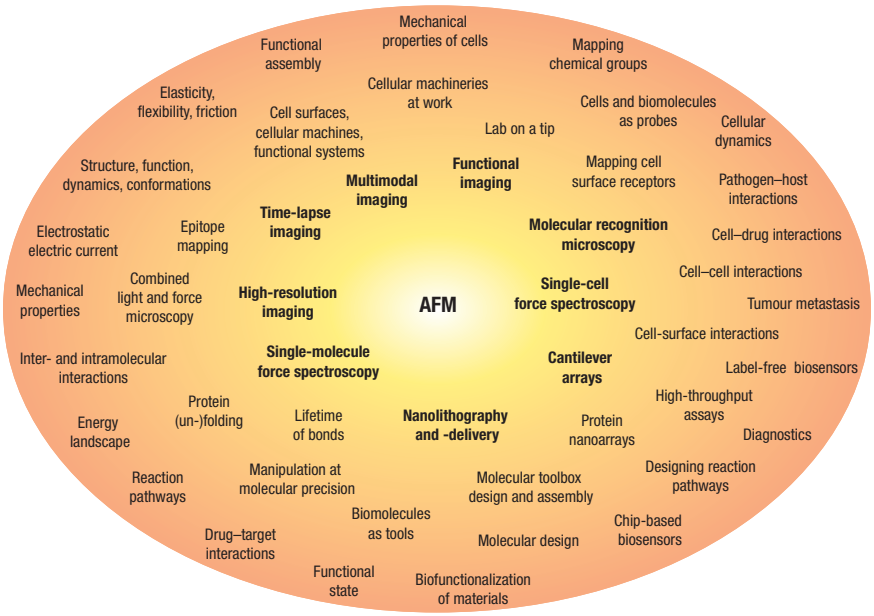

Figure 1 AFM techniques to characterize and manipulate biological systems on the nanometre scale. Originally invented to image the topography of surfaces, AFM has evolved into a multifunctional tool that makes it possible to characterize biological cells and their components with unprecedented resolution. AFM techniques allow single-molecule analyses and an understanding of various biochemical, physical and chemical properties and interactions in the cell.

rearrangements of the photosynthetic membrane (Fig. 2c). Simply tuning the light intensity optimizes the assembly of light-harvesting complexes for their conversion of light into electric energy. Similar resolution could be achieved on tubulin protofilaments that assemble into microtubuli, which are structures involved in cellular processes such as trafficking, migration and cell division. The anti-cancer agent taxol inhibits the polymerization of tubulin into microtubules and thus inhibits the division of cancer cells. AFM allows us to observe directly the action of taxol in slowing down the structural transitions of tubulin protofilaments by several orders of magnitude[23] (Fig. 2d). In the future, there is no doubt that AFM will equip most modern biological laboratories to characterize the assembly of the different functional components of the cell at nanoscopic resolution.

Watching cellular nanomachines at work provides a fascinating and direct way to access their structure–function relationships. Since the early days, time-lapse AFM has been used to observe dynamic processes ranging from the atomic, molecular and more microscopic scales[24]. In pioneering studies, time-lapse AFM was applied to observe directly the activity of RNA polymerase[25], the phospholipase-induced bilayer degradation[26] and the reversible closure of the pores of a bacterial surface layer[13]. Notably, AFM has been applied to characterize factors that trigger the function of the cell's machinery. For instance, by developing ultrafast small cantilevers of 10–30 μm in length, it was possible to monitor the formation of molecular chaperone complexes (GroEL/ES) in real time[27]. Addition of the 'biofuel' Mg-adenosine triphosphate (ATP) triggered the formation of these complexes and the fast cantilevers were able to measure their lifetimes. Developing even faster AFM cantilevers and feedback loops revealed the conformational states and lifetimes of GroEL, which were reversibly switching from the open to the closed state[28]. In another study[19], AFM revealed the reversible closure of communication channels in response to the ligand Ca^{2+}. In the presence of taurine — an aminosulphonate compound — a pH change again closes this channel, but apparently by a different gating mechanism (Fig. 2e)[29]. Whereas in the presence of Ca^{2+} the gap junction hemichannels moved their subunits centrally towards closing the channel entrance, the pH-induced closure twisted the subunits like a camera iris.

AFM is evolving from an imaging technique to a multifunctional toolbox. Conversion of the AFM stylus to a 'lab-on-a-tip', by means of specific surface modifications, makes simultaneous imaging and probing possible for a variety of additional traits such as antigen recognition, flexibility, elasticity or electric current[12,30]. This approach complements real-time structural observations with multifunctional data that are difficult to reveal by conventional approaches. In pioneering work, pH- and voltage-dependent conformational changes were demonstrated for the channel-forming protein OmpF porin[13]. The closure mechanism discovered for OmpF, whereby the flexible extracellular loops could reversibly gate the channel entrance, was later reported to gate other porins from the same family as well. Using the AFM stylus as a lab-on-a-tip has allowed the observation of individual OmpF porins at subnanometre resolution and the detection of the electrostatic field established by their channels[31]. This field was thought to tune the channel selectivity for charged ions. Recently, a conducting high-resolution AFM probe was introduced to detect electric currents in the femtoampere range, such as those gated by membrane proteins[32]. In the future, this approach may complement the 'patch clamp technique', which basically measures ion currents of single membrane channels. A modified AFM probe may simultaneously measure and locate ion channel currents of a given membrane protein and characterize functional related conformations at (sub)nanometre resolution.

SINGLE-MOLECULE MANIPULATION

Applying a mechanical load to molecules forces bonds formed through interactions within or between the molecules. As the externally applied force overcomes the stability of a bond it breaks. Gaub[33] and Lee[34] introduced the use of single-molecule force spectroscopy (SMFS) to probe the strength of receptor–ligand bonds (Fig. 3a). These experiments initiated many others which all demonstrated

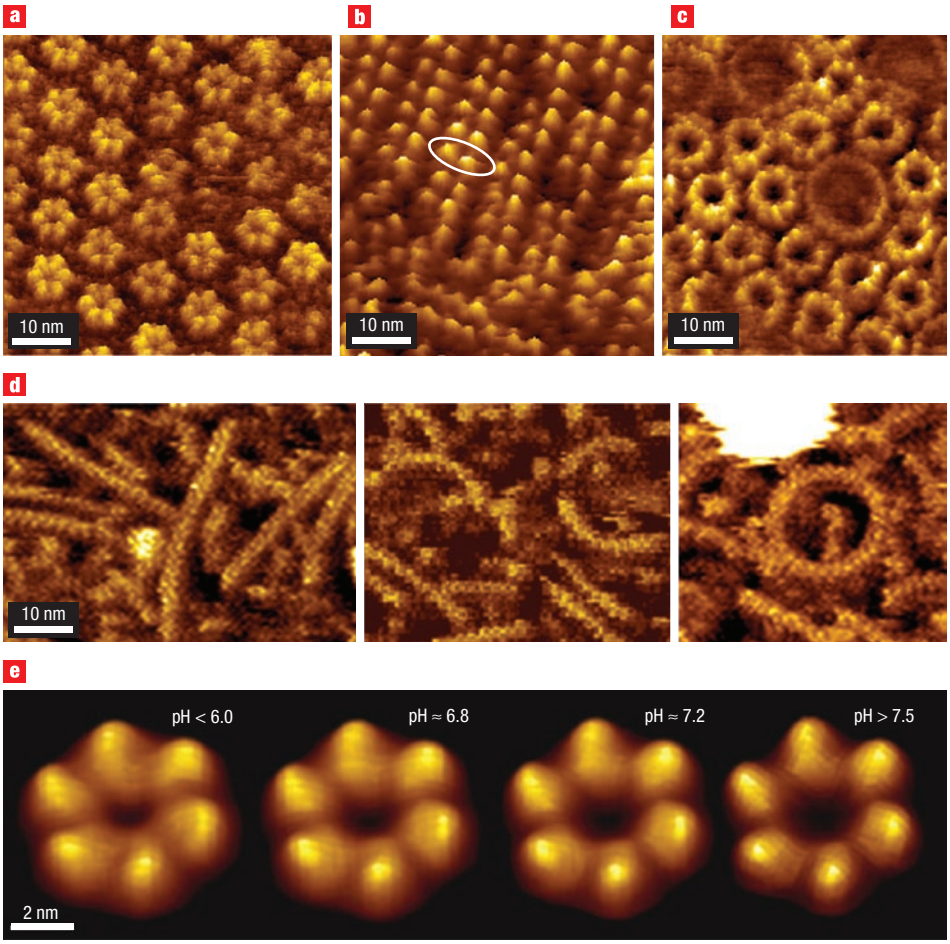

Figure 2 Observing the individuality of cellular machines at high resolution. **a**, Human communication channels known as gap junctions form hexameric pores. **b**, Bovine rhodopsin, the visual pigment of the eye, assembles into rows of dimers (circled in white). **c**, Assembly of light-harvesting II (small doughnuts) and light-harvesting I complexes (large doughnuts, surrounding the reaction centre) change with intensity of the incident light to optimize the collection of photons and their conversion into electric energy. **d**, The anti-cancer drug taxol slows down the tubulin hydrolysis of straight protofilaments to form rings of 22 nm in diameter (far right panel). **e**, In the presence of aminosulphonate compounds, human communication channels change from a closed (pH < 6.0) to an open state (pH > 7.5).

that the forces probed by SMFS reflect interactions established within or between molecules. Providing direct access to these inter- and intramolecular interactions, SMFS has produced answers to a number of biologically and medically pertinent questions. These include unravelling of protein folding and unfolding mechanisms, receptor–ligand interactions, and ligand-binding interactions that can switch a protein's functional state.

The first protein unfolded by SMFS, and probably the best-studied protein so far, is immunoglobulin titin[35]. In humans, the titin filament represents an adjustable molecular spring in muscle sarcomeres, the repeating unit of myofibrils of striated muscles. About 90% of titin is made of immunoglobulin domains, which provide mechanical elasticity to the filament[36]. SMFS showed that simply applying a mechanical pulling force to both peptide ends induced the fully reversible unfolding of an oligomeric titin construct. Recording the applied force over the stretching distance revealed a characteristic sawtooth-like pattern of force peaks. Each force peak of this pattern reflected the unfolding of a single immunoglobulin molecule and the sequence of force peaks described the unfolding pathways of all immunoglobulin molecules within the oligomeric titin construct. These unfolding pathways, predefined by the mechanical pulling direction using SMFS, mimicked a physiological process of an (over-)stretched sacromere. However, most other proteins unfold in complex three-dimensional trajectories[37]. Mechanically stretching proteins at different sites showed that their resistance to unfolding strongly depends on the pulling direction[38,39], suggesting that certain unfolding directions are preferred over others. To explore individual trajectories of unfolding pathways systematically, a group led by Rief[40] stretched and unfolded single green fluorescent proteins (GFP) in different directions. Other SMFS applications characterized the properties of cellular machineries that show functional related elastic properties such as nanosprings mediating protein–protein interactions[41], coiled coils being involved in structural tasks of the cell[42], spider silk[43] and elastomeric polyproteins[44].

Dynamic SMFS (DFS) probes molecular bonds at different force-loads (applied force over time), thereby allowing the approximation of the transition state and kinetic rate of the bond's energy barrier[45]. Such energy barriers form the energy landscape used to describe reaction pathways of protein unfolding, folding, binding and function[37]. Operated in the force-clamp mode, SMFS applies a constant pulling force to a molecular bond and measures the time until the bond breaks[46,47]. Unfolding steps of the clamped protein increase the

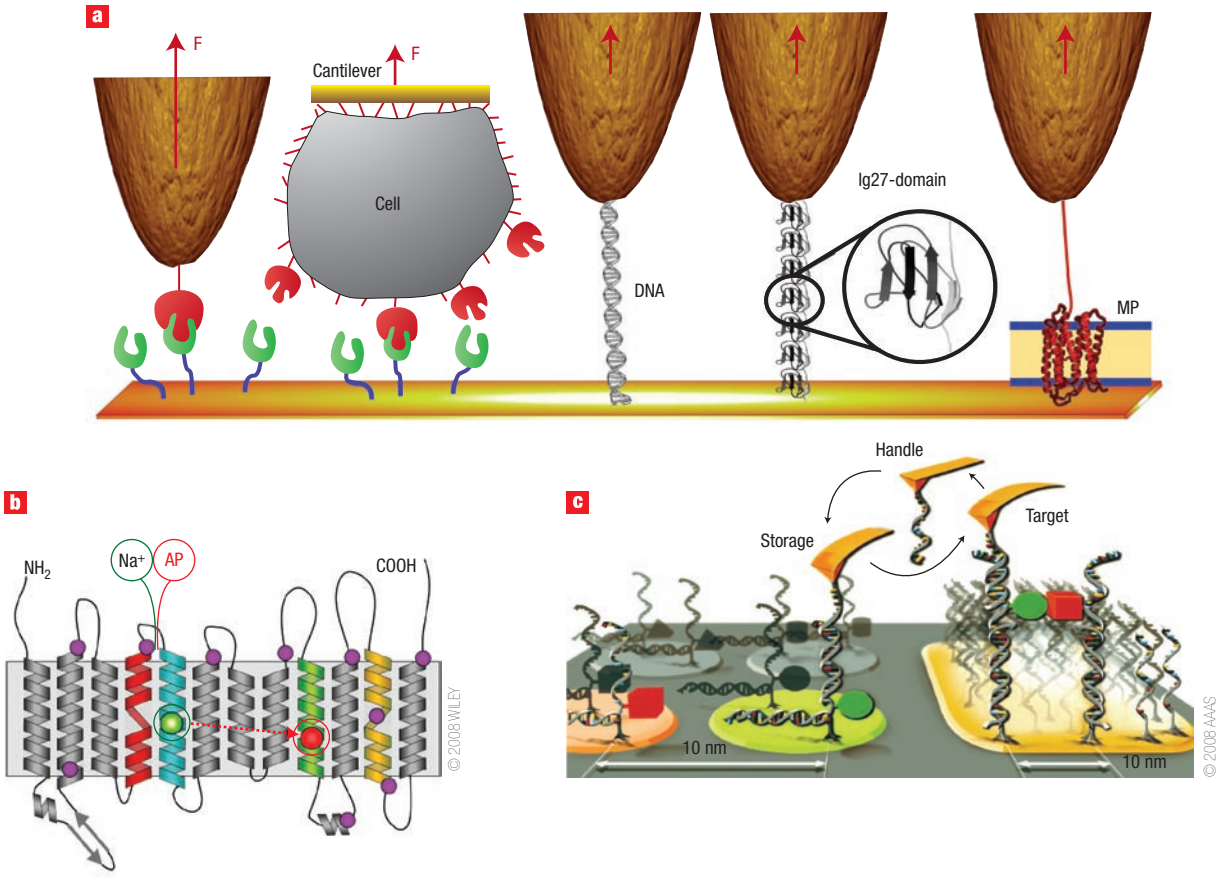

Figure 3 Single molecule manipulation, control and design. **a**, Applying AFM to probe interaction forces (F) of single biomolecules. These examples measure ligand–receptor interactions in their isolated form (left) and embedded in their cellular environment (probe replaced by a biological cell); stretching of a DNA molecule; unfolding of Ig27-titin; and unfolding of a membrane protein (MP). **b**, SMFS can detect and locate interactions (circles) on the structure of membrane proteins (here proton/sodium antiporter NhaA from *E. coli*). A ligand (Na^+ ion) or inhibitor (AP, 2-aminoperimidine) binding to the ligand-binding site (green circle) establishes different interactions activating (green circle) or deactivating (green and red circles) the antiporter (composed of 12 transmembrane helices). **c**, Single molecules can be mechanically assembled by picking up from discrete storage sites with a DNA oligomer at the AFM probe and depositing them at a target site with nanoscopic precision. Reproduced with permission from refs 52, 53 and 63.

length of the unfolded polypeptide and the sequence of these steps describes the protein's unfolding pathway. These single-molecule analyses offer exciting opportunities in biomedicine, particularly for studying the destabilization and misfolding of proteins involved in neurodegenerative diseases such as Alzheimer's, Parkinson's, Creutzfeldt–Jakob disease or diabetes[48]. We anticipate that in the future, SMFS will increasingly be applied to help understand how changing external conditions can guide proteins along different reaction pathways leading to their malfunction.

In contrast to water-soluble proteins, membrane proteins unfold in sequential steps with each force peak in the SMFS spectra denoting a structural unfolding intermediate[49]. Each unfolding intermediate denotes an energy barrier, with their sequence reflecting the unfolding pathway chosen by the membrane protein[50]. Using SMFS, researchers can observe how changes in temperature, pH, electrolyte and mutations can direct membrane proteins to choose certain unfolding pathways over others and thus to populate trajectories in their energy landscape differently. Binding of a ligand establishes a molecular interaction that can be detected by SMFS and located precisely on the membrane protein structure[51]. Interactions that activate the functional state of a membrane protein are different from those established on inhibitor-binding (Fig. 3b). Thus, SMFS spectra recording these interactions can identify whether a ligand or an inhibitor has bonded[52]. Moreover, once the characteristic SMFS spectrum has been assigned to the membrane proteins' functional state, conclusions can be drawn about possible interactions and the functional implications of a molecular compound. This possibility of using SMFS to screen the molecular interactions established on drug binding opens up fascinating avenues. Recent work using DFS has tracked how these interactions change the energy landscape of the membrane protein and thus the properties of certain structural regions relative to others[53]. These insights suggest that in the future, SMFS may be used to locate the binding of a drug to a target and to understand the mechanisms by which the drug functionally modulates the target[54].

Specific molecules or cells attached to the AFM cantilever can be used as reference probes for further single molecule manipulations. A prominent example is provided by oligomeric titin constructs engineered to sandwich a given protein. The known reference peaks of the SMFS spectra assigned to the unfolding of immunoglobulin titin molecules allow newly occurring force peaks to be attributed to the unfolding of the sandwiched protein[55]. This principle of sandwiching a protein between well-characterized ones is becoming a standard tool to characterize water-soluble proteins by SMFS. Whereas SMFS can detect the interactions of isolated receptors and ligands (Fig. 3a), single-cell force spectroscopy (SCFS) attaches a living cell to a cantilever to detect interactions of single-cell

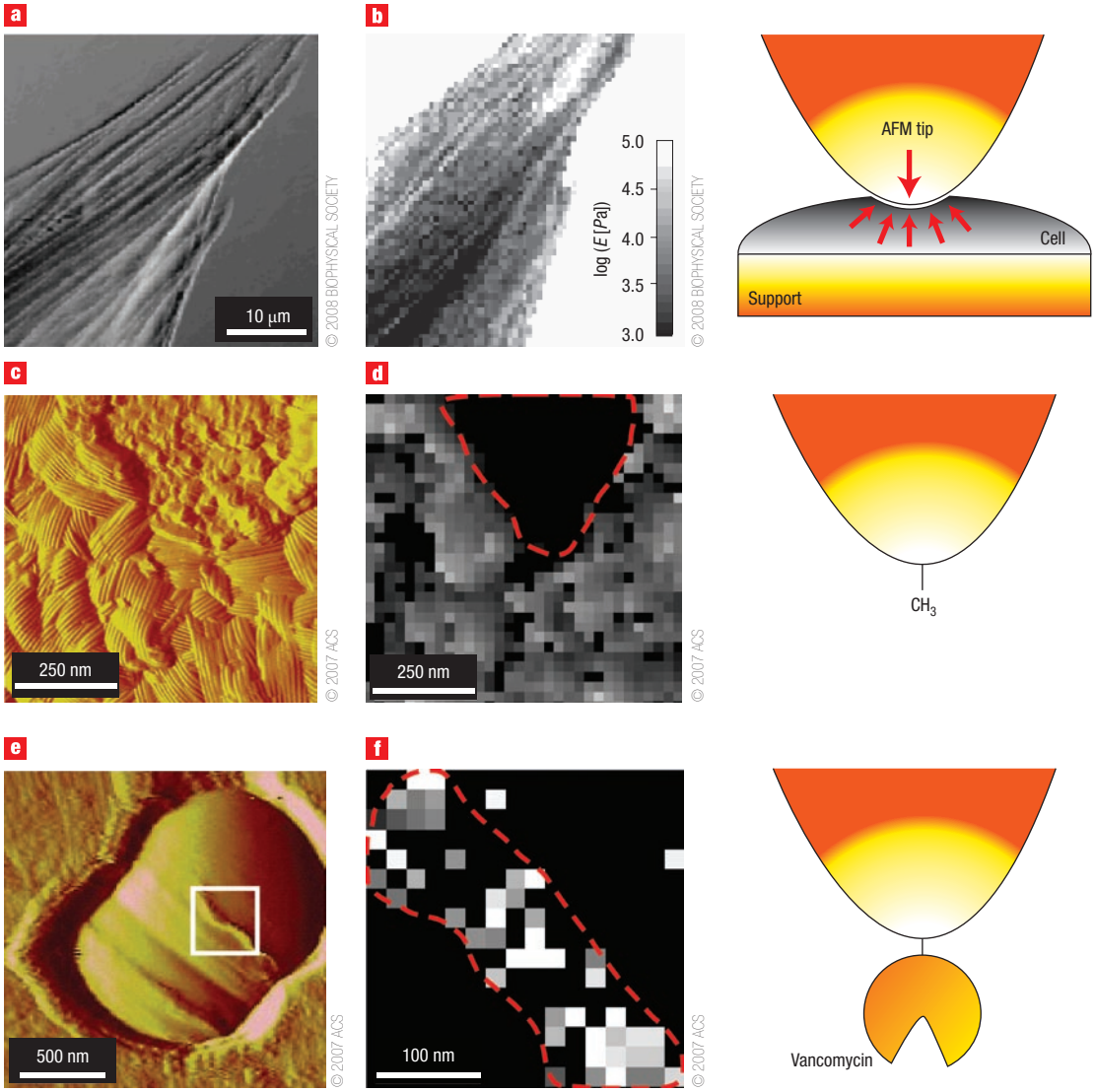

Figure 4 Nanoscale functional imaging of single live cells. **a**,**b**, Mapping cellular elasticity. AFM deflection image of a living fibroblast (**a**), and the corresponding elasticity map (**b**), obtained through nanomechanical force measurements (far right schematic) and showing the increasing stiffness of the fibrous structures. Reproduced with permission from ref. 65. **c**,**d**, Chemical force microscopy. AFM deflection image (**c**), and adhesion force map (**d**) recorded on the fungus *Aspergillus fumigatus* using a methyl-terminated tip (far right schematic), demonstrating that the nanostructured surface is globally hydrophobic (hydrophobins), with some hydrophilic nanopatches (polysaccharides, see area within dashed red line). Reproduced with permission from ref. 76. **e**,**f**, Imaging single receptors. AFM deflection image of *Lactococcus lactis* showing a well-defined division septum (**e**) and adhesion force map recorded with a vancomycin-terminated tip (far right schematic) on the septum region (**f**) (highlighted by the white box in **e**). Each bright pixel in the septum region (red line) reflects the detection of a single vancomycin binding site. Reproduced with permission from ref. 89.

surface receptors while they are embedded in their native cellular environment (Fig. 3a). This approach is of particular importance, as receptor–ligand interactions can be modulated by the cell's functional state. The accuracy of SCFS can detect adhesion of a cell to a support or to another cell and can resolve the contribution of individual receptors[56]. This provides the platform to study molecular adhesion mechanisms of living cells ranging from bacteria and plants to eukaryotes and prokaryotes. SCFS is of particular interest to cell biologists for characterizing cell–cell interactions at molecular resolution[57], as well as for medical researchers working in tumour metastasis[58] and for biotechnologists addressing cell adhesion to biofunctionalized surfaces[59,60].

AFM-based lithographies, particularly dip-pen nanolithography, offer a means of creating arrays of stable biomolecules with nanoscale resolution. They can present considerable advantages for biosensing applications, including short diffusion times, parallel detection of multiple targets and the requirement for only tiny amounts of sample[61,62]. In a pioneering work, Gaub's group established the first molecular toolbox in which they pick up single biomolecules with the AFM probe and drop them to places needed (Fig. 3c). Each pick and drop cycle can be monitored by fluorescence microscopy and by the characteristic force spectra during each pick-up and drop-off[63]. The ability to tinker with single biomolecules with nanoscopic precision allows the study of their structural and functional interactions. As a manipulating tool, AFM offers new ways to characterize biological matter from the cellular to the molecular level. Once characterized, each biomolecular system can easily become a potential probe that adds to the toolbox. A new era of nanobiotechnology has begun.

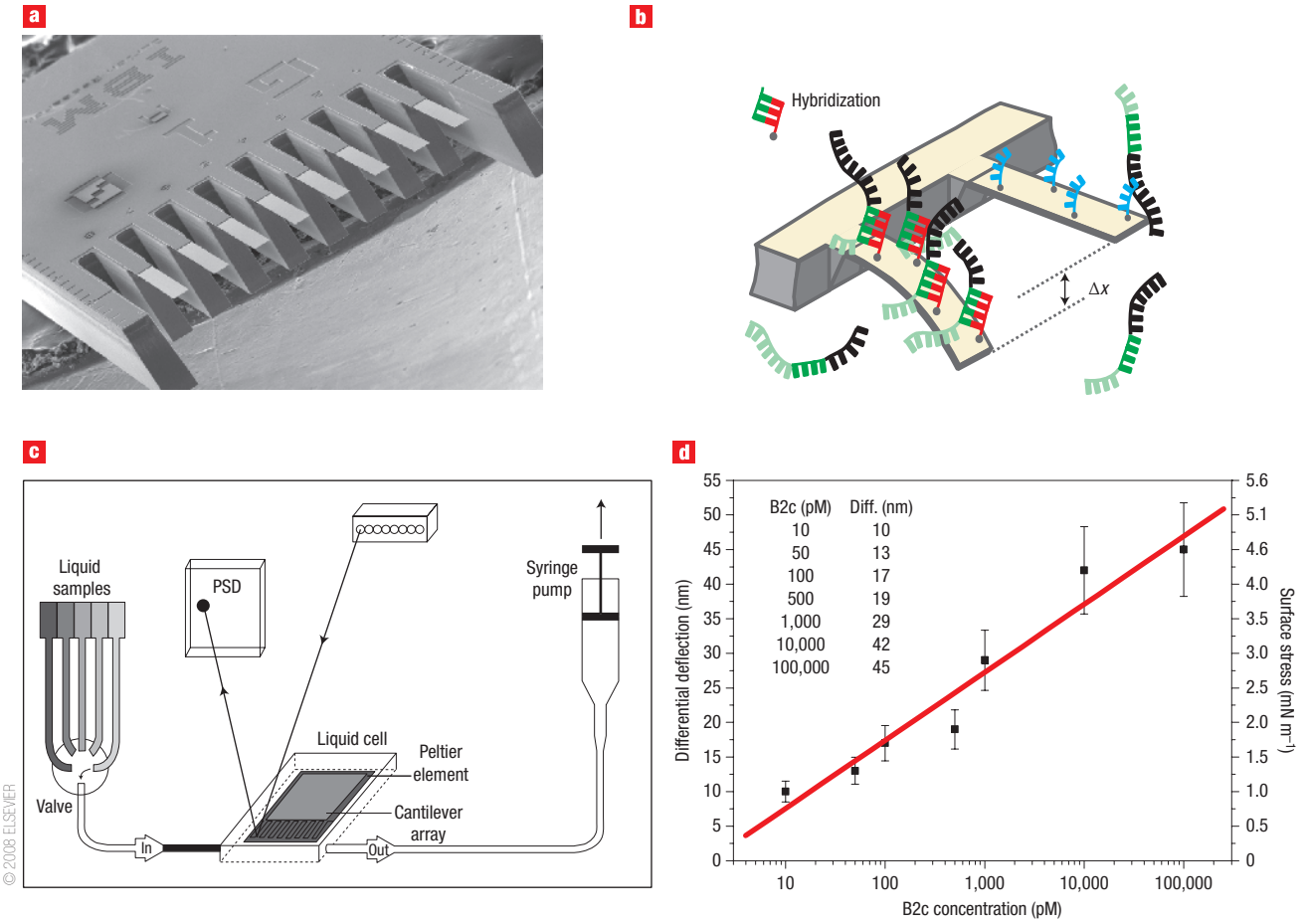

Figure 5 Microfabricated cantilever arrays as label-free biosensors. **a**, Cantilever array showing eight cantilevers that can be individually biofunctionalized. Courtesy of M. Hegner. **b**, Principle of differential readout using sensor and reference cantilevers. Two different probe oligonucleotides (in red and blue) are tethered to the two cantilevers. Nanomechanical bending of the sensor cantilever occurs on hybridization with the complementary oligonucleotides (in green). The cantilever that does not respond is used as a reference, leading to the differential readout (Δx). **c**, Set-up of the device. A laser beam is deflected at the end of a cantilever (see arrow) and its position is detected with a position-sensitive detector (PSD) to measure cantilever bending. Target solutions or buffer are circulated by means of a syringe pump. **d**, Example of differential cantilever response as a function of the concentration of complementary oligonucleotides B2c. Reproduced with permission from ref. 98 and 99.

NANOSCALE FUNCTIONAL IMAGING OF LIVE CELLS

Imaging the local properties of cells and localizing receptors on their surface is a basic challenge in cell biology and microbiology. AFM force spectroscopy may be applied to probe the elastic properties of the cell which play an important role in critical cellular functions, including migration, division and shape. The unique ability to detect and to map the cellular elasticity at the resolution of a few tens of nanometres provides information complementary to that obtained using other techniques such as magnetic or optical tweezers. Such nanoscale measurements involve analysing force–distance curves to provide quantitative information on the elasticity of the cell surface and of the cytoskeleton (that is, Young's modulus)[64]. Interestingly, two-dimensional mapping of elasticity can be obtained by recording arrays of curves over the cell surface (Fig. 4a and b)[65]. In this way, the mechanical properties of a variety of cells including fibroblasts[65,66], platelets[67], kidney cells[68], metastatic cancer cells[69], diatoms[70,71] and yeast cells[72] have been measured.

Of particular interest in medicine is the possibility of monitoring the changes in elasticity on incubation of the cells with drugs. For instance, Rotsch and Radmacher[65] investigated how various drugs that disrupt or stabilize actin or microtubule networks affect the elasticity of cells. Disaggregation of F-actin resulted in a loss of cell rigidity. However, treatment with drugs such as colchicine, colcemide or taxol that target microtubules yielded no effect, leading to the conclusion that the actin network mainly determines the elastic properties of living cells. More recently, Cross and co-workers[69] measured the stiffness of live metastatic cancer cells taken from the body fluids of patients with suspected lung, breast and pancreas cancer. Cancer cells were substantially softer than benign cells, indicating that nanomechanical analyses can distinguish cancerous cells from normal ones even when they have similar shapes. This suggests that AFM-based nanomechanical measurements have a strong potential in biomedicine for the detection of diseases such as cancer[73].

Non-covalent interactions, such as hydrophobic and electrostatic interactions, play essential roles in biology because they promote crucial events such as protein folding and cell adhesion. Chemical force microscopy (CFM)[74,75], in which AFM tips are modified with specific chemical groups, can now be used to map the spatial arrangement of chemical groups of live cells with nanoscale resolution[76–79]. In the first such study, Dague *et al.*[76] used methyl-terminated tips to map hydrophobic groups on the human opportunistic pathogen

Aspergillus fumigatus (Fig. 4c,d). Native spores were shown to be strongly hydrophobic, owing to the presence of 'hydrophobins', a family of small hydrophobic proteins. This supported the notion that hydrophobic forces may promote the adhesion of the pathogen to surfaces and tissues. Notably, using a temperature-controlled stage, progressive changes of surface hydrophobicity and structure could be monitored in real time upon spore germination[77]. Similarly, CFM was applied to demonstrate that treatment of mycobacteria with different antibiotics causes a sharp decrease in cell surface hydrophobicity, reflecting the removal of cell surface mycolic acids[78,79]. Thus, CFM provides unique opportunities to resolve the distribution of chemical groups on live cells as they grow or interact with drugs.

Understanding the specific interactions between ligand and receptor molecules represents a great challenge in current life science research and is critical for developing nanobiotechnology applications. During recent years, SMFS with biologically modified tips[80] has enabled researchers to measure the molecular forces and the dynamics of a variety of receptor–ligand interactions, including those associated with avidin/streptavidin[33,34], antibodies[81], DNA[82] and cell adhesion proteins[59]. DFS can provide estimates of kinetic and energetic parameters of the unbinding process such as the dissociation rate constants that characterize the kinetic stability of the bond. The use of spatially resolved SMFS[84] or dynamic recognition force mapping[85] makes it possible to map the distribution of individual receptors on cell surfaces. This is particularly relevant in current membrane biology because membrane proteins and lipids are known to compartmentalize into nanodomains ('lipid rafts'). In this context, Chtcheglova *et al.*[86] applied dynamic recognition force mapping to microvascular endothelial cells to demonstrate that cadherins, which are proteins involved in homophilic cell-to-cell adhesion, are organized into nanodomains ranging from 10 to 100 nm in diameter. The method has also made it possible to quantify both the binding kinetics and the distribution of growth factor receptors on human microvascular endothelial cells[87]. In microbiology, spatially resolved SMFS has been used to map the nanoscale distribution of single cell adhesion proteins[88] and antibiotic binding sites[89] on live bacteria (Fig. 4e,f), providing insight into pathogen–host and pathogen–drug interactions. In the future, we anticipate that the nanoscale functional imaging of live cells by AFM will find important applications in many fields, particularly for understanding the molecular organization of cell membranes and cell walls, the molecular basis of cellular interactions, and the modes of action of various drugs.

LABEL-FREE CANTILEVER BIOSENSORS

Another exciting application of AFM is to use microfabricated cantilever arrays as label-free biomolecular sensors (Fig. 5). Cantilever nanosensors have important advantages over other biomolecular techniques. First, sample preconditioning such as labelling and amplification is not required. Second, specific biomolecules can be detected with picomolar (~10 pM) sensitivity, which is comparable to current standard gene-chip technology. Third, cantilever arrays are not expensive because they are microfabricated by standard low-cost silicon technology. Fourth, cantilevers are highly suitable for parallelization into arrays, allowing the simultaneous detection of multiples bioanalytes within minutes.

The general idea behind AFM-based nanosensors is to translate specific biomolecular recognition directly into nanomechanical motion. For this purpose, receptor molecules, such as DNA, membranes, proteins, peptides and antibodies, are immobilized on the cantilevers (Fig. 5a), which are then incubated with specific ligands in a liquid environment. Specific molecular recognition reactions can be detected by monitoring either the resonance frequency shift (dynamic mode) or the cantilever bending (static mode). In the dynamic mode, the cantilever is used as a kind of microbalance to detect very small mass changes (<1 pg)[90]. The cantilever is oscillated, and mass changes on the functionalized cantilever surface are derived from shifts in the frequency of vibration. This dynamic method was used to detect viable eukaryotic cells (fungal spores), with a sensitivity of 10^3 colony-forming units per ml, opening up exciting opportunities for medical and environmental diagnostics, and for monitoring food and water quality[91,92]. Recently, Burg *et al.*[93] introduced microchannels into cantilevers, thereby making it possible to weigh single bacterial cells and submonolayers of adsorbed proteins in water with subfemtogram resolution.

In the static mode, only one side of the cantilever is functionalized with receptor molecules. A surface stress is generated on biomolecular recognition of the ligand and this bends the cantilever (Fig. 5b). The bending is usually detected optically, by monitoring the deflection of a laser beam (Fig. 5c). Because measurement of the deflection of a single cantilever can be misleading[90], it is essential to use multiple cantilevers with one reference cantilever (Fig. 5a,b). This parallel differential readout increases the sensor sensitivity and eliminates the influence of external factors such as thermal drift and unspecific interactions. In addition to providing an internal reference sensor, arrays of cantilevers enable multiple independent detection experiments to be performed simultaneously within minutes[94].

In the past few years, the general applicability of cantilever nanosensors has been demonstrated for probing DNA hybridization[94,95] and detecting medically important biomarkers, such as prostate-specific antigens[96], cardiac biomarker proteins[97], DNA-binding proteins[98] and mRNA markers for cancer progression[99]. In an elegant study, the biosensitivity of label-free cantilever-array sensors could be increased by orders of magnitude (~10 pM, which is comparable to current standard gene-chip technology) to detect mRNA biomarkers without amplification steps in total RNA derived from human or rat cell lines (Fig. 5d)[99]. The proposed nanosensors may become important medical diagnosis tools, leading to fast and reliable detection of biomarkers that reveal disease risk, progression or therapy response. In another recent report, DNA, RNA, protein or combinations thereof could be detected in parallel on a single cantilever array[100]. Thus, micromechanical cantilever arrays allow monitoring transcription and translation without creating artefacts, giving a more accurate picture of gene expression in either a healthy or a diseased cell.

PERSPECTIVES

AFM is revolutionizing nanobiotechnological research. Within a decade, this unconventional microscope has evolved into a multifunctional toolbox. For the first time, single cellular machineries can be watched at work at a spatial resolution of ~1 nm, and their folding and functional state explored. The technique has also been used to probe cell surface properties and to elucidate the molecular mechanisms of cell adhesion. Microfabricated cantilever arrays represent a powerful approach for the label-free, highly sensitive detection of bioanalytes. These AFM-based nanoscale analyses offer exciting opportunities in biomedicine. For instance, AFM should help refine our understanding of the destabilization of proteins involved in neurodegenerative diseases and the mechanisms by which drugs bind to and modulate the functional state of cell membrane and cell wall targets. It should also be very useful for characterizing cell–cell interactions involved in diseases such as cancer, and pathogen–host interactions. In diagnosis, nanosensors should be increasingly used for detecting biomarkers in blood samples.

But first there are technological challenges to be solved. At the molecular scale, most biological processes are much faster than the time required to record a high-resolution AFM image (~30 seconds). Nevertheless, remarkable advances have been made in developing fast scanning AFM. Improved time resolution (down to milliseconds) has

allowed a number of cellular machineries to be followed in a way that was not previously accessible[27,101,102].

The full potential of AFM in cell biology will be seen when combined with advanced light microscopy and spectroscopy techniques. This multi-faceted approach will let cellular structures be identified by fluorescence microscopy, while they are imaged at high resolution using AFM. Traditionally, the resolution of a light-focusing microscope is limited by the wavelength of light (λ), and is usually around 200 nm. In stimulated emission depletion microscopy, however, the resolution is no longer strictly limited by λ, meaning cellular structures with dimensions of a few tens of nanometres can be resolved[103]. Combining this technique with AFM will permit the imaging, manipulation and probing of biological matter from microscopic down to nanoscopic scales, thus helping us to cross borders currently limited by conventional microscopy techniques. This combined approach will allow us to observe and manipulate biological structures at an almost unlimited resolution (~1 nm) and simultaneously elucidate their sophisticated functions.

Advances in SMFS methodology will shed new light on the structures, functional states and reaction pathways of an increasing number of membrane proteins, which form targets for drug development in pharmacology[54]. In particular, malfunction, destabilization and misfolding of membrane proteins are involved in major pathologies such as neuropathological diseases[48]. Water-soluble proteins have been characterized much more frequently by SMFS, and most technological developments have been established based on standardized oligomeric protein constructs such as titin or fibronectin. These proteins are now well-studied references for the characterization of new proteins by SMFS. But the continuously growing number of publications shows that we are just beginning to understand the molecular interactions driving the structure and function relationship of these proteins and that the future will bring insights that we never expected.

First, SMFS experiments could detect, locate and differentiate interactions occurring upon ligand- and inhibitor-binding that switch the functional state of proteins. SMFS-based technologies could potentially dissect the contribution of different interactions such as van der Waals, hydrophobic, hydrogen and electrostatic interactions, revealing the spatial and temporal sequence of interactions that guide the binding of a drug to a protein and finally modulate the protein function. In the distant future it may even be possible to detect these interactions acting within and between cellular machineries without the need to unfold as we currently do.

Meanwhile, progress in molecular recognition SMFS and imaging will allow routine measuring of biomolecular interactions in biosystems ranging from single molecules to tissues. Decreasing the size of cantilevers should improve the force resolution, thereby permitting smaller forces to be measured, while finer nanotube tips may help improve the lateral resolution. Advances in nanodeposition techniques, such as dip-pen nanolithography[62], will enable nanoarrays with almost unlimited chemical and biochemical complexity to be created. These structures will be useful for designing new biological chips and nanomaterials, and will help elucidate surface binding events in single particles (proteins, cells)[62].

There are many open challenges in biosensing technology. High-throughput, multi-content sensing assays are needed, and these should be achievable using devices with thousands of cantilevers working in parallel. Another exciting challenge will be to develop assays that can be applied in biomedical laboratories for routine analysis. This will involve producing cantilever assays at low cost and making them easy to personalize and to use.

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Correspondence and requests for materials should be addressed to D.J.M. or Y.F.D.