Review

Atomic force microscopy: a nanoscopic window on the cell surface

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Atomic force microscopy (AFM) techniques provide a versatile platform for imaging and manipulating living cells to single-molecule resolution, thereby enabling us to address pertinent questions in key areas of cell biology, including cell adhesion and signalling, embryonic and tissue development, cell division and shape, and microbial pathogenesis. In this review, we describe the principles of AFM, and survey recent breakthroughs made in AFM-based cell nanoscopy, showing how the technology has increased our molecular understanding of the organization, mechanics, interactions and processes of the cell surface. We also discuss the advantages and limitations of AFM techniques, and the challenges remaining to be addressed in future research.

Measuring forces in biology

The past two decades have witnessed exciting progress in the application of single-molecule detection and manipulation techniques for the structural and functional investigation of biomolecules under physiological conditions [1–6]. Although classic fluorescence microscopy and spectroscopy methods can be used to detect the real-time position, distance, distribution and dynamics of single molecules, force-spectroscopy techniques exert and/or quantify forces to allow manipulation and characterization of the mechanical properties, functional state, conformations and interactions of biological systems to molecular resolution.

Single-molecule force-measuring techniques include flow-chamber experiments, microneedles, the biomembrane force probe (BFP), the optical and magnetic tweezers, and atomic force microscopy (AFM) [1–6]. These assays, which have in common that they measure forces exerted to and established by single molecules, cover a wide range of forces (approx. 0.1 pN to 100 nN) and length scales (approx. 0.1 nm to 100 μm) that are relevant to biology, ranging from small intermolecular interactions to strong covalent bonds and complex cellular interactions. There are basically two ways to exert force on molecules, either via mechanical force transducers, which directly use or sense forces such as microneedles, BFP and AFM, or via external fields acting on molecules from a distance, such as hydrodynamic flows, and optical and magnetic tweezers.

Optical and magnetic tweezers enable researchers to non-invasively manipulate biomolecules in solution, including within living cells. BFP is a surface technique, which offers the advantage that the transducer sensitivity can be tuned to measure a wide range of forces at various biological interfaces including cell surfaces. Of note, single-channel recording (patch-clamp technique) uses micropipettes to record the current contributions of individual ionic channels. Importantly, most single-molecule force experiments have to date been conducted *in vitro* (i.e. on isolated molecules, in which the biological system can be tightly controlled). The interactions guiding the assembly and functional state of the biomolecular machinery are dynamically controlled by the living cell, however, which emphasizes the need to move single-molecule experiments into living cells [5,6]. In recent years, much progress has been made in this direction using AFM techniques, thereby contributing to the growth of the exciting new field of live-cell nanoscopy.

Atomic force microscopy: a multifunctional molecular toolkit

Originally invented for topographic imaging [7], AFM has evolved into a multifunctional molecular toolkit (Figure 1; Box 1) [8], enabling researchers to observe the structural details of cell surfaces and to force-probe their individual molecules. Because of its outstanding signal-to-noise ratio, AFM can directly image single membrane proteins and live cells at nanometre resolution in buffer solution and at ambient temperature, which is a key advantage over electron- and light-microscopy techniques. Real-time AFM imaging (Figure 1b; Box 1) of single live cells can provide novel insight into dynamic processes, such as nanostructural changes that are caused by growth or drug interactions. AFM force-spectroscopy modes (Figure 1c; Box 1), including single-molecule force-spectroscopy (SMFS) [4,11,12], molecular recognition mapping (MRM) [9,18–20], and single-cell force spectroscopy (SCFS) [10,13] can be used to localize and quantify the interactions of biological systems over scales ranging from cells to single molecules [4,5,9–11]. In this technique, the tip of the AFM cantilever is functionalized with specific biomolecules or replaced by a living cell (Figure 1d), and the force interacting between the modified tip and sample is measured. Prominent applications of these AFM-based force spectroscopies include measuring interaction forces and dynamics between individual pairs of ligands and receptors, either *in vitro* or *in vivo* [14]; quantifying cell-adhesion forces, from the single molecule to the cellular level [10,15]; deciphering pathways of protein folding and

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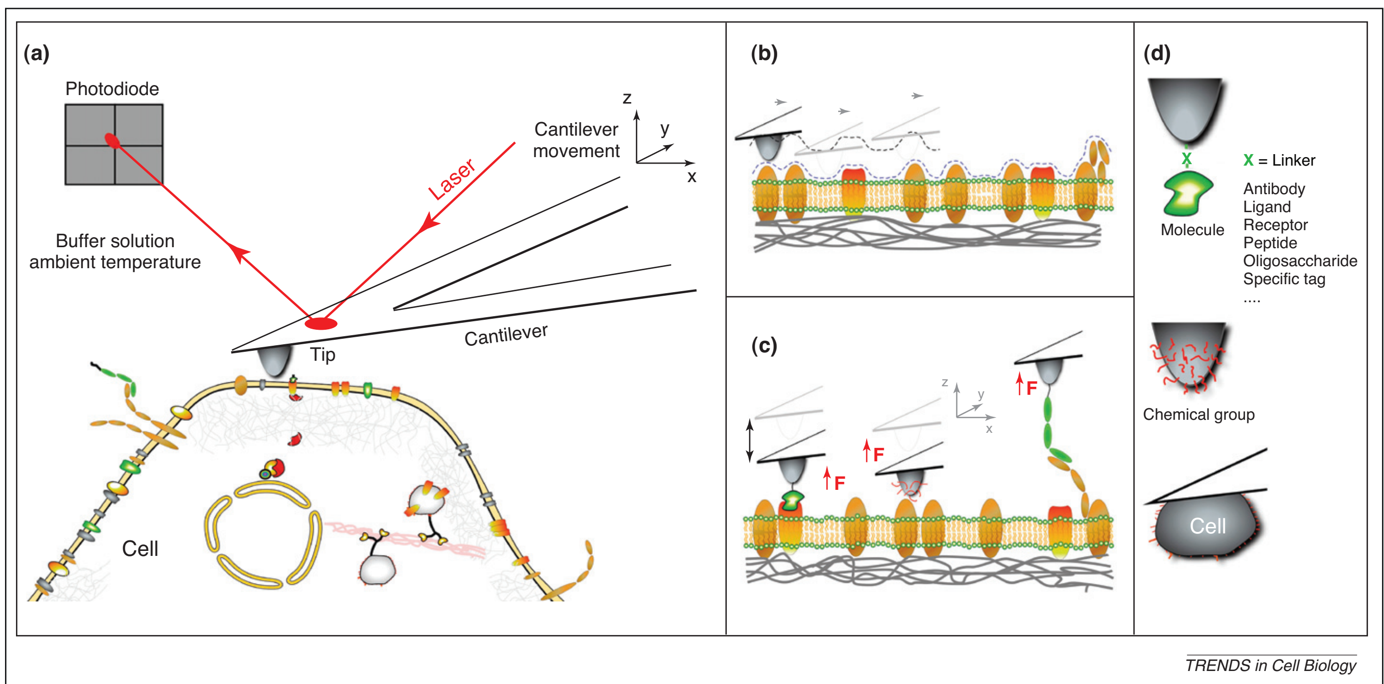

Figure 1. AFM-based nanoscopy of living cells. **(a)** Unlike other microscopy techniques, AFM works by sensing the tiny forces between a sharp tip and the cell surface. **(b)** In the imaging mode, the AFM tip is scanned across the cell (arrows) to contour its surface topography (dashed line). **(c)** In force spectroscopy techniques, the AFM tip is used to measure cell-surface interactions to single-molecule resolution. Examples (from left to right) show a tip functionalized with a ligand to probe interactions with its cognate receptor, a tip coated with chemical groups to detect chemical interactions, and a tip carrying cell-adhesion molecules to probe homo- or heterophilic interactions towards other cell-adhesion molecules. **(d)** Most force-spectroscopy applications imply functionalizing the AFM tip or cantilever to specifically probe biological, chemical or cellular interactions.

unfolding [11]; approaching molecular mechanisms regulating cell mechanics [16];measuring single molecules binding to a target protein or nucleic acid [11,17]; and mapping the spatial distribution of cell-surface receptors [18–20]. In this review, we provide a snapshot of some of the most exciting experiments that have been recently carried out in cell biology using AFM-based nanoscopy. We also discuss the principles of the various AFM techniques (Box 1), and their respective advantages and disadvantages for the cell biologist.

Imaging the cell: connecting structure and function

AFM topographic imaging is a powerful complement to fluorescence [21,22] and electron microscopies, offering new possibilities for visualizing the supramolecular organization of cell surfaces in buffer solution and in real time [23,24]. A recent example is the AFM imaging of living vascular endothelial cells, which revealed that the cells swell and soften when exposed to increased concentrations of extracellular potassium [25]. As a functional consequence, the cells undergo shear-stress-mediated deformations that result in enhanced release of nitric oxide. Currently, however, AFM imaging of living mammalian cells remains limited to resolutions in the 50–100 nm range, meaning that the individual components of the cell-surface machinery cannot be observed.

Compared with animal cells, high-resolution AFM imaging of living microbial cells has proved more straightfor-

Box 1. AFM principles

Unlike other microscopes, the AFM raster scans a sharp tip over the biological specimen to contour its surface (Figure 1a). For every point on the specimen surface, the tip locally senses forces that are used for feedback control to contour the specimen (Figure 1b). Interactions contributing to these forces are complex, and the contributions are biological, chemical and physical. A piezoelectric scanner allows high-resolution 3D positioning (1 Å) of the tip. The latter is attached to a soft cantilever that deflects and quantifies the force. Cantilever deflection is detected by a laser beam reflected from the free end of the cantilever into a photodiode.

Different AFM imaging modes are available, which differ mainly in the way in which the tip moves over the sample. The most widely used is the contact mode. Minimizing the force produced by the tip is often necessary to prevent sample damage. This can be achieved by use of the constant-force mode, in which the sample height is adjusted to keep the deflection of the cantilever, and thus the force used, constant, using a feedback loop. In dynamic or intermittent mode, an oscillating tip is scanned over the surface, and interactions between tip and sample change the cantilever amplitude used for feedback control.

In force spectroscopy techniques, such as chemical force microscopy (CFM), SMFS, MRM and single-cell force spectroscopy (SCFS), the AFM tip is functionalized with chemical groups, biological molecules or viruses, or even replaced by a living cell (Figure 1d). The modified tip is continuously approached towards and retracted from the biological sample, and the cantilever deflection measures the interaction force (Figure 1c). Such approach and retraction cycles are recorded by force–distance (F-D) curves. The characteristic adhesion (or unbinding) force observed during stylus retraction is the key parameter that provides information on specific receptor–ligand interactions (in SMFS), the spatial distribution of chemical groups (in CFM) or individual receptors (in MRM), and the forces that govern cell–cell and cell–substrate interactions (in SCFS). Lastly, topography and recognition (TREC) imaging is a recently introduced MRM mode in which molecular recognition signals are detected during dynamic force microscopy imaging, rather than through recording F-D curves. Importantly, all these AFM-based methods are fully compatible, and can be operated simultaneously with modern fluorescence microscopy at physiological conditions.

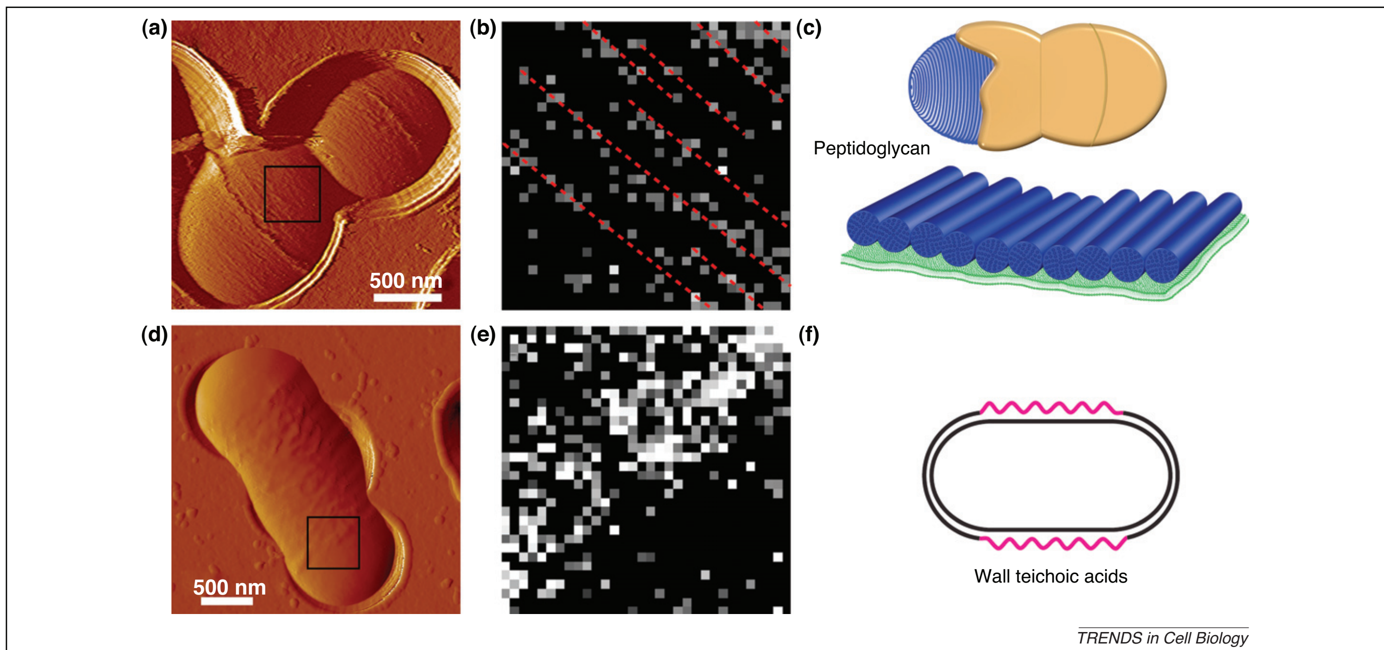

Figure 2. Imaging live cells: from structure to function. **(a–c)** Imaging the nanoscale organization of peptidoglycan in living *L. lactis* cells. **(a)** Topographic image of two dividing bacterial cells lacking cell-wall exopolysaccharides. **(b)** Single-molecule recognition map (400 × 400 nm) recorded with a LysM probe in the square area shown in the topographic image; peptidoglycan molecules were detected (bright pixels), and found to be arranged as lines running parallel to the short cell axis (red lines). **(c)** Schematic views of the architecture of the *L. lactis* cell wall: the top drawing emphasizes the two layers of the cell wall, that is, the periodic bands of peptidoglycan (blue) covered by cell-wall polysaccharides (brown), and the bottom drawing is an enlarged view of the peptidoglycan nanocables (blue) lying on the membrane (green). **(d-f)** Imaging the heterogeneous distribution of WTAs in *L. plantarum*. **(d)** Topographic image of a single *L. plantarum* cell revealing a highly polarized surface morphology, the poles being much smoother than the side walls. **(e)** Single-molecule recognition map (400 × 400 nm) recorded with a lectin probe in the square area shown in the topographic image; WTAs were often detected on the side walls (bright pixels) but were essentially lacking on the poles (dark pixels). **(f)** Schematic view of the architecture of the *L. plantarum* cell wall: WTAs localize exclusively to the side walls (red), correlating with a rough surface morphology. This polarized organization is important for cell morphogenesis. Figures reprinted with permission from **(a-c)** [31] and **(d–f)** [43].

ward and more reliable, because of the well-defined and rigid nature of the microbial cell surface [26]. AFM has allowed observation of the supramolecular organization of major cell-wall constituents on live cells, such as rodlets [27], surface-layer proteins [28], and peptidoglycan (Figure 2a–c) [29–31]. Living cells are highly dynamic, and continuously respond to environmental changes. Hence, an important asset of AFM is its ability to track the structural dynamics and remodelling of cells in response to environmental stimulants or therapeutics. A remarkable example of this is the real-time analysis of the structural dynamics of single *Bacillus atrophaeus* spores during germination [27]. AFM images revealed previously unrecognized germination-induced alterations in spore-coat architecture and in the disassembly of outer spore-coat rodlet structures. In biomedicine, the real-time imaging of pathogen–drug interactions opens up new possibilities for understanding the mode of action of antibiotics, and for screening new antimicrobial molecules [32–36]. As an example, incubation of mycobacteria with a series of drugs induced major ultrastructural alterations, reflecting the inhibition of the synthesis of major cell-wall constituents (mycolic acids, arabinans, and proteins) [35]. In another study, time-lapse AFM images of single *Staphylococcus aureus* cells exposed to lysostaphin, an enzyme that specifically cleaves peptidoglycan, documented progressive digestion of the cell wall, eventually leading to the formation of osmotically fragile cells [36]. The above studies used conventional AFM imaging, which is currently limited by its rather poor temporal resolution (approx. one image per minute). Remarkably, advances are being made in developing faster AFMs [37,38]. Although the use of high-speed AFM for live-cell imaging is not yet established, it was recently used to track structural changes induced by antimicrobial peptide CM15 on individual *Escherichia coli* cells [38].

Structural imaging by AFM lacks specificity, meaning that specific constituents cannot be identified or localized on live cells. Notably, MRM using biologically-modified tips (Box 1) enables researchers to map the distribution of single molecules on cell surfaces, thereby providing key insights into cell-surface heterogeneities, which had long been difficult to probe, because of their small size and transient nature. MRM has been used to map the location of vitronectin receptors on the surface of live osteoblastic cells [39], to probe the distribution of prostaglandin receptors on living Chinese hamster ovary (CHO) cells [40], to localize vascular endothelial cadherin binding sites and correlate their position with membrane topographical features [20], and to understand the organization and stiffness of neuron membrane domains containing glycosylphosphatidylinositol (GPI)-anchored proteins [41]. An important direction for future research is to combine those single-molecule experiments with other microscopy techniques. Recently, a new platform combining MRM and fluorescence was introduced for the improved localization of receptors on the same surface areas of CHO cells and of endothelial cells [42].

In microbiology, MRM has provided new insight into the spatial localization of major cell-wall constituents. As a complementary approach to structural imaging, MRM was applied to *Lactococcus lactis* to localize single peptidogly-

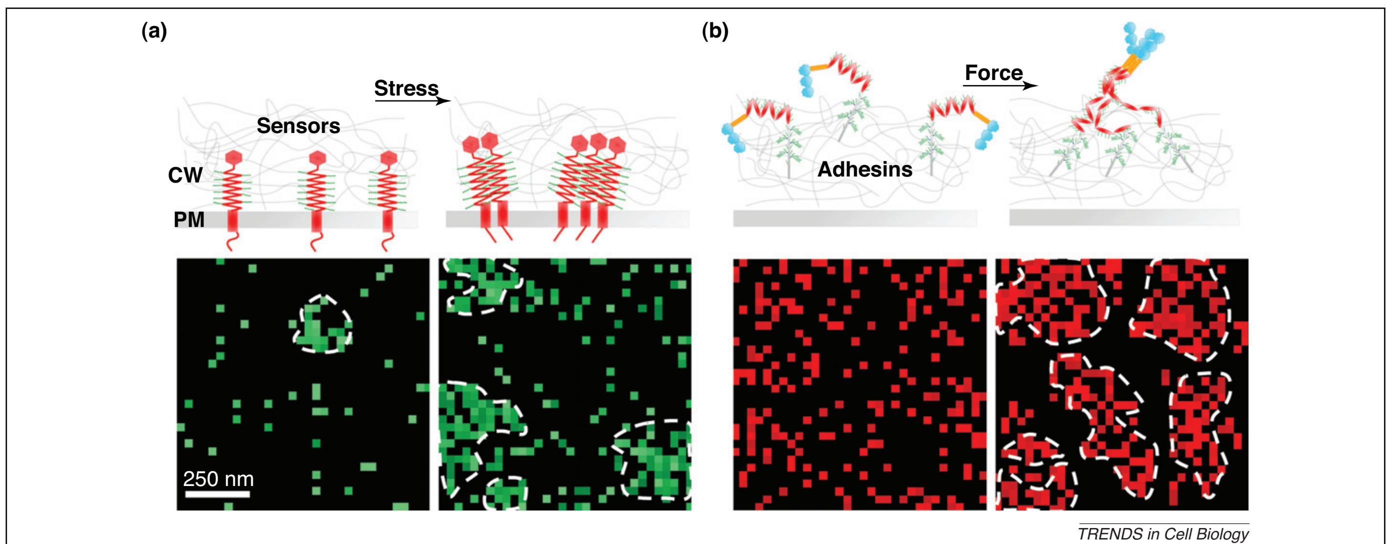

Figure 3. Single-molecule imaging unravels the dynamic clustering of cell-surface receptors [44,45]. **(a)** Molecular recognition maps demonstrating that clustering of the yeast sensor Wsc1 (green) is strongly enhanced by hypoosmotic shock; left and right maps were obtained in buffered solution and in deionized water, respectively; PM: plasma membrane, CW: cell wall. **(b)** Molecular recognition maps documenting the distribution of single Als5p adhesins (red) on a single yeast cell. Although native cells showed random arrangements of adhesins (left), cells that were mechanically stimulated displayed adhesion nanodomains (right).

can molecules [31] (Figure 2a–c). The correlation between structural and recognition images provided direct evidence that peptidoglycan localizes in the form of parallel cables in *L. lactis* (Figure 2c), thus supporting the classic model of peptidoglycan assembly. MRM and fluorescence microscopy were used to decipher the relationships between the spatial localization and the functional roles of cell wall teichoic acids (WTAs) in *Lactobacillus plantarum* [43]. MRM with specific lectin probes demonstrated that the polarized surface structure (Figure 2d) correlates with a heterogeneous distribution of WTAs (Figure 2e,f), and that this polarized cell-wall organization plays a key role in controlling cell morphogenesis.

MRM has also demonstrated that functional protein domains on live cells are able to grow under stress. Imaging single Wsc1 sensors in living yeast cells revealed that they form clusters of approximately 200 nm in size, and that clustering is strongly enhanced under stress conditions (e.g. osmotic stress, elevated temperature) (Figure 3a) [44]. These single-molecule observations indicate that signalling is coupled to the localized enrichment of sensors within membrane patches, for which the term 'nanosensosomes' was proposed. In another study, MRM showed that mechanical stimuli can trigger the formation of adhesion nanodomains on live cells [45]. Pulling on single Als5p cell-adhesion proteins (adhesins) from the pathogen *Candida albicans* with AFM tips functionalized with specific antibodies was shown to induce the formation and propagation of nanoadhesomes (i.e. adhesion domains 100–500 nm in size) (Figure 3b). Hence, clustering of adhesins in response to mechanical stimuli might be a general mechanism for activating cell adhesion in microbial pathogens.

Cellular mechanics

Cellular mechanics is of prime importance in many cellular processes (e.g. cell growth and division) and diseases (e.g. cancer). AFM force-spectroscopy could be used to quantify and map the elastic properties of the cell with nanoscale resolution. Using this approach, researchers tracked dynamic changes in the stiffness of the cortex of adherent cultured cells during M phase, from metaphase to cytokinesis, revealing furrow stiffening during cell division [46]. AFM has also been used to investigate how various drugs that disrupt or stabilize actin or microtubule networks affect the elasticity of cells [47], and to characterize the mechanisms by which stress reversibly softens actin networks and contributes to cell stiffness [48]. In another cell mechanics-based study, researchers using AFM showed that endothelial cells directly respond to small changes in extracellular sodium, which acts as a cell stiffener [49]. Recently, AFM was used for real-time monitoring of cell elasticity, and unveiled the cytoskeletal dynamics of living bronchial epithelial cells [50]. The data indicated that the collective activities of the myosin motor proteins induce fluctuations in cell mechanics.

AFM has been used to monitor the height of HEK2-93 cells to quantify how their mechanical properties change in response to hormone stimulation [51]. When stimulating the G-protein-coupled receptor angiotensin I with angiotensin II, well-known for its role in regulation of cell homeostasis, the cell showed a contractile response. Using confocal fluorescence microscopy, the researchers were able to attribute this contractile response to the reorganization of the actin cytoskeleton. Use of AFM detected this reorganization of the actomyosin cortex near the apical side of the cell membrane as nanoscopic height fluctuations of the cell surface. Inhibiting specific elements of the angiotensin I receptor signalling pathways suggested that these AFM measurements can be a reflection of cell mechanical responses. AFM was similarly used to study the mechanical properties of human umbilical vein endothelial cells (HUVEC) in response to two physiological agonists, namely thrombin and bradykinin [52].

Similarly, AFM was used to investigate the effect of retinol and conjugated linoleic acid (both of which have important roles in differentiation, proliferation and cell death) on the intracellular cytoskeleton, focal adhesions

and the nanomechanical properties of 3T3 fibroblasts [53]. The data revealed that both compounds disrupt formation of focal adhesions, leading to an increase in cell height and a significant decrease in stiffness. Lastly, cell mechanical measurements also offer potential applications in medicine for studying or detecting diseases. AFM force measurements revealed that live metastatic cancer cells were substantially softer than benign cells [54]. Quantitative differences in elasticity were observed between normal and cancerous human cervical epithelial cells, and attributed to differences in the spatial organization of the cell-surface brush layers [55].

In many mechanical studies, models that are generally used to derive parameters describing the elasticity of the cell are oversimplified, and barely approximate the structural and mechanical heterogeneity of a complex living cell. Ways to circumvent these problems include gluing μm-sized beads to the AFM cantilever, and probing the elastic cellular responses. In contrast to the nm-sized AFM tip, the microbead will sense the elastic properties averaged over a larger surface area, and will not deeply penetrate or disrupt the cell surface. Other approaches simply employ sensitive tipless cantilevers to gently touch the cell surface and sense the mechanical processes actively generated by the cell, such as cell rounding, fluctuation of the cell membrane, and migration.

Cell division and shape

Changes in cell shape are of central importance for cell growth, division and death. Control of cell shape relies on both tight regulation of intracellular mechanics and the cell's specific and nonspecific interactions with the environment [56]. When tissue culture cells enter mitosis, they undergo a dramatic shape change. The cells partially detach from the substrate and round up, leaving retraction fibers attached to the substrate. Mitotic cells remain round until cytokinesis, when cleavage furrow ingression divides them into two daughter cells. Mitotic cell rounding is thought to facilitate organization within the mitotic cell, and be necessary for the geometric requirements of division [57]. A role for actin-based processes in cortical retraction and stiffening during mitotic cell rounding has been demonstrated, but the forces and mechanisms that drive this drastic shape change remain largely unexplained. Using the AFM cantilever to confine a mitotic cell (Figure 4), it was found [58] that the mitotic rounding force of eukaryotic cells depends not only on the actomyosin cortex but also on the transmembrane ion gradient. This ion gradient generates an osmotic pressure that drives cell rounding for mitosis. Reducing this osmotic pressure induces cell shrinkage by actomyosin cortex contraction, whereas perturbing the actomyosin cortex contraction triggers transient increases in cell volume. These observations have led to a new model in which two opposing mechanisms facilitate cell rounding, with the osmotic gradient generating an outward directed pressure, and the contractile actomyosin cortex tension governing cell shape.

Single-molecule mechanics

At the molecular level, the mechanical properties of cellular proteins also play major roles in mediating physiological functions. Mechanosensing, for instance, involves the conversion of mechanical forces into biochemical signals via specific proteins. Although SMFS has been extensively used to measure the force response of cellular proteins *in vitro* [4,11,59,60], investigating how single proteins respond to forces in living cells has been a long-standing challenge. Recently, SMFS was used to stretch single Wsc1 sensors on living yeast cells to investigate their nanomechanical properties [61]. For many years, Wsc1 proteins had been suggested to act as mechanosensors activating stress pathways in response to physical changes in the cell wall, but direct evidence for such a mechanism was lacking. SMFS demonstrated that Wsc1 behaves like a nanospring capable of resisting high mechanical force and of responding to cell-surface stress [61]. In the cell adhesion context, stretching single Als5p adhesins revealed sawtooth patterns with well-defined force peaks, each peak corresponding to the force-induced unfolding of the secondary structures of individual tandem repeats engaged in cell

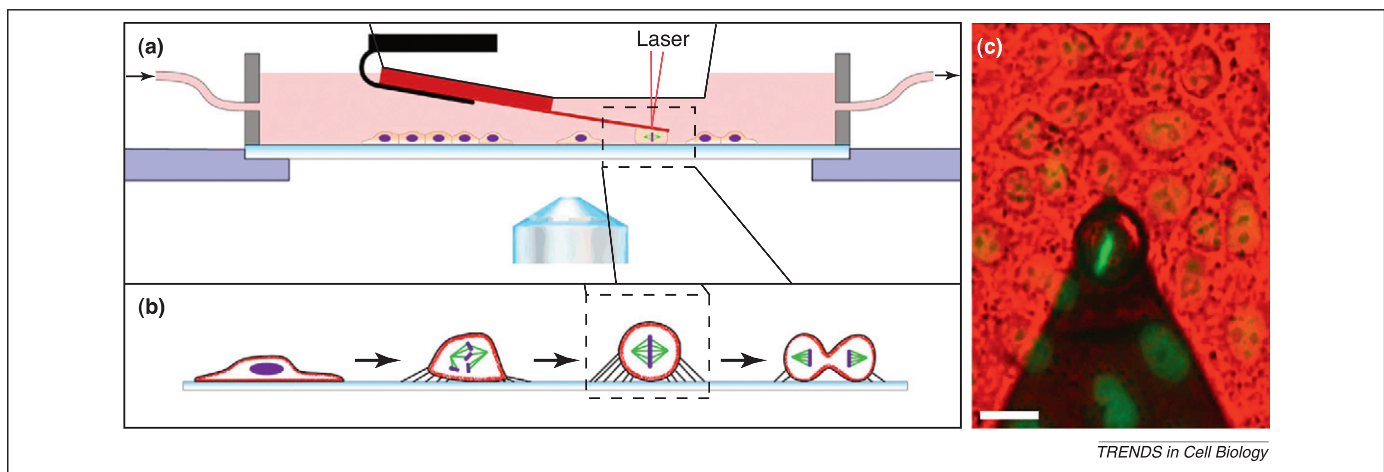

Figure 4. Probing a living cell rounding up for mitosis [58]. **(a)** A tipless AFM cantilever is placed above a single cell entering mitosis. The force generated by the cell rounding up against the cantilever is measured during the entire course of mitosis. **(b)** Adherent tissue culture cells (left cell) partially detach from the substrate and round up, leaving retraction fibers attached to the substrate (second cell). Mitotic cells remain round until cytokinesis (third cell), when cleavage furrow ingression divides them into two daughter cells (forth cell). **(c)** Combined phase contrast (red) and fluorescence (green) microscopy enables estimation of the mitotic state of green fluorescent protein (GFP)–histone labeled cells. The black triangle originates from the cantilever placed on top of the mitotic cell. Scale bar: 15 μm.

adhesion [62]. The unfolding probability increased with the number of tandem repeats expressed by the cells, and was correlated with the level of cell–cell adhesion, suggesting that these modular domains play a role in fungal adhesion. Presumably, the force-induced unfolding of Als proteins leads to extended conformations in which hydrophobic groups are freshly exposed, thus favouring hydrophobic interactions between cells. These single-molecule experiments demonstrate that, in the future, AFM will be a key tool to characterize the cell 'unfoldome' [60] (i.e. the set of cellular proteins that can be unfolded as part of their physiological function).

Assembly and crosstalk of cell-adhesion receptors

The regulation of cell-surface receptors can have manifold origins, and depends on their supramolecular assembly, the membrane composition (e.g. lipids and other molecules), the interaction with the extracellular matrix and with the actomyosin cortex, cell signalling, and the binding of molecular compounds [59]. Initiated by the first weak adhesion, the cell reorganizes its cytoskeleton and membrane receptors to increase adhesion strength [63]. Above, we have shown that MRM can detect and locate such receptor reassembly. However, AFM can also be used to quantify the extent to which such reassembly changes the adhesive state of a cell. In one such example, SCFS was recently used to quantify the adhesion of $\alpha_2\beta_1$-integrin-expressing CHO cells to collagen type I matrices [64]. During the first 60 seconds of contact, the adhesion increased only slowly, and rupture forces characterizing the smallest adhesion events reflected those of individual integrin–collagen bonds. After 60 seconds, the cells began switching into an activated adhesion state, increasing the overall cell-adhesion strength 10-fold. The smallest rupture events of this elevated adhesion mode were significantly enhanced, suggesting a change from single to cooperative receptor binding. This cooperative binding was supported by actomyosin contractility. Cellular interactions also initiate immune responses. In this context, SCFS was used to quantify the adhesion of single T cells in response to recognition of antigen-presenting cells (APCs). Compared with the initial binding-strength seen after 10 seconds of contact time, T cells increased this adhesion force 30-fold within 30 minutes [65]. This temporal increase of intercellular adhesion molecule-1 (ICAM-1)-dependent adhesion was correlated with an enrichment of lymphocyte function antigen-1 (LFA-1) at the adhesive interface between the cells, and also correlated with the kinetics of immune synapse formation.

Cells use specific and controllable adhesion mechanisms to interact with substrates, cells and tissue. Although most of the receptors involved in these adhesion mechanisms are known, the mechanisms by which they regulate adhesion remain largely unknown. The primary reason for this is that conventional cell-adhesion assays are not suited to providing quantitative insights into mechanisms that regulate cell adhesion. In a regulatory process termed 'crosstalk', one type of cell-surface receptor regulates the extent to which a specific cell-adhesion receptor contributes to cell adhesion. To characterize such crosstalk, stimulated SCFS (sSCFS) was developed [66]. sSCFS exposes a cell to a certain ligand (the first ligand) to functionally stimulate the cell. After the ligand has bound and activated a specific type of cell-surface receptor, the stimulated cell is used to probe adhesion to a different ligand (the second ligand). The ability of the first ligand–receptor pair to modulate cell adhesion mediated by the second ligand–receptor pair is evaluated by control measurements in which the cell has not been stimulated. In its first application, sSCFS detected a crosstalk between collagen-binding integrin $\alpha_1\beta_1$ and fibronectin-binding integrin $\alpha_5\beta_1$ in HeLa cells. In the future, such quantitative assays could be further developed to screen and quantify the various crosstalks of adherent cells, and to unravel their underlying regulatory pathways.

Cell migration, sorting and tissue formation

A hot topic in cell biology is that of the forces that drive cell migration, sorting and tissue formation. SCFS- and AFM-based elasticity measurements have been used to characterize the effect of adhesion and cortex tension in the sorting of ectoderm, mesoderm and endoderm progenitor cells from gastrulating zebrafish embryos [67]. It was found that in contrast to cell adhesion, cell-cortex tension is more important for germ-layer organization. The actomyosin-dependent cell-cortex tension is regulated by Nodal/transforming growth factor β (TGFβ) signalling, and is a key factor in directing progenitor-cell sorting. These quantitative insights led the authors to conclude that the differential adhesion hypothesis, which was more than 50 years old at the time, was insufficient to describe self-sorting of embryonic cells. SCFS and SMFS also revealed that cell-cortex tension and attachment are key parameters controlling directed cell migration during gastrulation of zebrafish embryos [68].

Another pertinent question in developmental biology is how the central lumen of blood vessels develops within a cord of vascular endothelial cells. Using SCFS and a bead-rolling assay in *in vivo* and *in vitro* models (Figure 5a–c), interaction mechanisms that separate the endothelial cell surfaces and drive lumen formation were characterized [69]. The interplay of electrostatic repulsive forces and adhesive forces between opposing endothelial cells were quantified, and showed that the sialic acids of apical glycoproteins localize to apposing endothelial cell surfaces, and generate repelling electrostatic fields within an endothelial cell cord.

Cell–cell adhesion also plays crucial roles in immune responses. T cells patrol the body and if their antigen-specific T cell receptors (TCRs) recognize foreign peptides on APCs, establish adhesive contacts to the APCs. Quantifying the adhesion between T cells and APCs, SCFS revealed that the adhesion strength significantly (approx. 15–30-fold) increased within the first 30 minutes after contact, and then decreased again after 60 minutes (Figure 5d) [70]. This peak of adhesion correlated with the kinetics of immune synapse formation, which reaches a maximum after 30 minutes.

These recent examples show nicely how AFM-based approaches can provide valuable insight into the complex interactions of biological systems, ranging from those between two living cells to those describing tissue formation

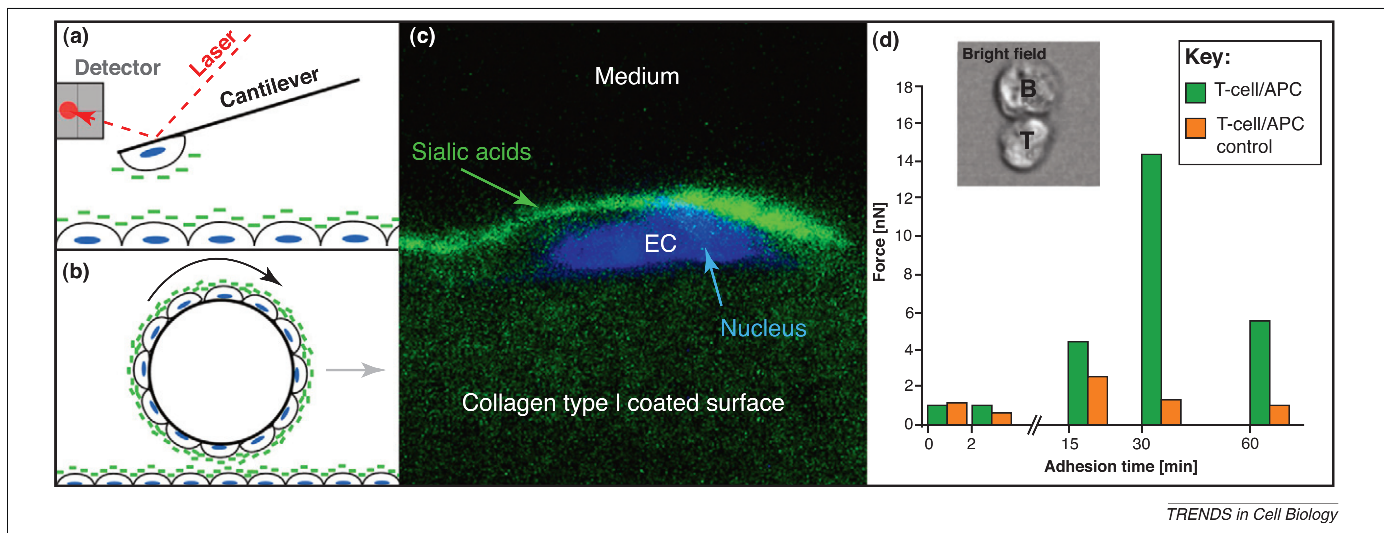

Figure 5. Probing the forces that drive tissue and synapse formation [69,70]. **(a,b)** Schematic diagram of **(a)** SCFS experiment and **(b)** bead-rolling assay. A HUVEC expressing sialic acids at the apical EC surface was grown onto a collagen I-coated AFM **(a)** cantilever or **(b)** bead. HUVECs expressing sialic acids at their apical EC surface were grown on collagen I-coated cell culture dishes. In both experiments, the expression of sialic acids at the apical EC surface significantly reduced adhesion between approaching HUVECs. **(c)** Confocal image of a transverse section through ECs grown on collagen I-coated beads and stained for sialic acids, as indicated in **(b)**. **(d)** Adhesion-force kinetics between a T-cell and an APC. The bright field image shows the T-cell (T) and the APC cell (B) adhering to each other. Using SCFS, the detachment force required to separate one T-cell from one APC was measured over different adhesion times. In the control experiments the specific adhesion of both cells was inhibited by a blocking HEL peptide. Images courtesy of (**c**) E. Lammert and (**d**, inset) J. Spatz. Figure 5c has been modified from [69] with permission.

and embryonic development. For each of these experiments, however, the AFM setup had to be adapted slightly. For example, for each of the cell types, different procedures had to be developed to attach the cell to the AFM cantilever. In all examples, cellular forces were measured the same way, by simply recording the deflection of the AFM cantilever. For broader use in biology, standardized protocols should be developed for the attachment of individual cell types.

Concluding remarks

The experiments reviewed here demonstrate that cell nanoscopy using AFM is revolutionizing the way in which biologists unravel the molecular details of the living cell. As an imaging tool, AFM deciphers the supramolecular architecture of cell surfaces, and localizes their individual constituents in relation to function (e.g. growth, division, adhesion and mechanosensing). As a force-measuring tool, SMFS and SCFS quantify cell-adhesion forces, from single molecules to whole cells, thereby addressing key questions in areas as diverse as cell adhesion, division and shape, embryonic and tissue development, and microbial pathogenesis.

Although powerful and versatile, AFM has several limitations. One issue concerns the technical difficulties associated with live-cell experiments. Although most AFM instruments are affordable and user-friendly, accurate data collection and interpretation are not trivial undertakings, and require extensive expertise and a great deal of patience, especially when dealing with living cells. The most crucial factors that will determine the outcome of an experiment are the quality of the sample and tip-preparation procedures, the accuracy of data collection and interpretation, and the control of the tip and sample integrity during the course of the experiment. Newcomers to AFM will generally need to practise for several months before obtaining good data. In force spectroscopy, current procedures for attaching biomolecules and cells to AFM cantilevers are labour-intensive and require specific expertise that is usually not found in cell biology laboratories. These expertises include chemical functionalization of the AFM cantilever for the attachment of the biomolecule or the living cell in its unperturbed state. In the future, defining simple standardized protocols for tip functionalization, data interpretation and automation of force-spectroscopy analyses, and making them readily available to the cell biology community will contribute to making force spectroscopy accessible for cell biologists.

Another problem with AFM is the rather limited imaging resolution typically achieved on mammalian cells (approximately 50–100 nm), meaning that the individual components of the cell surface cannot be observed. The main reason for this limitation is the very soft and dynamic nature of cell surfaces. Even when imaging at very low forces (<50 pN), the scanning AFM tip deforms the cell surface, increases the tip–sample contact area, and is easily contaminated by loosely bound macromolecules (glycocalyx). These effects limit the achievable resolution and thus, the structural information that can be obtained. One approach to circumvent these problems is to scan a nanopipette over the specimen surface without physical contact, a method known as scanning ion-conductance microscopy.

A third issue is that the interior of living cells cannot be accessed by conventional AFM, because, in essence, it is a surface-sensitive technique. Several approaches are being developed that will allow us to probe intracellular structures. First, by pressing the AFM tip onto the soft cell membrane, cytoskeletal structures can be accessed *in vivo*. These indentation experiments provide either images of the underlying cytoskeletal features, instead of the soft cell membrane, or a measure of the cell's mechanical properties. Second, new instruments are emerging for probing

intracellular structures, such as the photonic force microscope, in which the AFM cantilever is replaced by the 3D trapping potential of a laser focus, and scanning near-field ultrasonic holography, in which nanoparticles are imaged inside cells. Third, the use of a nanoneedle instead of an AFM tip enables researchers to push through the membrane to access the cytoplasm and deliver selected molecules.

Finally, time resolution is a crucial factor that currently limits cell imaging studies using AFM. Acquiring a high-resolution image takes minutes because of the highly corrugated character of the cell surface. This is much greater than the time scale at which dynamic processes usually occur in biology. Remarkable advances have been made in developing scanning probe instruments with increased imaging rates, giving access to unprecedented time scales (millisecond resolution). These ultrafast techniques open up fascinating new perspectives to explore cellular dynamics.

As AFM instrumentation continues to develop, we expect that many pertinent questions will be addressed in cell biology, including the molecular mechanisms guiding the functional state, shape, mitosis, adhesion, sorting and migration of live cells.

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