

Atomic Force Microscopy-Based Force Spectroscopy and Multiparametric Imaging of Biomolecular and Cellular Systems

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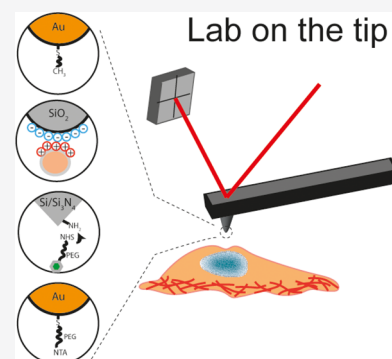


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ABSTRACT: During the last three decades, a series of key technological improvements turned atomic force microscopy (AFM) into a nanoscopic laboratory to directly observe and chemically characterize molecular and cell biological systems under physiological conditions. Here, we review key technological improvements that have established AFM as an analytical tool to observe and quantify native biological systems from the micro- to the nanoscale. Native biological systems include living tissues, cells, and cellular components such as single or complexed proteins, nucleic acids, lipids, or sugars. We showcase the procedures to customize nanoscopic chemical laboratories by functionalizing AFM tips and outline the advantages and limitations in applying different AFM modes to chemically image, sense, and manipulate biosystems at (sub)nanometer spatial and millisecond temporal resolution. We further discuss theoretical approaches to extract the kinetic and thermodynamic parameters of specific biomolecular interactions detected by AFM for single bonds and extend the discussion to multiple bonds. Finally, we highlight the potential of combining AFM with optical microscopy and spectroscopy to address the full complexity of biological systems and to tackle fundamental challenges in life sciences.



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1. INTRODUCTION

Plasma membranes, which permanently adapt their composition (lipids, proteins, sugars, etc.) and rearrange from the nano- to the micrometer scale by modulating the spatial distribution of molecules and domains, are a striking example of the functional complexity of living cells. The processes they execute are highly dynamic and play crucial roles in cellular functions, including signaling, adhesion, migration, sorting, molecular trafficking and transport (ions, water, molecules, peptides), or active cellular intake and secretion. Furthermore, as the plasma membrane provides a physical barrier separating the interior of the cell from the surrounding environment, it is also the first target of extracellular physical, chemical, and biological cues, including heat, chemical and mechanical stress, toxins, hormones, viruses, extracellular matrix proteins, and other cells. Characterizing the biophysical properties of complex biological systems remains a challenging task due to their heterogeneous and highly dynamic properties, as exemplified in many biological processes, including the assembly of functional domains in cellular membranes, actomyosin assembly and contraction, signal transduction, cell adhesion and migration, or adaptation of the cell shape to cell state and function. Moreover, such analysis requires fine-tuning the environmental conditions, because biological systems are sensitive to pH, electrolytes, temperature, humidity, pressure, and oxygen. Addressing this characterization challenge demands quantification of the biophysical properties of molecular and cellular systems at the nanoscale, along with simultaneous mapping of their structural properties.

Attempts to probe the biomolecular interactions and properties of complex biosystems have long been hampered by the lack of suitable techniques. Although optical tweezers,^{1–4} magnetic tweezers,^{5,6} micropipette manipulation,⁷ surface force apparatus (SFA),^{8–10} or flow-induced stretching¹¹ are very sensitive techniques to measure force, those techniques lack the ability to simultaneously quantify and structurally map these interactions over wide force ranges. Invented more than 30 years ago,¹² atomic force microscopy (AFM) combines high-resolution imaging from the micrometer to the (sub)nanometer range with the capability to apply and measure a wide range of forces from pico- to nanoNewtons; this ability is necessary to characterize the broad spectrum of biomolecular interactions occurring in living systems. In this review, we will focus on the exciting potential of multiparametric AFM-based approaches for shining light on the dynamic interplay between the structural, functional, and biophysical properties of biological systems. We further discuss possibilities and limitations of theoretical approaches to extract multiple parameters from AFM-based force spectroscopy and multiparametric imaging. We also showcase how AFM-based chemical imaging can be used to manipulate biological systems and be combined with optical microscopy to address their increasing complexity. Finally, we identify the challenges lying ahead and the need to integrate correlative nano- and microscopy techniques to address the chemical complexity of living biological systems at even higher spatial and temporal resolution, as well as chemical sensitivity.

2. MILESTONES TOWARD ESTABLISHING AFM AS A CHEMICAL IMAGING TOOLBOX

2.1. Invention of AFM and Application as a Nanotoolbox

Since its invention in 1986,¹² the technique and applications of AFM have quickly evolved, developed, and diversified. Originally invented to image solid surfaces at atomic resolution,¹² a basic AFM setup consists of a sharp tip attached to the free end of a microcantilever probe, a cantilever deflection sensor, a piezoelectric sub(nano)positioner, and an electrical feedback mechanism between the deflection sensor and the nanopositioner (Figure 1).¹³ Briefly, the working principle of

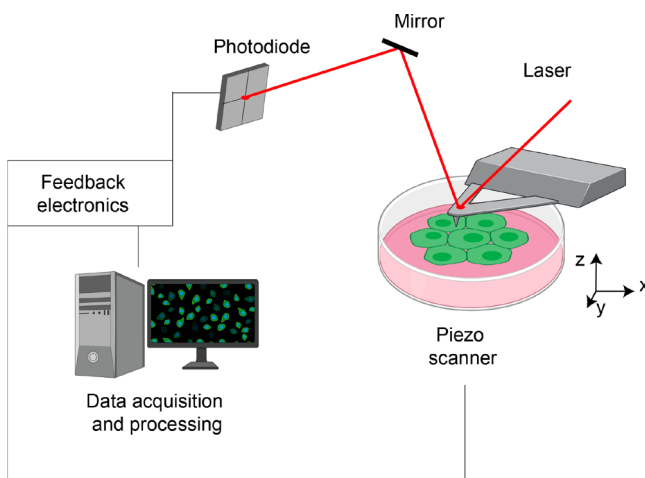


Figure 1. General principle of atomic force microscopy. AFM operates by scanning a very sharp tip across the sample surface, while the force of interaction between the tip and the sample is monitored with piconewton sensitivity. The sample is mounted on a piezoelectric scanner that ensures three-dimensional positioning with high resolution, and the force between tip and surface is monitored by measuring the cantilever deflection using an optical method (laser, photodiode).

AFM is based on monitoring the forces acting between the AFM tip and the sample surface. To monitor the forces acting between the AFM tip and the sample, a laser is focused on the end of the cantilever, from which it reflects to a quadrant, position-sensitive, photodiode, which is used to follow normal and torsional cantilever deflection simultaneously. The cantilever deflection is directly proportional to the force. Originally established to operate in vacuum, the AFM also functions in ambient atmosphere and in liquid¹⁴ at ambient temperature rendering it particularly suitable to characterize molecular and cellular biological systems in their functional state.

Since the very early years of AFM development, the cantilever was also used as a force sensor to measure interactions at surfaces and interfaces, including double-layer forces,^{15,16} surface-induced solvent ordering,¹⁷ and chemical forces, such as hydrophobic/hydrophilic interactions and hydrogen bonds.¹⁸ The applications were based on the acquisition of force–distance (FD) curves, in which the force experienced by a nanoscopic tip mounted at the free end of the AFM cantilever is monitored as a function of the distance between the tip and the sample surface. Such AFM-based applications opened the door to so-called force spectroscopy.

Pioneering measurements of adhesion forces opened the road to map the chemical properties of biological systems with chemically modified AFM tips. Already in the early 1990s, it was

AFM as a Chemical Lab-on-the-Tip

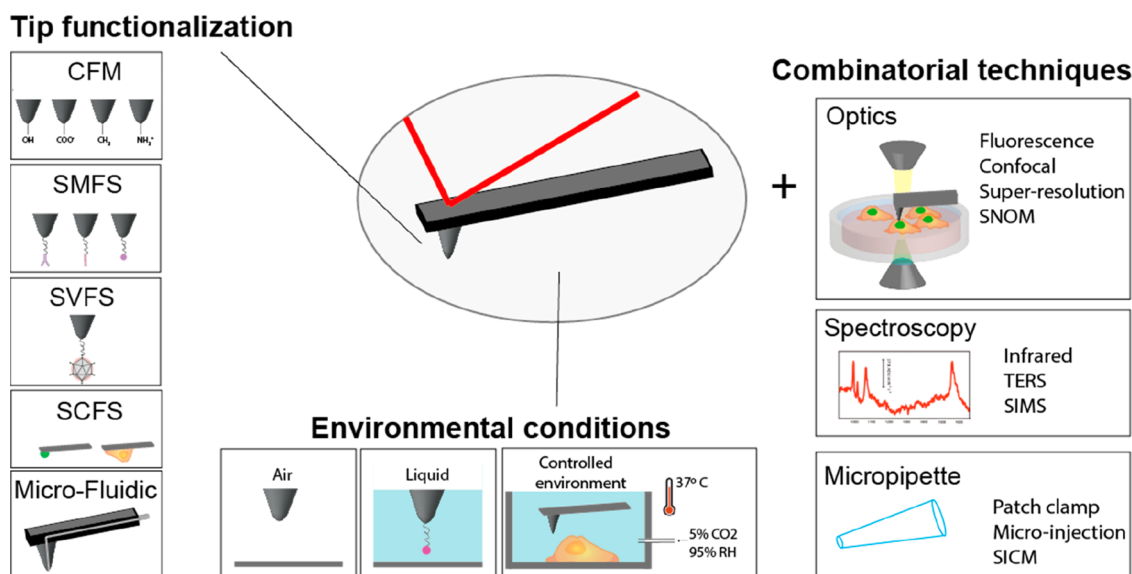


Figure 2. Overview of AFM as a (bio)chemical laboratory on the tip. Highlights of the current capabilities of AFM to study the morphological and biophysical properties of molecular and cellular biosystems. (Bio)chemical functionalization turns the AFM tip into a nanoscopic chemical toolbox. Chemical force microscopy (CFM) uses functional groups at the tip apex to probe hydrophilic ($-\text{OH}$ group), hydrophobic ($-\text{CH}_3$ group), or electrostatic ($-\text{COO}^-/-\text{NH}_3^+$) interactions. Single-molecule force spectroscopy (SMFS) uses the tip derivatized with antibodies, proteins, peptides, drugs, viruses (single-virus force spectroscopy; SVFS), and living cells (single-cell force spectroscopy; SCFS). AFM tips with microfluidic channels were introduced to manipulate individual cells and particles, as well as to inject or aspirate solutions. The precise adjustment of the environmental conditions (including air, liquid, humidity, and temperature) allows characterizing biological systems in the native state. Finally, the combination with complementary techniques allows us to better describe the complexity of living biological systems. These include optical microscopy (fluorescence microscopy, confocal microscopy, super-resolution nanoscopy, and scanning near-field optical microscopy (SNOM)), spectroscopy (infrared spectroscopy, tip-enhanced Raman spectroscopy (TERS), secondary ion mass spectroscopy (SIMS)), and micropipette (patch clamp, microinjection, and scanning-ion conductance microscopy (SICM)) techniques.

demonstrated that forces sensed by functionalized cantilevers can be exploited to simultaneously map the sample topography and to quantitatively extract physicochemical properties of every topographic pixel by recording force–distance curves. Representative examples in material science include mapping the differential adhesion of pristine and lubricant-coated surfaces,¹⁹ force mapping of phase-separated Langmuir–Blodgett monolayers,²⁰ the topographic mapping of elastic, electrostatic, and adhesive properties of polymers,²¹ and the investigation of electrical double-layer interactions on patterned surfaces.²² In early studies, force mapping was limited by long image acquisition times (see next section for technical developments). Developed as an alternative for chemical mapping, friction-force mapping keeps the cantilever in contact with the sample at constant force, while detecting its torsional movement, largely influenced by the chemical identity of the sample.^{18,23} This mode provided an alternative setup for mapping chemical variations of the surfaces. In one of the early examples of this chemical mapping strategy, AFM tips functionalized with self-assembled alkanethiol monolayers terminated with hydrophobic or hydrophilic groups were used to uncover hydrophilic and hydrophobic domains on topographically smooth surfaces.²³

Similar concepts and approaches were transferred to characterize biomolecular interactions by AFM. Thereby the exceptional signal-to-noise ratio, high spatial resolution, and compatibility with liquid environments of AFM provided unprecedented opportunities. Operating the AFM in the single-molecule force spectroscopy (SMFS) mode with biomolecule-functionalized tips provided invaluable insights into how molecular recognition contributes to the regulation of

biological systems. In this respect, the avidin–biotin interaction with its exceptionally high binding affinity was extensively used for proof-of-concept,^{24,25} setting the basis for the theoretical and technological development of SMFS. Several experimental setups were designed. Pioneering reports include for example the study of rupture forces between a biotinylated glass microsphere mounted at the AFM tip and surface immobilized streptavidin²⁴ and of the interactions between avidin-functionalized AFM tips and biotinylated agarose beads,²⁵ in which the multiplicity of binding was modulated by blocking biotin with avidin. These approaches were then applied to the investigation of antibody–antigen interactions, which required researchers to overcome difficulties that were different from those encountered with the biotin–avidin system due to the lower binding strength (i.e., rupture force), increased heterogeneity, and lower binding affinity. These challenges gave a decisive push toward the development of improved AFM cantilevers, tips, and sample functionalization strategies to fully explore the potential of SMFS. At the beginning of such applications of SMFS, it was difficult to discern nonspecific from specific interactions between the AFM tip and biological samples. Such nonspecific interactions mostly originate from the direct contact between AFM tip and sample, commonly at no tip–sample separation distance. A promising solution to discern specific from nonspecific interactions was developed by functionalizing AFM tips with hydrophilic, flexible linkers of longer length and lower packing density and to tether ligands or receptors to the free ends of the linkers. Upon separating the tip from the sample, the nonspecific interactions may have remained; however, the specific interactions were now detected in a region

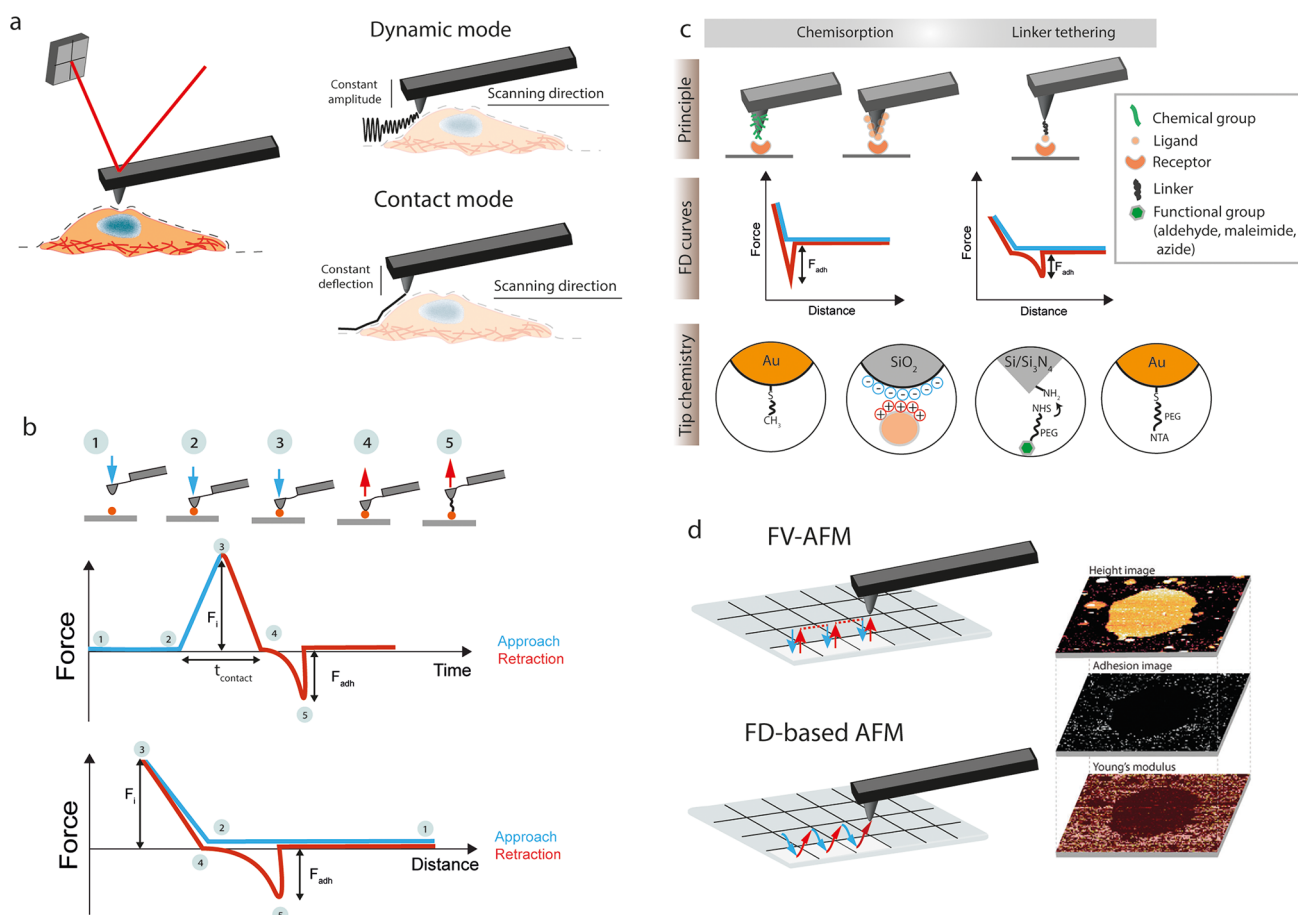


Figure 3. AFM-based characterization of topography and (bio)chemical properties of biological systems. (a) Principle of AFM contouring (dashed line) biointerfaces (here a mammalian adherent cell). The stylus of the cantilever is raster scanned across the sample to record topographic information. In the dynamic mode, AFM oscillates the cantilever close to or at its resonance frequency, while maintaining a constant oscillation amplitude, and the stylus only touches the sample surface intermittently. In the contact mode, AFM keeps the cantilever deflection constant (constant force) by adjusting the distance between stylus and sample. (b) Examples of force–time (FT) and force–distance (FD) curves. The AFM tip, initially away from the surface (#1), is brought into contact (approach curve in blue) with the surface (#2) to which it is pressed until reaching a certain force threshold (#3). Upon retraction (retraction curve in red) (#4), adhesive forces (F_{adh}) are observed (#5) at a separation distance depending on the presence of a polymeric linker tethering a molecular probe of interest (orange circle) to the AFM tip. (c) Protocols to functionalize the AFM stylus with chemical groups, ligands, or receptors through chemisorption or linker strategies are reviewed in the text and linked to the shape of FD curves expected. The first line presents a schematic of the principle of chemisorption (i.e., adsorption/physisorption of chemical groups or ligands directly to the tip) vs linker tethering (i.e., covalent attachment of the ligand through a linker). The second line highlights how the linker spacer will influence the position of the adhesive peak assessing the specificity of the interaction when using a linker. The third line provides details on the chemistry available to functionalize tip with chemical group, physisorbed ligand, or tethered ligands using PEG linker. (d) Force–volume (FV)-AFM and FD-based AFM can be used to contour the sample topography while measuring mechanical (here Young's modulus) and adhesive properties. For each pixel of the topography, the tip is approached and retracted using a linear (FV-AFM) or oscillating movement (FD-based AFM). Panel (d) has been adapted with permission from ref 59. Copyright 2018 Royal Society of Chemistry.

of the FD separated from the nonspecific interactions by the length of the stretched linker. This procedure for functionalizing AFM tips became the gold standard for studying ligand–receptor interactions using SMFS and other advanced AFM applications.^{26,27} Besides addressing the problem of steric effects, flexible linkers also impart greater orientational sampling to the biomolecule tethered to the AFM tip and hence increased binding probability. Furthermore, the characteristic nonlinear stretching of the linker before rupturing the ligand–receptor bond allows us to identify and analyze specific bond rupture events by classical polymer extension models such as the worm-like chain (WLC) model.²⁸ Other pioneering SMFS studies focused on surface opsonization,²⁹ cell adhesion by proteoglycans,³⁰ and DNA binding.³¹ Moreover, the development of

mechanical investigations of protein structure through protein stretching and unfolding began.^{32,33}

Besides using the AFM as a nanoscopic imaging tool or a nanoscopic force spectroscopy tool, both AFM modes can be combined to image a biological system at work and to simultaneously detect, quantify, and topographically map nonspecific or specific biomolecular interactions (Figure 2). In one of the first studies in this field, the acquisition of force curves while scanning lysozyme-functionalized surfaces enabled the acquisition of topography, adhesion force, and retraction velocity (related to viscosity) by postprocessing.³⁴ By functionalizing the tip with a specific binding partner for the biomolecule immobilized on a support, the approach was further advanced by “affinity imaging”,³⁵ which demonstrated the possibility of identifying and localizing specific biological

interactions while simultaneously acquiring the topography and mechanical properties of a sample.

The pioneering application of affinity imaging to red blood cells³⁶ opened avenues to development of AFM-based chemical and mechanical mapping applications on live cells. Exploiting the high specificity of a lectin for glycolipids of the membrane of group A red blood cells, correlating topography and affinity-based maps became possible, and discriminating between mixed populations of red blood cells having different groups became straightforward.³⁶ Force mapping was soon applied to a variety of bacterial^{37,38} and mammalian cells.^{39,40} In parallel, dynamic recognition modes started to be developed and applied to biological samples, the most prominent example being topography and recognition imaging (TREC) in which topography and molecular recognition signals are extrapolated from different regions of a magnetically driven cantilever oscillation cycle.^{41–44} High-speed FD curves were measured using torsional harmonic cantilevers, enabling the exploration of single-molecule processes on the microsecond time scale, as exemplified by the well-studied biotin–streptavidin complex.⁴⁵

Force spectroscopy and affinity mapping modes have been applied straightforwardly to study live prokaryotic cells thanks to their relatively high membrane stiffness and easy-to-implement native environments. However, live mammalian cells notoriously required much more controlled environments to maintain their homeostasis over timeframes required for AFM scanning, which strongly limited AFM-based live mammalian cell studies in the early years. Although protocols such as gentle cell fixation have been used to partially circumvent the issue,⁴¹ such experimental conditions are not fully representative of physiologically relevant scenarios, which restricts their biological significance. The development of environmental chambers to keep cells alive in physiological conditions for several days was a key-breakthrough toward AFM studies of live mammalian cells.⁴⁶ In parallel, the past decade has witnessed a series of instrumental developments in multiparametric AFM imaging, which has significantly reduced data acquisition times while allowing the multiparametric quantification of adhesion forces and manifold mechanical properties.⁴⁷ These advancements, together with the growing tendency to couple AFM setups to optical microscopy, such as fluorescence, confocal or super-resolution microscopy, currently enable biological questions to be approached with an increased level of detail and to further boost the realm of AFM-related biological applications.

In addition to acting as a probe of mechanical properties, the functionalized tip of the AFM cantilever offers the enticing possibility of applying mechanical forces to individual chemical bonds or enzymes, thus serving as an *in situ* actuator that is able to trigger (bio)chemical reactions, induce structural changes of cells and molecules, or control biological processes at the nanoscale.^{48,49}

2.2. AFM-Based Characterization of (Bio)chemical Properties

Today, AFM stands out among other scanning probe techniques as a uniquely suited and versatile tool due to its simple working principle and broad applicability:^{50,51} it relies on the use of a tiny tip mounted on a soft cantilever to detect localized interaction forces with piconewton sensitivity and Ångström precision (for further details on AFM working principles, please refer to refs 51–58). Initially developed as an imaging tool, meant to overcome inherent limitations of optical, electron, and scanning tunneling microscopy, AFM rapidly opened new horizons by

providing a variety of possibilities to physically or (bio)-chemically modify the tip and to manipulate individual biomolecules and chemical bonds.

As mentioned above, the working principle of AFM is based on monitoring the forces between the tip and the sample surface. Typically, these forces are measured via the vertical deflection of the cantilever (see ref 12, 54, and 55 for detailed information). In the imaging mode, AFM raster scans the tip across the sample surface and contours its topography, yielding the three-dimensional (3D) sample surface (Figure 3a). In the force spectroscopy mode, the tip is approached toward and retracted from the sample in a controlled manner. Cantilever deflection versus cantilever displacement are then converted into force versus distance (FD) or versus time (FT) curves (Figure 3b). An adhesion force between the tip and surface results in a downward deflection upon cantilever retraction, and with increasing deflection the adhesive bonds are increasingly tensed until they break. Hence, the AFM tip can probe nonspecific and specific biomolecular interactions, including those between biological macromolecules (proteins, lipids, nucleic acids, sugars) or receptors and ligands. At the single molecule-level, such interactions can be perceived as the sum of multiple noncovalent interactions acting in a concerted manner (e.g., hydrogen bonds, van der Waals forces, hydrophobic effects, π – π stacking, etc.), and their stability and lifetime can be determined. The strength of the single-molecule approach is that the FD or/and FT curves track individual dissociation events, intermediates, kinetics, and pathways, and can thus probe the heterogeneity of coexisting biomolecular populations. Such detailed insights are commonly difficult or impossible to characterize using biochemical bulk assays.

2.3. Single-Molecule Force Spectroscopy

AFM-based single-molecule force spectroscopy (SMFS) stands out among the fast growing single-molecule techniques as an ideal method for measuring intra- and intermolecular forces with high spatial resolution and force sensitivity ranging from ~ 1 pN to 100 nN.^{60,61} Nowadays, SMFS allows researchers to tackle a wide range of biological systems, characterizing the chemical and mechanical interactions contributing to cell adhesion, sorting and mechanics, membrane assembly, protein (un)-folding pathways, enzyme kinetics, ligand–receptor (un)-binding, and DNA mechanics.⁵³ In its earliest development, SMFS was setup to probe individual ligand–receptor pairs, including the widely studied avidin–biotin bond,^{24,25} complementary strands of DNA,³¹ and antibody–antigen²⁶ bonds, which were all grafted at low densities to the AFM tip and sample surface (Figure 3c). These pioneering experiments established the framework for subsequent SMFS studies by defining the prerequisites for measuring specific binding forces between single ligand–receptor pairs. These prerequisites include the need to (i) reduce nonspecific interactions between the tip and sample and (ii) use molecular linkers to tether the binding partners in order to provide them with sufficient flexibility for their interaction to be unperturbed by the linker and simultaneously reveal the “fingerprint” of a force that is specific (Figure 3c).^{62–66}

2.4. Force-Volume-AFM

AFM tips usually have small radii of curvature (typically in the range 1–100 nm) that can be positioned with nanometer accuracy above the biosystem of interest to record biomolecular interactions of a particular structural or morphological region. Thereto, SMFS and AFM imaging can be combined to record

FD curves for every pixel of the resulting sample topography. This combination of both AFM modes, which provides topographical interaction maps of the biological sample, is also known as affinity imaging AFM⁶⁷ or force-volume (FV)-AFM (Figure 3d). In FV-AFM, the tip-sample displacement is modulated by a triangular waveform. A topographic image is combined with the acquisition of FD curves at each pixel of the image. The resulting 3D interaction maps contrast the occurrence and magnitude (or time) of (non)specific interaction forces. As a proof-of-concept, the approach has been introduced by imaging a streptavidin pattern with a biotinylated tip and correlating topography and adhesion maps characterizing the rupture of single streptavidin-biotin bonds.⁶⁷ Similar experiments have used AFM tips functionalized with antibodies to map the distribution of a substrate covered with purified intracellular adhesion molecules (ICAM-1).⁶⁸ The ability to map the distribution of specific ligands at high-resolution has rapidly made FV-AFM a tool of choice for investigating surface receptors of living cells.^{69,70}

2.5. FD Curve-Based AFM

Despite its strengths, FV-AFM has drawbacks, including the relatively high forces applied during topographic imaging, long acquisition times (~15–90 min), and poor lateral resolution (>20–30 nm). Moreover, artifacts can be introduced when using high velocities due to discontinuities at the turning point between the approach and the retraction segments. These limitations triggered further developments and the introduction of sinusoidal waveforms as an alternative to the triangular waveforms characteristic of FV-AFM. With the implementation of FD curve-based AFM (FD-AFM), progress in terms of force control and data acquisition speed has allowed unprecedented progress in structurally mapping (bio)chemical interactions with molecular resolution.^{25,31,32,67,70–75} FD-AFM is an off-resonance mode, which oscillates the cantilever in a sinusoidal manner well below its resonance frequency, while the sample surface is contoured. For every pixel of the resulting sample topography, at least one approach-retraction cycle of the oscillating cantilever is recorded, from which multiple parameters can be extracted including sample height, Young's modulus, deformation, repulsion, and adhesion (Figure 3d). Because of its relative ease of use, FD-AFM quickly allowed the *in situ* characterization of chemical properties of complex living biological systems.^{59,76–92} FV-AFM and FD-AFM are also extensively used to map mechanical properties of biological samples;^{47,54,93} however, a full coverage of recent works covering nanomechanical measurements is beyond the scope of this review.

3. PRINCIPLES AND LIMITATIONS OF (BIO)-CHEMICAL AFM IMAGING

3.1. Physiologically Relevant Conditions and Sample Preparation

While AFM can operate in a wide range of conditions ranging from vacuum to liquid, key parameters should be carefully controlled to ensure the integrity of biological systems. When working on purified samples, such as isolated soluble proteins, nucleic acids, or enzymes, AFM measurements should be carried out in physiological solutions (pH, electrolyte) and temperature, while the immobilization strategy of the biological sample should not interfere with its functional state.^{94,95} Whereas water-soluble proteins may be adsorbed or tethered directly to a support, membrane proteins must be either reconstituted into

lipid membranes (lipid bilayers, liposomes or nanodiscs) or extracted in native cell membrane patches to provide physiologically relevant conditions that enable observing them at work.^{96,97} Because detergent-extraction approaches destroy naturally associated lipid bilayers of membrane proteins, alternative detergent-free approaches, based on nanoparticles⁹⁸ or membrane scaffolding proteins,⁹⁹ have been introduced to maintain their functional state during AFM measurements.¹⁰⁰

To characterize mammalian cells in the native state by AFM requires caution, because their homeostasis is governed by a fine-tuning of physical and chemical conditions maintained usually by the living system including many variables, such as pH, salts concentration, sugar, or oxygen. Therefore, evaporation of the buffer solution during AFM experiments should be avoided as it affects the osmolarity and pH of the medium, which can reduce cell viability. HEPES or carbonate buffers are widely used to maintain physiological pH despite changes in CO₂ concentration. However, phototoxic effects of some buffers should be accounted for. For example, HEPES buffer greatly increases the sensitivity of the cell culture media to phototoxic effects induced by light exposure,^{101,102} and mammalian cells start degrading and apoptosis after being exposed for a few hours to fluorescent light. This detrimental effect on the cell viability can be circumvented using an environmental AFM chamber that controls temperature (37 °C), CO₂ concentration (5% CO₂ in air), and relative humidity (>95%) to maintain cells in incubator-like conditions for extended periods of time.⁷⁷ It is also important to carefully choose the correct immobilization procedure for cells to avoid, alternatively, their detachment from the support and their functional impairment. Several methods have been established to guide the attachment of cells to AFM supports, including adjustment of electrostatic interactions,¹⁰³ coating the support by lectins or extracellular matrix proteins,¹⁰⁴ mechanical trapping,¹⁰⁵ or biotinylation of cell surface proteins.¹⁰⁶ When choosing an immobilization strategy, the user should keep in mind that this might affect the native state, structure, or functionality of the cell. For example, concanavalin A is known to elicit changes in the functional state of mononuclear phagocytes and lymphocytes,^{107,108} and the mechanical trapping of living cells can affect their mechanical properties.¹⁰⁹ Immobilization may be also facilitated by underpressure (suction), by, for example, using cantilevers with integrated microchannels,^{110,111} although cells can change state and adjust properties in response to pressure changes.¹¹²

3.2. Selection of AFM Cantilever and Tip

The choice of the AFM cantilever and tip is fundamental to detection of biomolecular interactions with single-molecule sensitivity. This choice is intrinsically linked to the characteristics of the investigated system, so an *a priori* evaluation of the type and range of the interaction forces, the desired lateral resolution, the size of the topographical features and kinetics should be carried out. Probing typical potential energy gradients of intermolecular interactions (10^{-12} – 10^{-7} N)¹¹³ requires using relatively soft AFM cantilevers with spring constants in the range of 0.01–1 N m⁻¹. For single-molecule studies, the time scale of conformational rearrangements or association–dissociation events will determine the resonance frequency f_R and quality factor Q of the cantilever to be chosen. For example, to study highly dynamic macromolecular complexes in the sub μ s range (i.e., protein conformational changes) requires the use of overdamped ($Q < 0.5$) fast cantilevers ($f_R > 100$ kHz). At such time ranges, underdamped cantilevers ($Q > 0.5$) oscillate, which

potentially introduces subtle conformational changes and affects the dynamics of the observed macromolecular complex.⁹¹ Conversely, if the goal of the experiment is to measure the mechanical properties of the sample, the spring constant of the cantilever should be similar to the stiffness of the sample. The AFM tip radius should be chosen depending on the application. For example, a sharp tip, having a radius of 2–10 nm, which reduces the probability that more than one molecule attaches to the functionalized AFM tip apex, may be chosen to measure the interactions of single molecules. In addition, sharp tips are required to collect AFM topographs at high resolution. However, pushing a sharp tip to a cell surface easily penetrates the cell. Thus, tip radii approaching several tens to hundreds of nanometers^{114,115} (tip to beads) may be applied to characterize the properties of larger surface areas such as of tissues or organs, albeit at a much lower resolution.

3.3. AFM Modes to Topographically Map Biomolecular Interactions

In AFM force-spectroscopy, the stylus is sequentially approached and retracted from the surface while monitoring the variation of the cantilever deflection (hence, the force) with respect to the tip–sample distance. Each approach–retract cycle generates force curves with piconewton sensitivity that can be plotted as a function of distance (FD curves) or of time (FT curves). Several regions can be distinguished in an FD curve, which can be associated with a variety of sample properties. (i) At the beginning of the approach cycle, the tip–sample distance is high enough for the cantilever deflection to be in an equilibrium, not interacting position. (ii) With the decrease of the tip–sample distance, various long- and short-range interactions start to be experienced by the tip. These interactions include electrostatic, double-layer and van der Waals forces, as well as surface-induced solvent ordering at short ranges, and their entity is largely dependent on the experimental conditions, notably the solvent (e.g., air or liquid, electrolyte concentration, solvent molecular size). This part of the curve also contains mechanical information, such as indentation, which becomes relevant in the case of soft samples. (iii) As the tip–sample distance is reduced further, the gradient of interaction force exceeds the cantilever force constant, causing the tip to jump into contact with the surface. (iv) The positive deflection of the cantilever in contact with the surface provides information on the sample elastic properties. (v) Upon retracting the tip, a negative deflection of the cantilever is observed if adhesive forces between the tip and the sample are established, until the restoring force of the cantilever prevails and the contact ruptures. Many applications focus on this region of the force–distance curves. By functionalizing AFM cantilevers with ligands or cells, adhesion forces are used to study chemical or biological bonds (single-molecule force-spectroscopy, SMFS), protein unfolding, and cell adhesion (single-cell force spectroscopy, SCFS). (vi) The cantilever comes back to its not interacting, equilibrium position.

In less common applications, the AFM tip is used as a force sensor in height clamp and force clamp modes. In height clamp mode, the tip is brought in contact with the sample and kept at a constant height, while monitoring the cantilever deflection and hence the force, which reflects the mechanical response of the sample. In force clamp mode, the tip is brought into contact with the sample, and the force is kept constant to monitor height changes. Among other applications, these modes have been used to monitor enzyme activity,¹¹⁶ to quantify the lifetime of

bonds,¹¹⁷ and to monitor biological responses on living cells *in situ*.¹¹⁸

A natural extension of force spectroscopy and the first important step toward the combination of AFM force spectroscopy and topography came with the development of force-volume mode (FV-AFM), in which the approach–retract movement of the cantilever in the *z*-direction is coupled to the *x*–*y* scanning.^{119,120} The resulting cantilever rectangular movement leads the simultaneous acquisition of force curves for each pixel of the scanned area and reconstruction of the topography of the sample. With the possibility of varying independently several experimental parameters, such as the tip approach and retraction velocities and the tip–sample contact time, this mode allows the acquisition of quantitative information of the sample topography on a pixel-by-pixel basis. Practically, however, the relatively high lateral forces applied during topographic imaging, long acquisition times (~15–90 min), and poor lateral resolution (>20–30 nm) pose some limitations to the applicability of this AFM mode for high resolution imaging.

3.4. Setting up AFM to Measure (Bio)chemical Parameters

Several commercially available FD-AFM modes were introduced over the past decade and differ in the way the cantilever or the sample are moved to record FD curves and reconstruct topographs (for a review please see ref 52). As mentioned above, the FD-AFM modes increase the sampling rate (i.e., the number of FD curves recorded per second) to ~0.1–10 kHz by vertically moving the AFM tip at speeds of up to 10–1000 $\mu\text{m s}^{-1}$ or even faster in high-speed force spectroscopy.^{45,121–123} While higher tip speeds enable faster data acquisition, they show drawbacks since they mostly probe biomolecular interactions in (far) out-of-equilibrium regimes. For example, too short tip–sample contact times can limit the formation of bonds (or interactions) having low association rates, whereas higher retraction speeds may prohibit intermediate states that would require conformational rearrangements to dissociate the bond.¹²⁴ Besides FD-AFM, other AFM modes can contribute complementary insight toward characterizing the complexity of biomolecular interactions. In AFM-based force-clamp spectroscopy, an active feedback maintains the force between, for example, a ligand–receptor pair at a constant value to measure the dissociation and association events of the bond.^{125,126} Force-clamp AFM has also been widely applied to study the mechanical un- and refolding of proteins,^{126–129} chemical reactions including formation,¹³⁰ cleavage of disulfide bonds,¹³¹ mechanical strength of catch bonds,¹³² the rupture of lipid bilayers,¹³³ and formation of membrane tethers.¹³⁴

3.5. Sensing Nonspecific Interactions

Nonspecific interactions are universal interactions for all potential targets and are usually characterized by low activity.¹³⁵ Despite their ubiquity in biology and chemistry, nonspecific interactions are generally overlooked, and a fundamental understanding and characterization of nonspecific interactions is lacking. Molecular chaperones have evolved to resist nonspecific interactions guiding proteins from a misfolded or unfolded conformation. Other examples include the electrostatic field and potential generated by transmembrane pores, and the adhesion of micro-organisms. For example, hydrophobic interactions play key roles in guiding protein folding, micelle formation, or cell adhesion. Historically, hydrophobic forces have been investigated with the SFA, but AFM has opened the possibility to characterize these forces at high spatial resolution

(~10 nm) and to unravel the hydrophobic properties of a variety of surfaces including colloids, oligopeptides, and living cells. To probe short-range hydrophobic forces at high spatial resolution, and to exclude other longer-range interactions such as electrostatic interactions, which may contribute, AFM tips carrying chemisorbed methyl-terminated monolayers of alkanethiols or ethoxysilanes can be used.^{62,63,136} In a recent work, multifrequency atomic force microscopy (MF-AFM) was used to topographically map the hydrophobic and hydrophilic regions and the mechanical properties of the membrane protein aquaporin-0 (AQP0).¹³⁷ Imaging using the fundamental eigenmode of the AFM cantilever highlighted a structural region that surrounds AQP0 tetramers and exposes a higher affinity for water, while the response of the second eigenmode, which is dominated by the mechanical response of the protein, provided submolecular details of the AQP0 surface and subsurface.¹³⁷

Electrostatic charges can be probed using bare¹³⁸ or functionalized tips.¹³⁹ By fine-tuning of the pH and salt concentration, negatively charged silicon tips were used to detect chemical and physical properties of the native purple membrane from *Halobacterium salinarum*^{140,141} and the native pore forming outer membrane protein F (OmpF) at sub-nanometer resolution.¹⁴² Such studies show that electrostatic interactions can be detected by AFM as apparent height differences or deformations of membrane proteins. Insulated conducting cantilevered nanotips together with a two-chamber recording system can be used to sense ions on biological membranes at high resolution¹³⁸ (<20 nm). This feature is of particular interest to detect ion currents (in the pA range) and topography at single-channel resolution.¹⁴³ In another study, a solid-state nanopore integrated in an AFM tip was used to sense molecule secretion and ion channel activity inside and outside of a cell in real time.¹⁴⁴

3.6. Sensing Specific Biological Interactions

To study specific biomolecular interactions, the AFM tip needs to be functionalized. Such chemical functionalization with a molecule of interest opens avenues to detect specific forces acting between single receptor–ligand pairs, biomolecules, or synthetic polymers. The unique ability of AFM to apply directional forces offers exciting possibilities to mechanically stress specific biological bonds along certain reaction coordinates until the bond ruptures. The application of mechanical stress reduces the time scale at which a bond can dissociate and associate along a given reaction coordinate.¹⁴⁵ Thus, probing the dissociation and association rates provides an approach to measuring the kinetic and energetic properties of the bond (see below for details). A critical step in such an experiment is being able to distinguish specific interaction forces from nonspecific ones.

A vast toolbox of chemical functionalization procedures is now available and allows researchers to isolate specific forces while minimizing nonspecific interactions in SMFS. Hereafter, the major AFM tips functionalization procedures are discussed (Figure 3c). For a specialized discussion on coupling strategies and recent developments, the reader is referred to previous works.^{146,147} The simplest functionalization strategy involves the chemical attachment of a molecule of interest to the AFM tip using short rigid linkers and blocking the rest of the tip surface from unspecific interactions with spacer molecules. Depending on the functional groups in the molecule and AFM tip material, which usually is made from silicon oxide/nitride or gold, a

variety of alkanethiols or polymeric linkers are used.⁶⁴ The presence of only one or a few molecules at the tip apex is critical to ensure that single molecules are probed. This can be ensured by adjusting the molecule/spacer ratio to about 1:100 or 1:1000. Evidence that the experiments are well-performed at the single-molecule level is to test if further dilution of the ligand onto the tip or/and the substrate only reduce the binding frequency without altering the binding force. Possible drawbacks of using short and rigid linkers include the difficulty in separating specific from nonspecific interactions and the limited orientational freedom of the molecule tethered to the AFM tip. In order to increase the orientational freedom of the molecule, elegant functionalization approaches have been developed in which the molecule of interest is tethered to the AFM stylus via a flexible polymeric linker (usually polyethylene glycol (PEG)).⁶⁵ The higher flexibility enhances the conformational freedom of the tethered molecule and thus maximizes its binding probability. Of equal importance, the length of the linker can be used to discard nonspecific from specific interaction forces by separating the pulling distance of a specific unbinding force from nonspecific forces, which mainly occur in the contact region of the tip and sample. Besides providing a signature for the specificity of the interaction, flexible linkers can also provide information about the number of bonds established in parallel.^{66,148} While most of the tethering options result in a random orientation of the molecule onto the AFM tip (through covalent attachment via amino residues or biotin tag randomly distributed), a controlled orientation can be achieved using adequate functionalization strategies. Taking advantage of the natural low abundance of surface-exposed cysteine residues, thiol-maleimide chemistry, represents one commonly used and easily implemented method to achieve site-specific and covalent immobilization, without the need for non-natural amino acids addition.^{147,149–152} Alternatively, a strategy inspired by metal ion affinity chromatography, nitrilotriacetic acid, and a tetradentate chelating of Ni²⁺ ions is used to immobilize histidine (His)-tagged proteins.^{59,153,154} Although being oriented, this approach has the disadvantage that it can only probe interactions being weaker than the immobilizing His-tag. To circumvent this limitation, novel strategies appeared using for example (i) the transpeptidase sortase A from *Staphylococcus aureus* to catalyze the formation of an amide bond between the carboxyl group of the threonine in a LPXTG motif and the amino group of the glycine in a pentaglycine motif,^{59,155} or (ii) the copper(I)-catalyzed/dibenzocyclooctyne copper-free alkyne–azide cycloaddition “click” reaction.^{156–158} Both strategies allow the tethering of molecules to the AFM tip with controlled orientation, enabling a well-defined geometry during binding and unbinding studies. The precise control over the geometrical loading configuration can result in dramatic effects on the observed mechanical response of protein domains and receptor–ligand complexes.^{87,159,160} Depending on the biological system studied, it may be important to study the native pulling geometry experienced by the protein *in vivo*. In addition to further validating the specificity of the interactions, blocking molecules (antibody, antagonist, free ligand) can be injected, although these molecules are not always available.

For some applications, it may be helpful to include a molecular fingerprint within the linker whose characteristic FD pattern allows the unmistakable identification of the unbinding or unfolding event. To this end, some protein domains are inserted into the linker tethering the tip and ligand such as

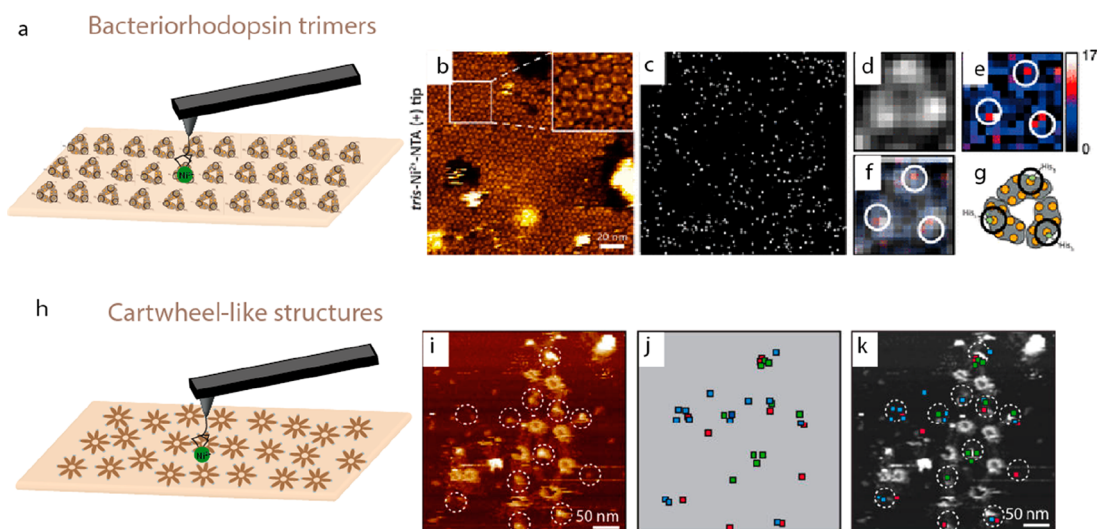


Figure 4. Toward establishing standardized procedures for AFM-based chemical imaging. Biological samples with well-defined lattices can be used as standards to characterize the ability of a tris- Ni^{2+} -NTA-functionalized AFM tips to image and localize specific groups at high-resolution (down to the sub-nanometer scale). Upper panel: High-resolution topography and chemical image of the cytoplasmic surface of purple membrane recorded using a tris- Ni^{2+} -NTA-functionalized AFM tip. (a) Naturally arranged into a hexagonal lattice (~ 6.2 nm lattice constant), each bacteriorhodopsin molecule of the bacteriorhodopsin trimer has been genetically modified to carry five histidine residues (His_5 -tag). (b) The topograph shows the bacteriorhodopsin molecules and trimers at sub-nanometer resolution, while (c) the adhesion map locates specific binding events of the tris- Ni^{2+} -NTA- His_5 interaction. The vertical scale highlights forces ranging from 30 pN (dark gray) to 310 pN (white). (d) The average bacteriorhodopsin topograph and (e) affinity map localize the tris- Ni^{2+} -NTA- His_5 interaction to the N-terminal end of bacteriorhodopsin (f, g). High-resolution of the SAS-6 cartwheel-like structure with rod domains radially emanating from the central ring formed by the N-terminal domains. At the distal end of each rod, the SAS-6 subunit exposes its C-terminus to which six histidine residues (His_6 -tag) were fused. (h, i) Topograph of SAS-6 cartwheel-like structures. The vertical scale ranges from 0 nm (dark brown) to 6 nm (yellow). (j) Localization of specific binding events of the tris- Ni^{2+} -NTA- His_6 interaction by overlapping three consecutive adhesion scans (blue, green, and red dots). (k) Superimposition of topography and adhesion map locates the specific binding events to the rod domains of the SAS-6 subunits at nanometer resolution. The gray scale corresponds to a vertical range of 6 nm. Lower panel: Panels (b–d) and (f–k) are adapted with permissions from ref 78 and 170. Copyright 2017 and 2014 American Chemical Society.

immunoglobulin-like (Ig) domains¹⁶¹ or domain 4 of the F-actin cross-linker filamin from *Dictyostelium discoideum* (ddFLN4).¹⁶²

3.7. Establishing Standardized AFM Procedures

To compare the specific and nonspecific forces detected by chemical AFM, experimental and data analysis procedures must be standardized. A standardized nanomechanical AFM procedure (SNAP) was recently proposed to reliably determine the Young's moduli of soft samples.¹⁶³ The main sources of error in AFM-based nanomechanical measurements are uncertainties in determining the deflection sensitivity and spring constant of the cantilever. To eliminate this uncertainty, the cantilever spring constant is determined by a vibrometer and its deflection sensitivity calculated from the thermal fluctuations of the free cantilever. This reduces the variability in elastic moduli of hydrogels down to 1% and increases the consistency of living cells elasticity measurements by a factor of 2, thus rendering highly reproducible elasticity measurements. An accurate estimation of the spring constant is mandatory in force spectroscopy, and calibration procedures for AFM cantilevers have been extensively compared and reviewed in previous works.^{164–167} For AFM-based molecular recognition studies, bacteriorhodopsin (BR) from *H. salinarum* was used as molecular template because its structure and function have been extensively characterized,¹⁶⁸ and it acts as a model system for high-resolution AFM imaging and force spectroscopy.¹⁶⁹ BR natively assembles in trimers that assemble in highly ordered two-dimensional lattices, called a purple membrane. Fusing a His-tag to the terminal end of each BR renders a well-defined two-dimensional lattice, which allows systematic mapping and structural localization of binding sites at subnanometer precision

(Figure 4a–g). This possibility of engineering molecularly defined standard samples for AFM-based high-affinity and -resolution imaging was recently demonstrated by using tris-N-nitrilotriacetic acid (tris-NTA) functionalized AFM tips to localize tris-NTA- His_5 -tag bonds on BR trimers and to quantify the thermodynamic and kinetic parameters of the Ni^{2+} -mediated bonds.⁷⁸ Similarly, the spindle assembly abnormal protein 6 homologue (SAS-6), a protein involved in centriole duplication that assembles into cartwheel-like structures can be used to standardize molecular recognition measurements (Figure 4h–k).¹⁷⁰ SAS-6 C-terminal domains form ~ 40 nm long parallel two-stranded coiled coil rods that emanate radially from the central ring formed by the N-terminal domains. At the distal end of each coiled coil rod, the SAS-6 subunit exposes its C-terminus to which six histidine residues were fused (C-terminal His_6 -tag). The approach offers the advantage that in principle any tag can be fused to the terminal end of BR, SAS-6, or another structurally well-defined and assembled protein.¹⁷⁰

4. CONTOURING THE BINDING FREE-ENERGY LANDSCAPE OF (BIO)CHEMICAL BONDS

When a bond or molecular complex is pulled apart under various mechanical loads, the bond is shifted from equilibrium to nonequilibrium. The force applied to the molecule of interest increases with time at a rate r termed the force-loading rate. Plotting the forces required to rupture the bond over a wide spectrum of force loading rates shows a crossover from *near-equilibrium* to *far-from-equilibrium* dissociation, followed by ascending regimes of strength.¹⁷¹ This presentation of the rupture force on loading rate is named a dynamic force

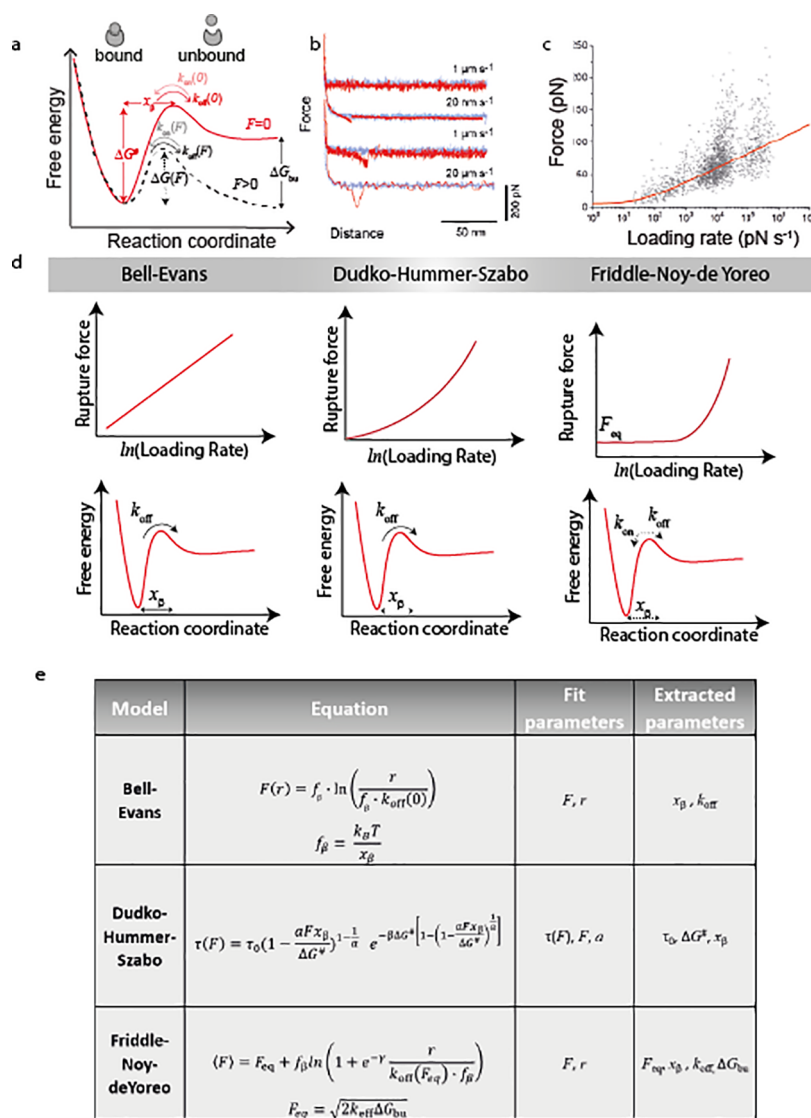


Figure 5. Detecting and extracting kinetic and thermodynamic properties of specific biomolecular interactions. (a, b) Probing the interaction forces of a (bio)molecular bond over a wide range of loading rates allows determination of the parameters describing the free-energy barrier stabilizing the bond. These parameters include the free-energy difference ΔG_{bu} between the bound state and the unbound state, the distance x_β separating the bound from the transition state, and the transition rates k_{on} and k_{off} . To explore a wide spectrum of loading rates, the retraction speed is varied upon recording FD curves. (c) Dynamic force spectroscopy plot of Wnt7b peptide-Reck receptor bonds, as representative example to show the dependence of the rupture force on the loading rate. Each dot represents a single ligand–receptor bond rupture event. The Friddle–Noy–de Yoreo model (red line) is used to fit the experimental data. (d) Several biophysical models are available to interpret DFS data. On the basis of various hypothesis discussed in the text, the resultant theoretical force versus loading rate relationships are schematically drawn. (e) Using the equations summarized in the table, kinetic and thermodynamic parameters are extracted depending on the model. In the Bell–Evans model, F is the most probable rupture force at a loading rate r , k_B is Boltzmann’s constant, T is temperature, x_β indicates the location of the energy barrier, and k_{off} is the off-rate constant at zero force. In the Dudko–Hummer–Szabo model, $\tau(F)$ is the force-dependent lifetime of the bond, a is a scaling factor that specifies the nature of the free-energy profile, $\Delta G^\ddagger$ is the apparent free energy of activation at zero force and τ_0 is the intrinsic lifetime of the bond. In the Friddle–Noy–De Yoreo model, F_{eq} indicates the force at which the dissociation and association are in equilibrium, and $k_{\text{off}}(F_{\text{eq}})$ is the off-rate constant at F_{eq} . Panels a and c are adapted with permission from ref 90. Copyright 2018 American Association for the Advancement of Science.

spectroscopy (DFS) plot and explores the free-energy landscape of the dissociating and associating bond, which is usually inaccessible in bulk assays. Here, we overview the prevalent theoretical frameworks available to analyze the stochastic process of bond dissociation and association and discuss the limitations of some of the biophysical models used to extract the kinetic and thermodynamic parameters of the bond from out-of-equilibrium and close-to-equilibrium AFM-based single-molecule force spectroscopy measurements.^{85,172–177}

4.1. Probing the Kinetics of Single Bonds

From a biochemical perspective, ligand–receptor bonds have limited lifetimes and dissociate under almost any level of force when pulled on for a sufficient period of time. Noncovalent bonds in a receptor–ligand complex are considered as “ideal bonds” if their dissociation lifetimes grow strictly exponentially with the applied force. An ideal bond is modeled as a bound state confined by a single energy barrier located at a fixed distance x_β from the bound state (Figure 5a). The transition between bound and unbound states exhibits, for an ideal bond, first-order

kinetics in their association (k_{on}) and dissociation (k_{off}) rates. The thermodynamic bond strength is usually described as the free-energy difference ΔG_{bu} between bound and unbound state. The rates k_{on} and k_{off} decrease exponentially with the height of the crossed entrance barrier (ΔG_{b}) and exit barrier ($\Delta G^{\#}$), respectively, relative to the unbound state and the primary minimum stabilizing the bond. The difference $\Delta G^{\#} - \Delta G_{\text{b}}$ in free energy barriers equals the binding free energy ΔG_{bw} , while their ratio $k_{\text{on}}/k_{\text{off}}$ refers to the dissociation constant at equilibrium. From a classical mechanics perspective, the strength of an isolated bond is related to the force required to separate the two binding partners. For a macromolecule in solution, the equivalent quantity is the free-energy gradient in the direction of pulling. If one assumes that compared to the motion in this direction all molecular motions and the cantilever response are fast, then the molecular partners should separate “spontaneously” once the externally applied force exceeds the steepest free-energy gradient. Hence, from such a purely mechanical perspective, bond strength would appear to be independent of the height of the free-energy barrier and from features of the free-energy landscape other than the maximum gradient, meaning that some hidden part of the free-energy landscape would appear not to affect bond strength.

Practically, the application of an external force to a ligand–receptor bond adds a mechanical potential that tilts the free-energy landscape and lowers the free-energy barrier to dissociation, thereby increasing the rate of unbinding. Pulling on a bond with forces of a few times the thermal force ($f_{\beta} = k_{\text{B}}T/x_{\beta}$), i.e., the force acting on the bond originating from the thermal fluctuations, will cause a bond to dissociate orders of magnitude faster than it would spontaneously. However, breaking the bond within a very short time ($\sim$ ns) can require the application of, say, 40–50 times the thermal force. The different models presented below enable one to extract the kinetic and thermodynamic parameters of the bond from such *out-of-equilibrium* measurements. To this end, the bond strength is explored over a wide range of loading rates r , either by varying the retraction speed upon recording FD curves (Figure 5b) or by using oscillating modes such as an FD-based AFM mode where the cantilever is withdrawn in a sinusoidal manner. The nonlinear movement of the oscillation probes the bond over a wide range of pulling speeds (up to mm s^{-1} for an oscillation frequency of 0.25 kHz and amplitude of 1 μm). The loading rate is calculated from the FT curve, as the slope of the force before rupture of the bond. Next, the variation F vs r is represented in a DFS plot (Figure 5c). Loading rates typically explored in SMFS vary between 10^1 and 10^7 pN s^{-1} .

4.2. Bell–Evans Model

As with most of the established theoretical frameworks currently used to interpret DFS plots (Figure 5d–e), the Bell–Evans (BE) model is based on Kramers’ analytically tractable reaction rate theory.¹⁷⁸ This theory recovers the Arrhenius dependence of the dissociation rate on the height of the free-energy barrier ($k_{\text{off}} \propto \exp(-\Delta G/k_{\text{B}}T)$) and relies on the assumption that the activation energy is large compared with the thermal energy scale, $\Delta G \gg k_{\text{B}}T = 1/\beta$. Bell introduced the phenomenological description of how an external force pulling on a bond reduces the activation free-energy barrier toward dissociation.¹⁷² According to Bell, the presence of an external pulling force F along the reaction coordinate “tilts” the internal binding potential and reduces the lifetime of the bond.

One assumption of the Bell model is that the distance between the bound state and the transition state along the reaction coordinate, x_{β} , remains constant under the applied external force. The force-dependent unbinding rate is described as

$$k_{\text{off}}(F) = k_{\text{off}}(0) \exp(Fx_{\beta}/k_{\text{B}}T) \quad (1)$$

Further, the external applied force is assumed to be constant in time; however, in AFM experiments molecular bonds are dissociated using cantilevers exhibiting Hookean stretching behavior that exert forces increasing linearly with the retraction distance.

Starting from the Bell model, Evans and Ritchie assumed an external force increasing linearly in time and derived the survival probability of the bond^{145,173,179} (i.e., the probability that the bond is still associated at time t). This led to the derivation of a general expression for the distribution of rupture forces measured by SMFS. Focused on the bond dissociation kinetics, the BE model for a single dominant barrier to rupture predicts that the most probable rupture force F^* (i.e., the mode of the rupture-force distribution) in the thermally activated regime scales linearly with the logarithm of the loading rate as

$$F^*(r) = f_{\beta} \ln \left(\frac{r}{f_{\beta} k_{\text{off}}(0)} \right) \quad (2)$$

The dependence $F^* \approx \ln r$ can take into account nonlinear stretching of elastic force transducers, such as polymeric linkers.^{180,181} The BE model has been widely used to elucidate receptor–ligand unbinding kinetics,^{90,145} protein unfolding,¹⁸² and virus detachment from cell surfaces.^{148,183,184} The BE model does, however, have certain limitations, and deviations from the predicted behavior have been observed with the increasing dynamic range of experiments. In particular, the assumption of a force-independent x_{β} is not always justified at high forces, where the transition barrier and the well necessarily move closer until they eventually merge at a critical force when the barrier to rupture vanishes.

4.3. Dudko–Hummer–Szabo Model

SMFS experiments spanning increasingly wide ranges of r have observed deviations from the BE model, in particular, curved DFS plots and variances in the rupture force that depend on the loading rate.¹⁷⁴ These deviations motivated new interpretations including that multiple transition free-energy barriers are crossed upon dissociating the bond,^{145,185} a shortening of the distance between bound and transition state at high forces,¹⁸⁶ and a nonlinear behavior induced by the force transducer itself^{85,177,187,188} or the transition from a probabilistic thermally activated regime to a deterministic regime.^{121,176} Dudko, Hummer, and Szabo (DHS) revisited the assumption in the BE model that the distance to the transition state, x_{β} , does not depend on the external applied force.¹⁸⁹ Focusing on an external force that is ramped up linearly in time, the DHS model assumes that the free-energy potential well and barrier will eventually merge at a critical force when the barrier to rupture vanishes. The DHS model includes the BE model as a limiting case, but predicts that in general both the logarithm of the lifetime at constant force and the mean rupture force are nonlinear functions of the force and the logarithm of the r , respectively.¹⁸⁹ The DHS formalism adds a parameter to the BE model that enables a smooth interpolation between different potential shapes, which is the apparent free energy of activation $\Delta G^{\#}$. The DHS model was validated as a simple method to determine

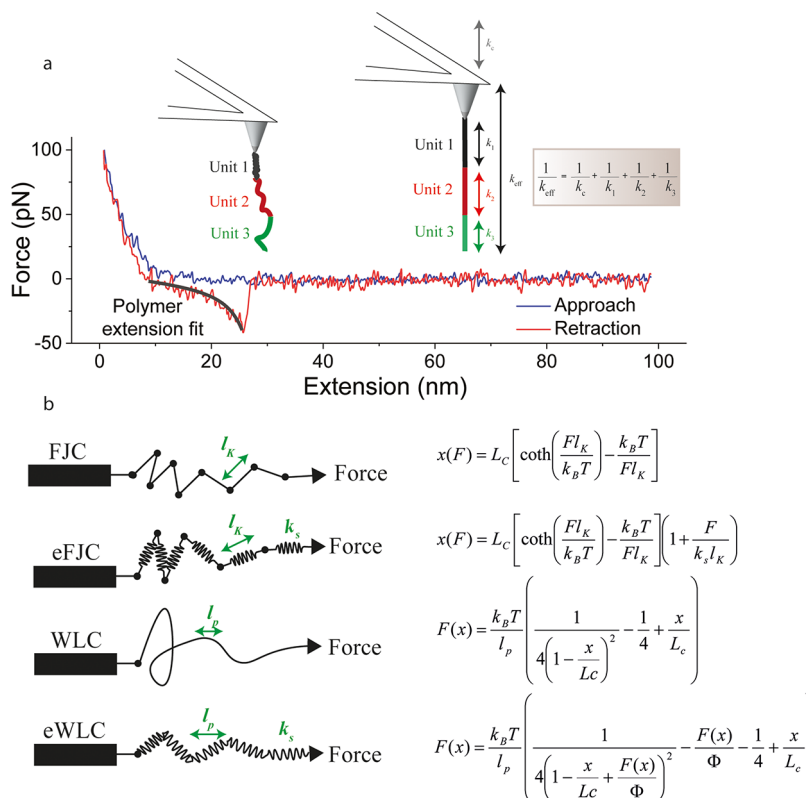


Figure 6. Overview of theoretical frameworks modeling the force extension relationship of complex systems. (a) A typical force–distance curve for stretching a flexible PEG linker tethering a polypeptide-based ligand to an AFM tip. The unbinding event in the retraction curve can be fit with a polymer extension model to estimate the contour length of the complex tethered to the AFM tip using a suitable elastic model. The effective spring constant k_{eff} of the cantilever-PEG-polypeptide linker system is the result of combining the cantilever stiffness k_c and the stiffness of the PEG-polypeptide complex k_{1-3} . (b) Four polymer elasticity models are described: the freely jointed chain (FJC) model assumes a single flexible polymer chain consisting of a series of n rigid segments of Kuhn length l_k connected by freely rotating joints. The extended FJC (eFJC) model assumes that a molecule can be overstretched beyond its contour length at high stretching forces due to an increasingly enthalpic molecular response. An additional segment elasticity parameter k_s to account for the elasticity of individual segments when stretched is introduced in the equation. The WLC model describes the polymer as a homogeneous stiff elastic rod without discrete structure and is characterized by the contour length L_c of the polymer and its persistence length l_p ; the extended WLC (eWLC) model assumes an enthalpic deformability of the chain segments, in the same fashion as described for the eFJC model. As an additional stretching term, the stiffness of the chain k_s is introduced to quantify the increase of the chain extension. Long PEG flexible linkers can be modeled using the eFJC model, while polypeptides can be described well using the WLC model. By combining both models, the force–distance relationship of the PEG-polypeptide linker can be obtained.

force-dependent lifetimes $\tau(F)$ from rupture-force histograms collected at different pulling speeds:¹⁸⁹

$$\tau(F) = \tau_0 \left(1 - \frac{aFx_\beta}{\Delta G^\#} \right)^{1-1/a} e^{-\beta\Delta G^\#} \left[1 - \left(1 - \frac{aFx_\beta}{\Delta G^\#} \right)^{1/a} \right] \quad (3)$$

The scaling factor a specifies the nature of the underlying free-energy profile:¹⁸⁶ $a = 1/2$ corresponds to a cusp-shaped potential, while $a = 2/3$ corresponds to a linear-cubic binding potential. The BE expression is recovered for $a = 1$. The advantage of the DHS model lies in the fact that it largely eliminates the need to make model-dependent assumptions about the functional form of $\tau(F)$. Another advantage is that the DHS model absorbs the influence of flexible polymeric linkers into a force-dependent loading rate,

$$\frac{\nu}{r} = \frac{1}{k_c} + \frac{dl(F)}{dF} \quad (4)$$

where ν is the retraction velocity, k_c is the cantilever spring constant, and $l(F)$ is the force-dependent extension of the linker. The linker extension can be modeled using the WLC or freely

jointed chain (FJC) model or a combination of these, depending on the experimental setup (see Figure 6 for details).^{28,66,190,191}

In summary, the DHS model provides an excellent approximation over a wide kinetic regime of a bond. Despite being developed for irreversible rupture events of the bond, the model is applicable also to reversible transitions, as long as the association and dissociation events can be separated in the analysis process, e.g., based on their molecular extensions.¹⁸⁹ However, the DHS model assumes that a significant barrier $> k_B T$ exists and that the applied force stays below a critical value at which the transition barrier vanishes $F < \Delta G^\# / \nu x_\beta$, which may not be the case under extreme loading rates.

4.4. Friddle–Noy–De Yoreo Model

The BE and DHS models are currently being used to extract kinetic information from SMFS experiments, while fluctuation theorems such as the Jarzynski equality (JE)¹⁹² and its generalization, the Crooks fluctuation theorem,¹⁹³ provide a rigorous theoretical framework¹⁹⁴ for the estimation of thermodynamic parameters.^{195,196} Previous studies have shown that the free-energy difference between the initial bound and the final unbound equilibrium states could be estimated via a nonequilibrium, irreversible process that

connects them, thus bridging the gap between equilibrium and nonequilibrium statistical mechanics.¹⁹⁶ In a recent theoretical approach, the Friddle–Noy–de Yoreo (FNdY) model¹⁷⁴ bridges the gap between the thermal and kinetic regimes and challenges the irreversibility assumptions made by BE and DHS models. The FNdY model predicts that the DFS plot of a ligand–receptor bond attached to a force transducer consists of two primary regimes, a near-equilibrium regime of the bond-transducer system where rebinding events can occur and which is characterized by an equilibrium force F_{eq} and a kinetic regime, which is characterized by a fast rupture of a bond at nonequilibrium.¹⁷⁴ According to this theory, the transition rates of the rebinding and unbinding intersect at F_{eq} :

$$F_{\text{eq}} = \sqrt{2k_{\text{eff}}\Delta G_{\text{bu}}} \quad (5)$$

The mean rupture force is defined as

$$\begin{aligned} \langle F \rangle &= F_{\text{eq}} + f_{\beta} \ln(1 + e^{-\gamma R(F_{\text{eq}})}) \text{ where } R(F_{\text{eq}}) \\ &= \frac{r}{k_{\text{off}}(F_{\text{eq}})f_{\beta}} \end{aligned} \quad (6)$$

γ is the Euler's constant, k_{eff} is the effective spring constant of the transducer, ΔG_{bu} is the equilibrium free energy, and $k_{\text{off}}(F_{\text{eq}})$ is the unbinding rate scaled by the Boltzmann-weighted energy of a spring extended between the barrier location and the relative displacement of the spring minimum under F_{eq} (for further details, see ref 174).

A possible shortcoming of the FNdY model is that a stiff transducer or linker system is assumed, which is not a realistic description of AFM-based force spectroscopy experiments on biomolecular systems or living cells. Therefore, a key point when applying the FNdY model is the accurate estimation of the effective spring constant of the force transducer system, k_{eff} , for a correct calculation of the equilibrium free energy. In a simplistic approximation, k_{eff} is equivalent to the stiffness of the whole force transducer including the spring constant of the cantilever k_{c} and the stiffness of the polymeric linker k_{linker} attaching the binding partner to the AFM tip:

$$k_{\text{eff}} = \frac{k_{\text{c}}k_{\text{linker}}}{k_{\text{c}} + k_{\text{linker}}} \quad (7)$$

In principle, an experimental estimation of k_{eff} can be achieved by reconstructing the dependency of the effective spring constant of the transducer from the rupture event in force–distance curves and extrapolating to F_{eq} . However, values of F_{eq} for most receptor–ligand bonds lie at the lower end of the instrument detection limits (50 pN or below), which makes the extrapolation of k_{eff} values at F_{eq} quite difficult, at least with conventional AFM-based force spectroscopy tools. Therefore, for a rigorous calculation of k_{eff} , the use of appropriate biophysical models that take into account polymeric linkers elasticity and nonlinear stretching behavior is preferable^{28,197,77,191,198} (see Figure 6a). For more complex systems where besides the cantilever and PEG linker the force transducer includes other components such as viruses, nanoparticles, or cell membrane, the estimation of k_{eff} becomes a nontrivial problem, and assumptions have to be made regarding the stiffness of unknown elements (Figure 6b). This estimation can add considerable errors in calculating k_{eff} and upon propagating k_{eff} to estimate ΔG_{bu} .

The extraction of kinetic rate coefficients from DFS on a molecular bond gives (using the above-mentioned theoretical

models) a rate of dissociation, k_{off} , and a pseudo-first-order rate of association, k'_{on} . Both rate coefficients have units of inverse time. To relate the measurements obtained at the single molecule level to bulk measurements, k'_{on} needs to be converted into a concentration-dependent on-rate k_{on} with units of $\text{M}^{-1} \text{s}^{-1}$. We thus need to multiply the pseudo on-rate by a volume, which can be estimated from the effective spring constant k_{L} of the molecular construct with intact bond at zero force as $v = (2\pi k_{\text{B}}T/k_{\text{L}})^{3/2}$. The product $k_{\text{on}} = N_{\text{A}}v k'_{\text{on}}$, with N_{A} Avogadro's number, gives the bimolecular on-rate per mole of ligand. The dissociation constant for the binding reaction then becomes $K_{\text{D}} = (k_{\text{off}}/k_{\text{on}}) = k_{\text{off}}/(N_{\text{A}}v k'_{\text{on}})$. In this form, K_{D} has the units of M. The FNdY model has been extensively used in recent FD-based AFM studies mapping (bio)chemical information and where nonlinear DFS plots were obtained.^{77,78,191,199}

4.5. Dissecting Multivalent Interactions

An ideal SMFS experiment probes a single bond. However, in practice, despite careful sample preparation and tip functionalization, a percentage of the rupture events detect multiple bonded structures. These multivalent interactions can prove difficult or impossible to interpret when the partition of force and degree of cooperativity among the binding sites is unknown. Fortunately, biophysical models have been developed to account for multiple bond formation assuming generic types of multivalent interactions. These predictions help discriminate if the bonds are correlated or not, i.e., if all the bonds are equivalent or if the first binding event induces an allosteric modulation of the second binding event.^{174,198,200} In a seminal work on dynamic force spectroscopy of multiple bonds,¹⁷¹ Williams and Evans describe three simple mechanical scenarios for multiple bonds: (i) a *series* of bonds, where the force is experienced fully by each bond in the system; (ii) a *zipper*, in which bonds are connected in series but the force is sensed by a lead bond: once this bond fails, the force load propagates to the next bond in a domino effect, and (iii) *parallel* bonds where the load is equally distributed among the existing bonds in an attachment. The zipper is actually a specific case of bonds loaded in series, where attachments are loaded and broken consecutively. Two general architectures of multivalent interactions can thus be considered: a series connection where one or several bonds sustain the load and a parallel configuration where the force is shared equally across the connections (for further details please refer to ref 171). It should be noted that when polymeric linkers are involved, their nonlinear stiffness has to be taken into account when applying the Williams–Evans model to analyze the rupture of multiple identical bonds.

4.6. High-Speed DFS

Recent developments in AFM instrumentation have tackled rapid dynamics of bond breakage by accessing high r (up to 10^8 N/s) and thus to rupture bonds very far from equilibrium.^{85,121} To extend the information on experimental DFS plots, the studies combined SMFS with atomistic molecular dynamics simulations. Such computational force spectroscopy approaches usually operate at the limit of very high r (10^8 – 10^{13} pN/s), where bond breaking becomes deterministic.²⁰¹ This approach acted as a stimulus for the development of new unified analytical theories,^{176,187,202} such as Brownian dynamics simulations,^{85,186} which cover both slow and fast r regimes. However, the pulling apparatus and the linkers can influence the observed dynamics and the deduced bond-rupture rates.²⁰³

5. PROBING BIOLOGICAL SYSTEMS FROM SINGLE-MOLECULES TO LIVING CELLS

Over the years, (bio)chemical imaging by AFM has been instrumental in mapping and analyzing the interactions and properties of biosystems, thereby answering key questions in a variety of fields, including microbiology, virology, immunology, or cell biology.

5.1. Strength of Single Biomolecular Bonds: From Pico- to NanoNewtons

SMFS measured that the rupture forces of biomolecular complexes such as antibody–antigen or between DNA strands typically range from 20–150 pN and depend on the sample and experimental conditions.⁵⁸ For many years, the streptavidin–biotin bond had been considered as being the strongest receptor–ligand interaction in nature, with a rupture force approaching ~ 200 pN. However, recent studies have demonstrated that the binding strength of staphylococcal adhesion proteins (adhesins), including SdrG, ClfA, and ClfB, bind their ligand with ultrastrong forces of up to ~ 2000 pN, which is in the range of forces needed to rupture covalent bonds.²⁰⁴ This insight highlights the fact that, during evolution, bacterial pathogens evolved sophisticated strategies to firmly attach to protein-coated biomaterials and host cells. Ligand binding by SdrG and related proteins involves the multistep dock, lock, and latch (DLL) mechanism, where conformational changes in adhesins greatly stabilize the adhesion complex (Figure 7a). Molecular dynamics simulations show that the ligand (fibrinogen) is confined in a screw-like manner in the binding pocket of SdrG, and the binding strength of the complex results from numerous hydrogen bonds between the peptide backbone and SdrG, independent of the peptide side chains. Consequently, separation of the binding partners involves the simultaneous, cooperative rupture of numerous hydrogen bonds, leading to an extremely high mechanostability.⁸⁶

5.2. Imaging Chemical and Biological Sites

Using chemically functionalized tips, AFM is also a powerful method for imaging distributions of chemical and biological groups. For example, AFM can map the physicochemical properties (hydrophobicity, charge, ...) of biological samples ranging from living cells to single proteins. AFM showed the spore surface of the fungal pathogen *A. fumigatus* to be strongly hydrophobic, in agreement with the presence of hydrophobic proteins on the surface.²⁰⁵ Similarly, AFM can also map remarkable hydrophobic properties of mycobacteria, and their alteration upon treatment with drugs.⁶² Furthermore, the use of biochemical probes combined with the technological progresses of FD-AFM in the last years now enables localization of specific binding sites at high-resolution approaching 1 nm or better. FD-AFM with Ni^{2+} -NTA functionalized tips was used to image soluble or membrane proteins and allowed structurally locating specific interaction sites (histidine tags) on the spindle assembly abnormal protein 6 homologue (SAS-6)¹⁷⁰ and on the light-driven proton pump bacteriorhodopsin of purple membrane at (sub)-nanometer resolution and quantifying the binding strength of the Ni^{2+} -NTA groups to histidine residues.⁷⁸ The same strategy has been used to record multiparametric images of the structure, adhesion, and elasticity of *E. coli* bacteria infected by His-tagged bacteriophages revealing the virus assembly machinery in the form of soft nanodomains surrounded by stiff cell wall material.²⁰⁶ Recently, the combination of AFM topography and nanomechanical mapping with specific probes for molecular recognition of lipids was used to identify

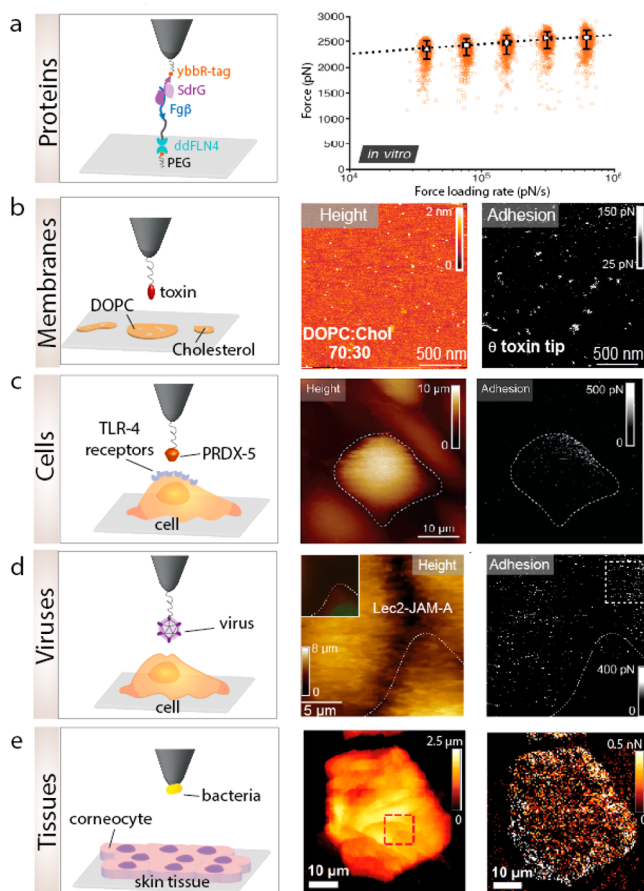


Figure 7. AFM-based chemical imaging of biomolecular and cellular systems. (a) Single-molecule force spectroscopy between Staphylococcal SdrG adhesin and fibrinogen (Fg) reveals that the complex withstands enormous forces, as shown in the DFS plot, thanks to a dock-lock and latch mechanism. The experimental AFM setup includes a ddFLN4 fingerprint domain (cyan) and covalent attachment to the surface and tip via PEG linkers and the ybBR-tag (yellow dots). (b) High-resolution mapping of lipid distribution on lipid bilayers. Cholesterol domains are localized by an AFM tip derivatized by a covalently linked θ -toxin through a PEG linker. Topography and corresponding adhesion map showing specific adhesion events (brighter pixels). (c) Peroxiredoxin-5 binding to toll-like receptor-4 (TLR-4) is probed directly on living cells. Specific interactions are observed on the adhesion map on the cell in the center that has been transfected to overexpress TLR4 receptors, while surrounding cells are not transfected and serve as internal control of the specificity. (d) A single virion is covalently linked to the AFM tip, and interactions force are probed on living cells expressing the specific JAM-A receptors. The fluorescently labeled cell (JAM-A deficient) in the lower right corner serves as an internal control. (e) The forces between *Staphylococcus aureus* and human skin are quantified at the nanoscale. Topography (vertical scale 0–2.5 μm) and corresponding adhesion map (vertical scale 0–0.5 nN) show that the *S. aureus*–skin adhesion is strong. Part of panel a was adapted with permission from ref 87. Copyright 2018 American Association for the Advancement of Science. Panel b was adapted with permission from 59. Copyright 2018 John Wiley and Sons. Panel c was adapted with permission from ref 205. Copyright 2018 American Chemical Society., Panel d was adapted with permission from ref 183. Copyright 2019 Springer Nature. Panel e was adapted with permission from ref 206. Copyright 2016 John Wiley and Sons.

cholesterol and sphingomyelin-enriched domains in supported lipid bilayers (Figure 7b).⁵⁹

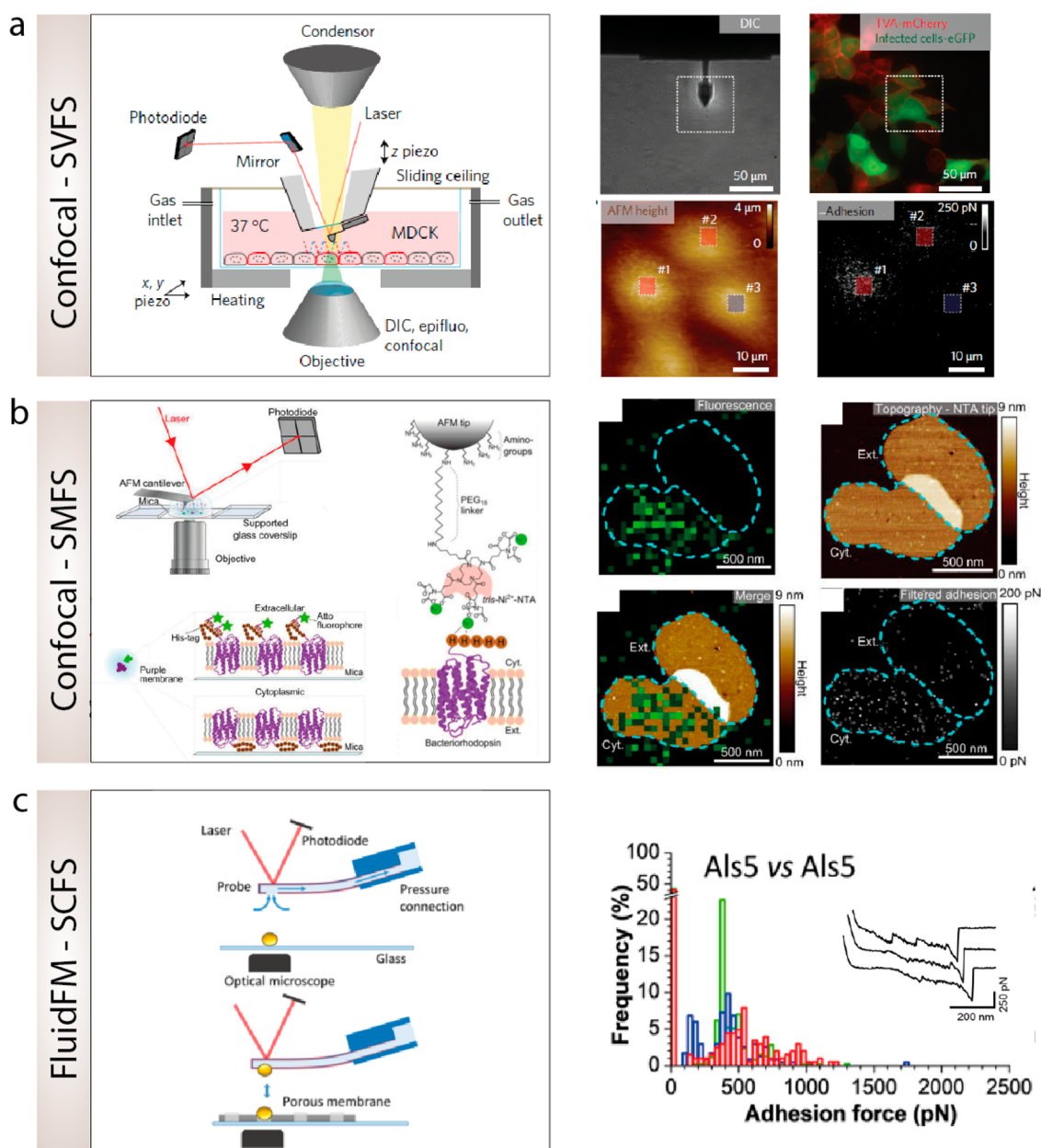


Figure 8. Combining AFM with confocal microscopy or microfluidic hollow cantilevers. (a) An AFM tip functionalized with EnvA-pseudotyped single rabies virus is scanned over a mixed confluent layer of MDCK cells expressing or not expressing the fluorescently tagged receptor (mCherry-TVA receptor). Specific interactions are only observed on cells expressing TVA receptors facilitating the binding of the viral EnvA receptor. (b) Confocal microscopy discriminates the orientation of purple membrane adsorbed on mica, while AFM images at high-resolution its topography and extracts specific interactions between a NTA-Ni²⁺-derivatized tip and bacteriorhodopsin displaying histidine-tags (His₁₀). (c) HollowAFM cantilevers (FluidFM) can be used to manipulate individual cells and to probe cell–cell interactions at high-throughput. Specific interactions between yeast *Saccharomyces cerevisiae* expressing AlsSp adhesins are measured. Partial unfolding of the tandem repeat of AlsSp is observed in the FD curves in the inset. Panel a was adapted with permissions from ref 77. Copyright 2017 Springer Nature. Panels b and c were adapted with permissions from refs 83 and 110. Copyright 2017 and 2019 American Chemical Society.

5.3. AFM Mapping of Mammalian Cells

Tremendous efforts have been realized over the last few years to investigate mammalian cells in physiologically relevant conditions and preferably without invoking phototoxic buffers. Roduit et al. for example observed a relative stiffening of glycosylphosphatidylinositol (GPI) domains on hippocampal neurons derived from rat embryos using AFM tips functionalized with aerolysin.²⁰⁷ The nano-organization of human gonadotropin-releasing hormone receptor was analyzed on fixed human bladder cancer cells revealing a tendency to

assemble in clusters irregularly distributed on the cell surface.²⁰⁸ Similarly, Fc gamma receptors that are widely expressed on effector cells such as macrophages and that play an important role in clinical cancer immunotherapy were localized on the surface of macrophage using an AFM tip functionalized with rituximab, the first monoclonal antibody developed to treat non-Hodgkin's lymphoma.²⁰⁹ Recently, for the first time in immunology, AFM combined with functionalized probes compellingly demonstrated that the specific binding of human peroxiredoxin-5 with the cell surface Toll-like receptor 4 (Figure

7c) leads to the stiffening of mammalian cells, likely via the release and action of interleukin IL-1 β .²¹⁰ In virology, AFM tips bearing single virions appeared as useful tools to investigate the first binding steps to cell surface receptors.¹⁴⁸ Studying the binding of the pseudotyped rabies virus EnvA to TVA cell surface receptors at the single virus level unraveled a cooperative mechanism by which the viral glycoproteins establish multiple bonds to cell surface receptors.¹⁸⁴ Similarly, a regulation mechanism of the number of bonds established between herpes virus and cellular attachment factors (including heparin and chondroitin sulfate) was deciphered by FD-AFM mapping the viral interactions with living cells at high force sensitivity.⁸⁹ Very recently, the direct interplay between attachment factors of reoviruses binding to entry cell surface receptors was observed for the first time by AFM¹⁸³ (Figure 7d). It was revealed that binding of viral σ 1 glycoproteins to sialic acid induces σ 1 to adopt a more extended conformation, leading to a higher avidity of the σ 1 head domain to its specific entry receptor, the junctional adhesion molecule A.⁷⁶

5.4. Single-Cell Force Spectroscopy

As many biological processes require controlled cell adhesion (assembly of tissues, development, inflammation, metastasis, viral and bacterial infection, ...), the use of AFM for single-cell force spectroscopy (SCFS) offers unique perspectives.^{58,73,211} By combining AFM and optical microscopy, the AFM can position cells at selected locations of a functionalized surface, tissue, or on another cell and to assess cellular interactions.⁷³ The basic principle of SCFS is straightforward and is based on a single cell attached to the free end of an AFM cantilever. Conventionally, the cell is attached either by biochemical interactions (including lectins,²¹² biotinylated cell surface proteins,²¹³ extracellular matrix proteins,^{214,215} or cell-TAK polyphenolic proteins²¹⁶) or by a nanofluidics-AFM combination, also called fluidic force microscope (FluidFM).²¹⁷ The attachment converts the living cell into a probe enabling detection of cellular interactions with various substrates. The challenge, however, lies in interpreting the deadhesion signature recorded in FD or FT curves because specific as well as unspecific adhesion events can be detected. For mammalian cells, the retraction FD curve can be divided into three parts. At low separation, the largest adhesion force is usually recorded (referred to the detachment force) that represents the maximum strength of the cell binding the substrate, while the area under the curve refers to the work that is required to detach the cell. The detachment force depends on many different cellular properties including cell elasticity, cortex tension, cell geometry, and last but not least on the binding of cell surface receptors to the environment properties.²¹⁸ After the detachment force has been overcome, individual deadhesion events can be observed on the FD curves. In these events the cell surface receptors either detach from their ligand or are pulled away from the cell cortex at the tip of a membrane tether.²¹⁹

Interestingly, multiparametric imaging can also be carried out using single bacterial probes as AFM tips. This approach was initially developed to spatially map bacterium–host adhesive interactions on skin surfaces²²⁰ (Figure 7e). Adhesion images showed that *Staphylococcus aureus* exhibits strong adhesion forces toward skin corneocytes, involving specific interactions between bacterial adhesins and their ligands. In the clinical context, adhesion of *S. aureus* to skin corneocytes plays a role in atopic dermatitis, also known as eczema. Reduced levels of natural moisturizing factor in the stratum corneum have been

shown to be associated with more severe atopic dermatitis symptoms. By means of imaging with single bacterial probes, a correlation was found between the level of natural moisturizing factor in the skin and the strength of bacterium–corneocyte adhesion. This study suggests that AFM-based affinity imaging with single bacterial probes should find utility for studying host–microbe interactions and could represent a potential tool to test new therapeutic approaches.

6. MULTIMETHODOLOGICAL CHARACTERIZATION

With increasing complexity of the biological system, AFM faces the difficulty of identifying the imaged morphological and structural details. Although chemical imaging can identify and localize one or a few components, the complexity of, for example, a cell surface with its thousands of different biomolecules (e.g., sugars, lipids, membrane proteins, associated water-soluble proteins) is extremely high. This situation is further complicated by the fact that, depending on the functional state of the cell, biomolecules dynamically assemble and disassemble macromolecular complexes. It thus becomes challenging to correlate the chemical information detected by AFM to cell state, morphology, and compartments. To overcome this problem, it is advisable to combine AFM-based chemical imaging with complementary micro- or nanoscopic methods.^{49,76,83,110} Combined with optical microscopy, such as differential interference or phase contrast, AFM topographs and chemical maps can be correlated with rather large cellular structures such as nucleus, organelles, lamellipodia, and cytoskeleton. Details of smaller cellular compartments and structures and of their specific composition can be tackled by combining AFM with fluorescence microscopy, among which confocal microscopy is particularly suited to imaging cellular structures interfaced with the AFM tip or to monitor and localize processes in the cell. Thereto fluorescence markers, either attached chemically (chemical fluorophores) or genetically (e.g., fluorescent proteins), are needed to label cellular components. In one such example, AFM was used to image single binding events of pseudotyped rabies viruses to MDCK cells that have been engineered to express the EnvA receptor to facilitate virus binding and entry⁷⁷ (Figure 8a). The AFM topography, virus-binding maps, and confocal microscopy images revealed rabies viruses only binding to cell surfaces expressing the fluorescently labeled receptor, while they could not bind to adjacent cells lacking the receptor. Similarly, AFM and confocal microscopy were combined to determine the sidedness of purple membrane adsorbed on mica and to localize specific His₁₀-tags fused the extracellular N-terminal end of bacteriorhodopsins (Figure 8b).⁸³ After fluorescently localizing purple membranes exposing extracellular His-tags, high-resolution AFM was used to image the purple membrane topography at (sub-) nanometer resolution and to assign the topographic structures to the extracellular surface of purple membrane. Similarly, it was possible to fluorescently label purple membrane carrying cytoplasmic His-tags. In addition, FD-AFM could be applied to monitor and localize specific binding events of an NTA-functionalized AFM tip to His-tagged bacteriorhodopsin molecules residing in purple membrane.

The resolution limit of conventional optical microscopy, ranging between 250 and 400 nm, is generally much lower than that of AFM, which can image cell surfaces at resolutions $\sim$ 50 nm. However, super-resolution optical microscopy can also approach resolutions of $\sim$ 50 nm.²²¹ Among the super-resolution optical microscopy family, including PALM, STORM, and

STED, STED-based approaches are possibly most suitable to imaging live cells because they can focus on selected cellular areas and monitor individual fluorophores or groups thereof at high temporal resolution approaching minutes to seconds.^{221–223} Combining AFM with optical super resolution is a challenging goal. Odermatt et al. designed a correlated single molecule localization microscope (SMLM)/AFM that allows localizing specific, labeled proteins within high-resolution AFM images in a biologically relevant context.²²⁴ Particularly, the newly invented STED-based MINFLUX nanoscopy allows the imaging of cellular processes at <5 nm lateral and <10 ms time resolution.²²⁵ Although a combination of MINFLUX with AFM does not yet exist, it would allow imaging of cellular processes in “real-time” while probing their chemical properties or stimulating the cells using biochemical compounds (e.g., ligands, proteins, viruses, drugs) attached to the AFM tip.

Other promising AFM-based approaches to spectroscopically characterizing the biological complexity include tip-enhanced infrared (IR) spectroscopy or scanning near-field optical microscopy (SNOM).^{82,84} In SNOM, the tip is replaced by an optical fiber with a nanoscale aperture, meaning topographic and optical images are simultaneously recorded, revealing the spatial distribution of fluorescently labeled molecules.²²⁶ Progress in photonic antennas and plasmonics is expected to offer nanoimaging and nanospectroscopy of single molecules in living cells.²²⁷ Tip enhanced Raman spectroscopy (TERS) combines the spatial resolution of AFM with the chemical selectivity of Raman spectroscopy.^{228–230} These approaches have begun delivering useful insights into the chemical compositions and thus to chemically identify the composition of complex biological systems, such as membranes. Similarly, microfluidic devices coupled to hollow AFM cantilevers, also named Fluid-FM, can be used to manipulate cells or to inject or extract a small amount of solutions into cells (Figure 8c).²¹⁷ Combined with chemical imaging, such AFM-based devices may be applicable to directly sense, localize, and trigger specific (bio)chemical compounds and reactions.

7. PERSPECTIVE

AFM-based chemical sensing brings a unique (bio)chemical laboratory to a nanoscopic probe. The possibility to sense, quantify, image, and manipulate noncovalent molecular interactions, reactions, and processes opens avenues to groundbreaking experiments in life sciences. Such experiments include, for example, localizing cell surface receptors of living cells and triggering cellular responses via control of the ligand binding to the receptor. Moreover, measuring the force and time of ligand binding to a receptor allows determination of the free-energy landscape of the ligand–receptor bond and, in principle, investigation of how the cell regulates its receptors to bind ligands. As we highlighted, the first successful attempts to address this biological challenge have been achieved, and we expect that many more examples will follow flanked by a deepening understanding of how cells regulate cell surface receptors. We also see a considerable potential for using photoswitchable ligands or compounds tethered to the AFM tip, which can be switched in their state to binding their receptor of interest or to undergo specific interactions with the biological system of interest. Such applications further extending the applicability of the AFM as chemical toolbox may be soon developed and established. However, AFM-based chemical imaging is not trivial, as it requires several chemical, technological, and theoretical prerequisites to be able to detect

and analyze specific or nonspecific biomolecular interactions. We have outlined the most important steps toward reaching this goal. Nevertheless, bringing the field toward the full potential range of applications requires that standards be set. Such standards involve the use of reference samples, which are structurally and chemically well-defined. One such reference could be the atomically well-defined purple membrane, where bacteriorhodopsin molecules assemble hexagonally and can be genetically modified to carry the chemical tags of interest.

In the future, we expect that AFM will be combined with other ultrasensitive force techniques to measure the forces associated with native biological systems ranging from tissues, cells to biomolecules, including microfluidic devices, microneedles, biomembrane force probe, or optical and magnetic tweezers.^{231–235} Molecular dynamics simulations will become increasingly used tool complement such experiments, contributing to understand the molecular mechanisms underlying for example cellular functions at atomistic detail. Infrared and Raman spectroscopy combined with scanning probe methods, i.e., photothermal induced resonance (PTIR) and tip-enhanced Raman spectroscopy (TERS), will offer new possibilities for the chemical imaging and spectroscopy of biosystems at the nanoscale.^{230,236,237} Such chemical imaging may in the future allow us to analyze the complex composition of dynamically changing cellular compartments, such as organelles, membranes, or cytoskeleton to name only a few.

For decades, AFM-based approaches, including AFM-based chemical imaging, have been mainly applied to characterization of isolated biological systems, such as individual or complexed peptides, sugars, proteins, nucleic acids, and membrane patches. Such *in situ* approaches are important to develop and establish AFM-based chemical imaging. However, biology also teaches us that most of these systems are highly regulated and that their structural and functional state depends on the biological environment, which is the cell, tissue, or organ. Thus, to approach how biological systems work *in vivo* also requires their characterization in the complexity of the living system. To fully address this complexity requires the combination of AFM-based chemical imaging with complementary microscopic, nanoscopic, and spectroscopic tools that allow one to characterize and link as many parameters as possible to describe and understand living systems. As one major complementary tool apparently involves fluorescence microscopy, one may think to soon use “intelligent” fluorescent dyes to localize, switch, and sense signal transduction pathways, cell membrane tension, intracellular pressure or pH, or other cellular parameters and processes relating to the (bio)chemical information sensed by AFM. In this respect it will be beneficial to combine AFM-based methods with advanced optical methods like near-field scanning optical microscopy (NSOM), 4Pi microscopy, saturated pattern excitation microscopy (SPEM), stimulated emission depletion (STED) microscopy, photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), or MinFlux microscopy and to localize and track single molecules in complex biological systems, including the interior of live cells, at high spatial and temporal resolutions.^{238–240}

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Peter Hinterdorfer is department head of experimental applied biophysics at the Johannes Kepler University in Linz, Austria, where he also obtained his master's and Ph.D. degrees, followed by a postdoctoral stay at the University of Virginia, VA. The research of Peter Hinterdorfer is focused around nanoscopic techniques in life science, bionano technology, and medical diagnostics. In particular, his research covers molecular recognition and transport in several molecular and cellular systems. His department has its core competence in the application and development of atomic force microscopy (AFM) based methods in life science (Biological atomic force microscopy (Bio-AFM)). AFM techniques and modes, such as single molecule force spectroscopy, molecular recognition force microscopy, elasticity mapping, recognition imaging, fluorescence microscopy, and high-speed AFM are used to characterize molecular interactions in various biological systems.

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REFERENCES

- (1) Ashkin, A.; Dziedzic, J. M.; Yamane, T. Optical Trapping and Manipulation of Single Cells Using Infrared Laser Beams. *Nature* **1987**, *330*, 769–771.
- (2) Kuo, S.; Sheetz, M. Force of Single Kinesin Molecules Measured with Optical Tweezers. *Science* **1993**, *260*, 232–234.
- (3) Svoboda, K.; Schmidt, C. F.; Schnapp, B. J.; Block, S. M. Direct Observation of Kinesin Stepping by Optical Trapping Interferometry. *Nature* **1993**, *365*, 721–727.
- (4) Ashkin, A.; Dziedzic, J. M.; Bjorkholm, J. E.; Chu, S. Observation of a Single-Beam Gradient Force Optical Trap for Dielectric Particles. *Opt. Lett.* **1986**, *11*, 288–290.
- (5) Wang, N.; Butler, J.; Ingber, D. Mechanotransduction Across the Cell Surface and Through the Cytoskeleton. *Science* **1993**, *260*, 1124–1127.
- (6) Smith, S.; Finzi, L.; Bustamante, C. Direct Mechanical Measurements of the elasticity of Single DNA Molecules by Using Magnetic Beads. *Science* **1992**, *258*, 1122–1126.
- (7) Evans, E.; Berk, D.; Leung, A.; Mohandas, N. Detachment of Agglutinin-Bonded Red Blood Cells. II. Mechanical Energies to Separate Large Contact Areas. *Biophys. J.* **1991**, *59*, 849–860.
- (8) Israelachvili, J. N. In *Intermolecular And Surface Forces*, 3rd ed.; Academic Press: San Diego, 2011; Chapter 14 - Electrostatic Forces Between Surfaces in Liquids
- (9) Yoshizawa, H.; Chen, Y. L.; Israelachvili, J. Fundamental Mechanisms of Interfacial Friction. 1. Relation Between Adhesion and Friction. *J. Phys. Chem.* **1993**, *97*, 4128–4140.
- (10) Chen, Y. L.; Helm, C. A.; Israelachvili, J. N. Molecular Mechanisms Associated with Adhesion and Contact Angle Hysteresis of Monolayer Surfaces. *J. Phys. Chem.* **1991**, *95*, 10736–10747.
- (11) Kim, S.; Blainey, P. C.; Schroeder, C. M.; Xie, X. S. Multiplexed Single-Molecule Assay for Enzymatic Activity on Flow-Stretched DNA. *Nat. Methods* **2007**, *4*, 397–399.
- (12) Binnig, G.; Quate, C. F.; Gerber, C. Atomic Force Microscope. *Phys. Rev. Lett.* **1986**, *56*, 930–933.
- (13) Hansma, H. G.; Hoh, J. H. Biomolecular Imaging with the Atomic Force Microscope. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 115–140.
- (14) Marti, O.; Drake, B.; Hansma, P. K. Atomic Force Microscopy of Liquid-Covered Surfaces: Atomic Resolution Images. *Appl. Phys. Lett.* **1987**, *51*, 484–486.
- (15) Ducker, W. A.; Senden, T. J.; Pashley, R. M. Direct Measurement of Colloidal Forces Using an Atomic Force Microscope. *Nature* **1991**, *353*, 239–241.
- (16) Lin, X. Y.; Creuzet, F.; Arribart, H. Atomic Force Microscopy for Local Characterization of Surface Acid-Base Properties. *J. Phys. Chem.* **1993**, *97*, 7272–7276.
- (17) O'Shea, S. J.; Welland, M. E.; Pethica, J. B. Atomic Force Microscopy of Local Compliance at Solid–Liquid Interfaces. *Chem. Phys. Lett.* **1994**, *223*, 336–340.
- (18) Noy, A.; Frisbie, C. D.; Rozsnyai, L. F.; Wrigton, M. S.; Lieber, C. M. Chemical Force Microscopy: Exploiting Chemically-Modified Tips to Quantify Adhesion, Friction, and Functional Group Distributions in Molecular Assemblies. *J. Am. Chem. Soc.* **1995**, *117*, 7943–7951.
- (19) Miyamoto, T.; Kaneko, R.; Ando, Y. Interaction Force Between Thin Film Disk Media and Elastic Solids Investigated by Atomic Force Microscope. *J. Tribol.* **1990**, *112*, 567–572.
- (20) Berger, C. E. H.; Van Der Werf, K. O.; Kooyman, R. P. H.; De Grooth, B. G.; Greve, J. Functional Group Imaging by Adhesion AFM Applied to Lipid Monolayers. *Langmuir* **1995**, *11*, 4188–4192.
- (21) Rosa-Zeiser, A.; Weilandt, E.; Hild, S.; Marti, O. The Simultaneous Measurement of Elastic, Electrostatic and Adhesive Properties by Scanning Force Microscopy: Pulsed-Force Mode Operation. *Meas. Sci. Technol.* **1997**, *8*, 1333–1338.
- (22) Miyatani, T.; Horii, M.; Rosa, A.; Fujihira, M.; Marti, O. Mapping of Electrical Double-Layer Force Between Tip and Sample Surfaces in Water with Pulsed-Force-Mode Atomic Force Microscopy. *Appl. Phys. Lett.* **1997**, *71*, 2632–2634.
- (23) Frisbie, C. D.; Rozsnyai, L. F.; Noy, A.; Wrigton, M. S.; Lieber, C. M. Functional Group Imaging by Chemical Force Microscopy. *Science (Washington, DC, U. S.)* **1994**, *265*, 2071–2074.
- (24) Lee, G. U.; Kidwell, D. A.; Colton, R. J. Sensing Discrete Streptavidin-Biotin Interactions with Atomic Force Microscopy. *Langmuir* **1994**, *10*, 354–357.
- (25) Florin, E.; Moy, V.; Gaub, H. Adhesion Forces Between Individual Ligand-Receptor Pairs. *Science* **1994**, *264*, 415–417.
- (26) Hinterdorfer, P.; Baumgartner, W.; Gruber, H. J.; Schilcher, K.; Schindler, H. Detection and Localization of Individual Antibody-Antigen Recognition Events by Atomic Force Microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 3477–3481.
- (27) Dammer, U.; Hegner, M.; Anselmetti, D.; Wagner, P.; Dreier, M.; Huber, W.; Guntherodt, H. J. Specific Antigen/Antibody Interactions Measured By Force Microscopy. *Biophys. J.* **1996**, *70*, 2437–2441.
- (28) Bustamante, C.; Marko, J. F.; Siggia, E. D.; Smith, S. Entropic Elasticity of Lambda-Phage DNA. *Science* **1994**, *265*, 1599–1600.
- (29) Chen, X.; Patel, N.; Davies, M. C.; Roberts, C. J.; Tendler, S. J. B.; Williams, P. M.; Davies, J.; Dawkes, A. C.; Edwards, J. C. Application of Protein-Coated Scanning Force Microscopy Probes in Measurements of Surface Affinity to Protein Adsorption. *Appl. Phys. A: Mater. Sci. Process.* **1998**, *66*, S631–S634.
- (30) Dammer, U.; Popescu, O.; Wagner, P.; Anselmetti, D.; Guntherodt, H. J.; Misevic, G. N. Binding Strength Between Cell

Adhesion Proteoglycans Measured by Atomic Force Microscopy. *Science (Washington, DC, U. S.)* **1995**, *267*, 1173–1175.

(31) Lee, G.; Chrisey, L.; Colton, R. Direct Measurement of the Forces Between Complementary Strands Of DNA. *Science* **1994**, *266*, 771–773.

(32) Rief, M.; Gautel, M.; Oesterhelt, F.; Fernandez, J. M.; Gaub, H. E. Reversible Unfolding of Individual Titin Immunoglobulin Domains By AFM. *Science (Washington, DC, U. S.)* **1997**, *276*, 1109–1112.

(33) Rief, M.; Oesterhelt, F.; Heymann, B.; Gaub, H. E. Single Molecule Force Spectroscopy on Polysaccharides by Atomic Force Microscopy. *Science (Washington, DC, U. S.)* **1997**, *275*, 1295–1297.

(34) Radmacher, M.; Fritz, M.; Cleveland, J. P.; Walters, D. A.; Hansma, P. K. Imaging Adhesion Forces and Elasticity of Lysozyme Adsorbed on Mica with the Atomic Force Microscope. *Langmuir* **1994**, *10*, 3809–3814.

(35) Ludwig, M.; Dettmann, W.; Gaub, H. E. Atomic Force Microscope Imaging Contrast Based on Molecular Recognition. *Biophys. J.* **1997**, *72*, 445–448.

(36) Grandbois, M.; Dettmann, W.; Benoit, M.; Gaub, H. E. Affinity Imaging of Red Blood Cells Using an Atomic Force Microscope. *J. Histochem. Cytochem.* **2000**, *48*, 719–724.

(37) Dupres, V.; Menozzi, F. D.; Loch, C.; Clare, B. H.; Abbott, N. L.; Cuenot, S.; Bompard, C.; Raze, D.; Dufrêne, Y. F. Nanoscale Mapping and Functional Analysis of Individual Adhesins on Living Bacteria. *Nat. Methods* **2005**, *2*, 515–520.

(38) Gilbert, Y.; Deghorain, M.; Wang, L.; Xu, B.; Pollheimer, P. D.; Gruber, H. J.; Errington, J.; Hallet, B.; Haulot, X.; Verbelen, C.; et al. Single-Molecule Force Spectroscopy and Imaging of the Vancomycin/D-Ala-D-Ala Interaction. *Nano Lett.* **2007**, *7*, 796–801.

(39) Lehenkari, P. P.; Charras, G. T.; Nykänen, A.; Horton, M. A. Adapting Atomic Force Microscopy for Cell Biology. *Ultramicroscopy* **2000**, *82*, 289–295.

(40) Almqvist, N.; Bhatia, R.; Primbs, G.; Desai, N.; Banerjee, S.; Lal, R. Elasticity and Adhesion Force Mapping Reveals Real-Time Clustering of Growth Factor Receptors and Associated Changes in Local Cellular Rheological Properties. *Biophys. J.* **2004**, *86*, 1753–1762.

(41) Chtcheglova, L. A.; Waschke, J.; Wildling, L.; Drenckhahn, D.; Hinterdorfer, P. Nano-scale dynamic recognition imaging on vascular endothelial cells. *Biophys. J.* **2007**, *93*, L11–L13.

(42) Raab, A.; Han, W.; Badt, D.; Smith-Gill, S. J.; Lindsay, S. M.; Schindler, H.; Hinterdorfer, P. Antibody recognition imaging by force microscopy. *Nat. Biotechnol.* **1999**, *17*, 901–905.

(43) Stroth, C. M.; Ebner, A.; Geretschläger, M.; Freudenthaler, G.; Kienberger, F.; Kamruzzahan, A. S. M.; Smith-Gill, S. J.; Gruber, H. J.; Hinterdorfer, P. Simultaneous Topography and Recognition Imaging Using Force Microscopy. *Biophys. J.* **2004**, *87*, 1981–1990.

(44) Stroth, C.; Wang, H.; Bash, R.; Ashcroft, B.; Nelson, J.; Gruber, H.; Lohr, D.; Lindsay, S. M.; Hinterdorfer, P. Single-molecule recognition imaging microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 12503–12507.

(45) Dong, M.; Sahin, O. A Nanomechanical Interface to Rapid Single-Molecule Interactions. *Nat. Commun.* **2011**, *2*, 247–253.

(46) Alsteens, D.; Newton, R.; Schubert, R.; Martinez-Martin, D.; Delguste, M.; Roska, B.; Muller, D. J. Nanomechanical Mapping of First Binding Steps of a Virus to Animal Cells. *Nat. Nanotechnol.* **2017**, *12*, 177–183.

(47) Dufrêne, Y. F.; Ando, T.; Garcia, R.; Alsteens, D.; Martinez-Martin, D.; Engel, A.; Gerber, C.; Muller, D. J. Imaging Modes of Atomic Force Microscopy for Application in Molecular and Cell Biology. *Nat. Nanotechnol.* **2017**, *12*, 295–307.

(48) Serdiuk, T.; Steudle, A.; Mari, S. A.; Manioglou, S.; Kaback, H. R.; Kuhn, A.; Müller, D. J. Insertion and Folding Pathways of Single Membrane Proteins Guided by Translocases and Insertases. *Sci. Adv.* **2019**, *5*, eaau6824.

(49) Kufer, S. K.; Puchner, E. M.; Gump, H.; Liedl, T.; Gaub, H. E. Single-Molecule Cut-And-Paste Surface Assembly. *Science* **2008**, *319*, 594–596.

(50) Gerber, C.; Lang, H. P. How the Doors to the Nanoworld Were Opened. *Nat. Nanotechnol.* **2006**, *1*, 3–5.

(51) Müller, D. J.; Dufrêne, Y. F. Atomic Force Microscopy as a Multifunctional Molecular Toolbox in Nanobiotechnology. *Nat. Nanotechnol.* **2008**, *3*, 261–269.

(52) Dufrêne, Y. F.; Martinez-Martin, D.; Medalsy, I.; Alsteens, D.; Muller, D. J. Multiparametric Imaging of Biological Systems by Force-Distance Curve-Based AFM. *Nat. Methods* **2013**, *10*, 847–854.

(53) Alsteens, D.; Gaub, H. E.; Newton, R.; Pfreundschuh, M.; Gerber, C.; Müller, D. J. Atomic Force Microscopy-Based Characterization and Design of Biointerfaces. *Nat. Rev. Mater.* **2017**, *2*, 17008.

(54) Krieg, M.; Fläschner, G.; Alsteens, D.; Gaub, B. M.; Roos, W. H.; Wuite, G. J. L.; Gaub, H. E.; Gerber, C.; Dufrêne, Y. F.; Müller, D. J. Atomic Force Microscopy-Based Mechanobiology. *Nat. Rev. Phys.* **2019**, *1*, 41–57.

(55) Dufrêne, Y. F.; Ando, T.; Garcia, R.; Alsteens, D.; Martinez-Martin, D.; Engel, A.; Gerber, C.; Müller, D. J. Imaging Modes of Atomic Force Microscopy for Application in Molecular and Cell Biology. *Nat. Nanotechnol.* **2017**, *12*, 295–307.

(56) Lo Giudice, C.; Dumitru, A. C.; Alsteens, D. Probing Ligand-Receptor Bonds in Physiologically Relevant Conditions Using AFM. *Anal. Bioanal. Chem.* **2019**, *411*, 6549–6559.

(57) Garcia-Manyes, S.; Beedle, A. E. M. Steering Chemical Reactions with Force. *Nat. Rev. Chem.* **2017**, *1*, 0083.

(58) Müller, D. J.; Helenius, J.; Alsteens, D.; Dufrêne, Y. F. Force Probing Surfaces of Living Cells to Molecular Resolution. *Nat. Chem. Biol.* **2009**, *5*, 383–390.

(59) Dumitru, A. C.; Conrard, L.; Lo Giudice, C.; Henriët, P.; Veiga-Da-Cunha, M.; Derclaye, S.; Tyteca, D.; Alsteens, D. High-Resolution Mapping and Recognition of Lipid Domains Using AFM with Toxin-Derivatized Probes. *Chem. Commun.* **2018**, *54*, 6903–6906.

(60) Junker, J. P.; Ziegler, F.; Rief, M. Ligand-Dependent Equilibrium Fluctuations of Single Calmodulin Molecules. *Science* **2009**, *323*, 633–637.

(61) Churnside, A. B.; Sullan, R. M. A.; Nguyen, D. M.; Case, S. O.; Bull, M. S.; King, G. M.; Perkins, T. T. Routine and Timely Sub-Piconewton Force Stability and Precision for Biological Applications of Atomic Force Microscopy. *Nano Lett.* **2012**, *12*, 3557–3561.

(62) Alsteens, D.; Dague, E.; Rouxhet, P. G.; Baulard, A. R.; Dufrêne, Y. F. Direct Measurement of Hydrophobic Forces on Cell Surfaces Using AFM. *Langmuir* **2007**, *23*, 11977–11979.

(63) Ma, C. D.; Acevedo-Vélez, C.; Wang, C.; Gellman, S. H.; Abbott, N. L. Interaction of the Hydrophobic Tip of an Atomic Force Microscope with Oligopeptides Immobilized Using Short and Long Tethers. *Langmuir* **2016**, *32*, 2985–2995.

(64) Berquand, A.; Xia, N.; Castner, D. G.; Clare, B. H.; Abbott, N. L.; Dupres, V.; Adriaenssen, Y.; Dufrêne, Y. F. Antigen Binding Forces of Single Antilysozyme Fv Fragments Explored by Atomic Force Microscopy. *Langmuir* **2005**, *21*, 5517–5523.

(65) Wildling, L.; Unterauer, B.; Zhu, R.; Rupprecht, A.; Haselgrübler, T.; Rank, C.; Ebner, A.; Vater, D.; Pollheimer, P.; Pohl, E.; Hinterdorfer, P.; Gruber, H. J. Linking of Sensor Molecules with Amino Groups to Amino-Functionalized AFM Tips. *Bioconjugate Chem.* **2011**, *22*, 1239–1248.

(66) Sulchek, T.; Friddle, R. W.; Noy, A. Strength of Multiple Parallel Biological Bonds. *Biophys. J.* **2006**, *90*, 4686–4691.

(67) Ludwig, M.; Dettmann, W.; Gaub, H. E. Atomic Force Microscope Imaging Contrast Based on Molecular Recognition. *Biophys. J.* **1997**, *72*, 445–448.

(68) Willemssen, O. H.; Snel, M. M. E.; Van Der Werf, K. O.; De Grooth, B. G.; Greve, J.; Hinterdorfer, P.; Gruber, H. J.; Schindler, H.; Van Kooyk, Y.; Figdor, C. G. Simultaneous Height and Adhesion Imaging of Antibody-Antigen Interactions by Atomic Force Microscopy. *Biophys. J.* **1998**, *75*, 2220–2228.

(69) Gad, M.; Itoh, A.; Ikai, A. Mapping Cell Wall Polysaccharides of Living Microbial Cells Using Atomic Force Microscopy. *Cell Biol. Int.* **1997**, *21*, 697–706.

(70) Grandbois, M.; Dettmann, W.; Benoit, M.; Gaub, H. E. Affinity Imaging of Red Blood Cells Using an Atomic Force Microscope. *J. Histochem. Cytochem.* **2000**, *48*, 719–724.

- (71) Rief, M.; Oesterhelt, F.; Heymann, B.; Gaub, H. E. Single Molecule Force Spectroscopy on Polysaccharides by Atomic Force Microscopy. *Science* **1997**, *275*, 1295–1297.
- (72) Grandbois, M.; Beyer, M.; Rief, M.; Clausen-Schaumann, H.; Gaub, H. E. How Strong is a Covalent Bond? *Science* **1999**, *283*, 1727–1730.
- (73) Benoit, M.; Gabriel, D.; Gerisch, G.; Gaub, H. E. Discrete Interactions in Cell Adhesion Measured by Single-Molecule Force Spectroscopy. *Nat. Cell Biol.* **2000**, *2*, 313–317.
- (74) Lee, G. U.; Kidwell, D. A.; Colton, R. J. Sensing Discrete Streptavidin Biotin Interactions with Atomic Force Microscopy. *Langmuir* **1994**, *10*, 354–357.
- (75) Moy, V. T.; Florin, E.-L.; Gaub, H. E. Intermolecular Forces and Energies Between Ligands and Receptors. *Science* **1994**, *266*, 257–259.
- (76) Newton, R.; Delguste, M.; Koehler, M.; Dumitru, A. C.; Laskowski, P. R.; Müller, D. J.; Alsteens, D. Combining Confocal and Atomic Force Microscopy to Quantify Single-Virus Binding to Mammalian Cell Surfaces. *Nat. Protoc.* **2017**, *12*, 2275–2292.
- (77) Alsteens, D.; Newton, R.; Schubert, R.; Martinez-Martin, D.; Delguste, M.; Roska, B.; Müller, D. J. Nanomechanical Mapping of First Binding Steps of a Virus to Animal Cells. *Nat. Nanotechnol.* **2017**, *12*, 177–183.
- (78) Pfreundschuh, M.; Harder, D.; Ucurum, Z.; Fotiadis, D.; Müller, D. J. Detecting Ligand-Binding Events and Free Energy Landscape while Imaging Membrane Receptors at Subnanometer Resolution. *Nano Lett.* **2017**, *17*, 3261–3269.
- (79) Oh, Y. J.; Koehler, M.; Lee, Y.; Mishra, S.; Park, J. W.; Hinterdorfer, P. Ultra-Sensitive and Label-Free Probing of Binding Affinity Using Recognition Imaging. *Nano Lett.* **2019**, *19*, 612–617.
- (80) Prystopiuk, V.; Feuillie, C.; Herman-Bausier, P.; Viela, F.; Alsteens, D.; Pietrocola, G.; Speziale, P.; Dufrene, Y. F. Mechanical Forces Guiding Staphylococcus Aureus Cellular Invasion. *ACS Nano* **2018**, *12*, 3609–3622.
- (81) Herman-Bausier, P.; Labate, C.; Towell, A. M.; Derclaye, S.; Geoghegan, J. A.; Dufrene, Y. F. Staphylococcus Aureus Clumping Factor A is a Force-Sensitive Molecular Switch that Activates Bacterial Adhesion. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 5564–5569.
- (82) Yang, Q.; Ma, Q.; Herum, K. M.; Wang, C.; Patel, N.; Lee, J.; Wang, S.; Yen, T. M.; Wang, J.; Tang, H.; et al. Array Atomic Force Microscopy for Real-Time Multiparametric Analysis. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 5872–5877.
- (83) Laskowski, P. R.; Pfreundschuh, M.; Staufer, M.; Ucurum, Z.; Fotiadis, D.; Müller, D. J. High-Resolution Imaging and Multiparametric Characterization of Native Membranes by Combining Confocal Microscopy and an Atomic Force Microscopy-Based Toolbox. *ACS Nano* **2017**, *11*, 8292–8301.
- (84) Giliberti, V.; Polito, R.; Ritter, E.; Broser, M.; Hegemann, P.; Puskar, L.; Schade, U.; Zanetti-Polzi, L.; Daidone, I.; Corni, S.; et al. Tip-Enhanced Infrared Difference Nanospectroscopy of the Proton Pump Activity of Bacteriorhodopsin in Single Purple Membrane Patches. *Nano Lett.* **2019**, *19*, 3104–3114.
- (85) Rico, F.; Russek, A.; González, L.; Grubmüller, H.; Scheuring, S. Heterogeneous and Rate-Dependent Streptavidin-Biotin Unbinding Revealed by High-Speed Force Spectroscopy and Atomistic Simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 6594–6601.
- (86) Milles, L. F.; Unterauer, E. M.; Nicolaus, T.; Gaub, H. E. Calcium Stabilizes the Strongest Protein Fold. *Nat. Commun.* **2018**, *9*, 4764.
- (87) Milles, L. F.; Schulten, K.; Gaub, H. E.; Bernardi, R. C. Molecular Mechanism of Extreme Mechanostability in a Pathogen Adhesin. *Science* **2018**, *359*, 1527–1533.
- (88) Sedlak, S. M.; Schendel, L. C.; Melo, M. C. R.; Pippig, D. A.; Luthey-Schulten, Z.; Gaub, H. E.; Bernardi, R. C. Direction Matters: Monovalent Streptavidin/Biotin Complex Under Load. *Nano Lett.* **2019**, *19*, 3415–3421.
- (89) Delguste, M.; Peerboom, N.; Le Brun, G.; Trybala, E.; Olofsson, S.; Bergström, T.; Alsteens, D.; Bally, M. Regulatory Mechanisms of the Mucin-Like Region on Herpes Simplex Virus During Cellular Attachment. *ACS Chem. Biol.* **2019**, *14*, 534–542.
- (90) Eubelen, M.; Bostaille, N.; Cabochette, P.; Gauquier, A.; Tebabi, P.; Dumitru, A. C.; Koehler, M.; Gut, P.; Alsteens, D.; Stainier, D. Y. R.; et al. A Molecular Mechanism for Wnt Ligand-Specific Signaling. *Science* **2018**, *361*, e.
- (91) Yu, H.; Siewny, M. G. W.; Edwards, D. T.; Sanders, A. W.; Perkins, T. T. Hidden Dynamics in the Unfolding of Individual Bacteriorhodopsin Proteins. *Science* **2017**, *355*, 945.
- (92) Edwards, D. T.; Perkins, T. T. Optimizing Force Spectroscopy by Modifying Commercial Cantilevers: Improved Stability, Precision, and Temporal Resolution. *J. Struct. Biol.* **2017**, *197*, 13–25.
- (93) Garcia, R. Nanomechanical Mapping of Soft Materials with the Atomic Force Microscope: Methods, Theory and Applications. *Chem. Soc. Rev.* **2020**, *49*, 5850–5884.
- (94) Bill, R. M.; Henderson, P. J. F.; Iwata, S.; Kunji, E. R. S.; Michel, H.; Neutze, R.; Newstead, S.; Poolman, B.; Tate, C. G.; Vogel, H. Overcoming Barriers to Membrane Protein Structure Determination. *Nat. Biotechnol.* **2011**, *29*, 335–340.
- (95) Harayama, T.; Riezman, H. Understanding the Diversity Of Membrane Lipid Composition. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 281–296.
- (96) Mio, K.; Sato, C. Lipid Environment of Membrane Proteins in Cryo-EM Based Structural Analysis. *Biophys. Rev.* **2018**, *10*, 307–316.
- (97) Früh, V.; Ijzerman, A. P.; Siegal, G. How to Catch a Membrane Protein in Action: A Review of Functional Membrane Protein Immobilization Strategies and their Applications. *Chem. Rev.* **2011**, *111*, 640–656.
- (98) Qiu, W.; Fu, Z.; Xu, G. G.; Grassucci, R. A.; Zhang, Y.; Frank, J.; Hendrickson, W. A.; Guo, Y. Structure and Activity of Lipid Bilayer Within a Membrane-Protein Transporter. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 12985–12990.
- (99) Dörr, J. M.; Koorengevel, M. C.; Schäfer, M.; Prokofyev, A. V.; Scheidelaar, S.; Van Der Cruysen, E. A. W.; Dafforn, T. R.; Baldus, M.; Killian, J. A. Detergent-Free Isolation, Characterization, and Functional Reconstitution of a Tetrameric K⁺ Channel: The Power of Native Nanodiscs. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 18607–18612.
- (100) Voskoboinikova, N.; Mosslehy, W.; Colbasevici, A.; Ismagulova, T. T.; Bagrov, D. V.; Akovantseva, A. A.; Timashev, P. S.; Mulkidjanian, A. Y.; Bagratashvili, V. N.; Shaitan, K. V.; et al. Characterization of an Archaeal Photoreceptor/Transducer Complex from Natronomonas Pharaonis Assembled Within Styrene-Maleic Acid Lipid Particles. *RSC Adv.* **2017**, *7*, 51324–51334.
- (101) Lepe-Zuniga, J. L.; Zigler, J. S.; Gery, I. Toxicity of Light-Exposed Hepes Media. *J. Immunol. Methods* **1987**, *103*, 145.
- (102) Otero, D. H.; Wilbekin, F.; Meyer, E. M. Effects of 4-(2-Hydroxyethyl)-1-Piperazine-Ethanesulfonic Acid (AH5183) on Rat Cortical Synaptosome Choline Uptake, Acetylcholine Storage and Release. *Brain Res.* **1985**, *359*, 208–214.
- (103) Dumitru, A. C.; Poncin, M. A.; Conrard, L.; Dufrene, Y. F.; Tyteca, D.; Alsteens, D. Nanoscale Membrane Architecture of Healthy and Pathological Red Blood Cells. *Nanoscale Horiz.* **2018**, *3*, 293–304.
- (104) Mandriota, N.; Friedsam, C.; Jones-Molina, J. A.; Tatem, K. V.; Ingber, D. E.; Sahin, O. Cellular Nanoscale Stiffness Patterns Governed by Intracellular Forces. *Nat. Mater.* **2019**, *18*, 1071–1077.
- (105) Alsteens, D.; Garcia, M. C.; Lipke, P. N.; Dufrene, Y. F. Force-Induced Formation and Propagation of Adhesion Nanodomains in Living Fungal Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20744–20749.
- (106) Uhl, E.; Donati, A.; Reviakine, I. Platelet Immobilization on Supported Phospholipid Bilayers for Single Platelet Studies. *Langmuir* **2016**, *32*, 8516–8524.
- (107) Keshervani, V.; Sodhi, A. Differential Activation of Macrophages in Vitro by Lectin Concanavalin A, Phytohemagglutinin and Wheat Germ Agglutinin: Production and Regulation of Nitric Oxide. *Nitric Oxide* **2007**, *16*, 294–305.
- (108) Sundqvist, K. G.; Otteskog, P.; Wanger, L.; Thorstensson, R.; Utter, G. Morphology and Microfilament Organization in Human Blood Lymphocytes: Effects of Substratum and Mitogen Exposure. *Exp. Cell Res.* **1980**, *130*, 327–337.

- (109) Turner, R. D.; Thomson, N. H.; Kirkham, J.; Devine, D. Improvement of the Pore Trapping Method to Immobilize Vital Coccoid Bacteria for High-Resolution AFM: A Study of *Staphylococcus Aureus*. *J. Microsc.* **2010**, *238*, 102–110.
- (110) Dehullu, J.; Valotteau, C.; Herman-Bausier, P.; Garcia-Sherman, M.; Mittelviehhaus, M.; Vorholt, J. A.; Lipke, P. N.; Dufrêne, Y. F. Fluidic Force Microscopy Demonstrates that Homophilic Adhesion by *Candida Albicans* Als Proteins is Mediated by Amyloid Bonds Between Cells. *Nano Lett.* **2019**, *19*, 3846–3853.
- (111) Dörig, P.; Stiefel, P.; Behr, P.; Sarajlic, E.; Bijl, D.; Gabi, M.; Vörös, J.; Vorholt, J. A.; Zambelli, T. Force-Controlled Spatial Manipulation of Viable Mammalian Cells and Micro-Organisms by Means of FluidFM Technology. *Appl. Phys. Lett.* **2010**, *97*, 023701.
- (112) Holstein, T. W.; Benoit, M.; Herder, G. V.; David, C. N.; Wanner, G.; Gaub, H. E. Fibrous Mini-Collagens in *Hydra* Nematocysts. *Science* **1994**, *265*, 402–404.
- (113) Israelachvili, J. N. In *Intermolecular And Surface Forces*, 3rd ed.; Academic Press: San Diego, 2011; Chapter 10 - Unifying Concepts in Intermolecular and Interparticle Forces.
- (114) Harjumäki, R.; Zhang, X.; Nugroho, R. W. N.; Farooq, M.; Lou, Y.-R.; Yliperttula, M.; Valle-Delgado, J. J.; Österberg, M. AFM Force Spectroscopy Reveals the Role of Integrins and their Activation in Cell-Biomaterial Interactions. *ACS Appl. Bio. Mater.* **2020**, *3*, 1406–1417.
- (115) Chighizola, M.; Previdi, A.; Dini, T.; Piazzoni, C.; Lenardi, C.; Milani, P.; Schulte, C.; Podestà, A. Adhesion Force Spectroscopy with Nanostructured Colloidal Probes Reveals Nanotopography-Dependent Early Mechanotransductive Interactions at the Cell Membrane Level. *Nanoscale* **2020**, *12*, 14708–14723.
- (116) Alegre-Cebollada, J.; Perez-Jimenez, R.; Kosuri, P.; Fernandez, J. M. Single-Molecule Force Spectroscopy Approach to Enzyme Catalysis. *J. Biol. Chem.* **2010**, *285*, 18961–18966.
- (117) Harder, A.; Moller, A. K.; Milz, F.; Neuhaus, P.; Walhorn, V.; Dierks, T.; Anselmetti, D. Catch Bond Interaction Between Cell-Surface Sulfatase Sulf1 And Glycosaminoglycans. *Biophys. J.* **2015**, *108*, 1709–1717.
- (118) Renard, H.-F.; Tyckaert, F.; Lo Giudice, C.; Hirsch, T.; Valades-Cruz, C. A.; Lemaigre, C.; Shafaq-Zadah, M.; Wunder, C.; Wattiez, R.; Johannes, L.; et al. Endophilin-A3 and Galectin-8 Control the Clathrin-Independent Endocytosis Of CD166. *Nat. Commun.* **2020**, *11*, 1457.
- (119) Radmacher, M.; Cleveland, J. P.; Fritz, M.; Hansma, H. G.; Hansma, P. K. Mapping Interaction Forces with the Atomic Force Microscope. *Biophys. J.* **1994**, *66*, 2159–2165.
- (120) Heinz, W. F.; Hoh, J. H. Spatially Resolved Force Spectroscopy of Biological Surfaces Using the Atomic Force Microscope. *Trends Biotechnol.* **1999**, *17*, 143–150.
- (121) Rico, F.; Gonzalez, L.; Casuso, I.; Puig-Vidal, M.; Scheuring, S. High-Speed Force Spectroscopy Unfolds Titin at the Velocity of Molecular Dynamics Simulations. *Science* **2013**, *342*, 741–743.
- (122) Rigato, A.; Miyagi, A.; Scheuring, S.; Rico, F. High-Frequency Microrheology Reveals Cytoskeleton Dynamics in Living Cells. *Nat. Phys.* **2017**, *13*, 771–775.
- (123) Nievergelt, A. P.; Banterle, N.; Andany, S. H.; Gönczy, P.; Fantner, G. E. High-Speed Photothermal Off-Resonance Atomic Force Microscopy Reveals Assembly Routes of Centriolar Scaffold Protein SAS-6. *Nat. Nanotechnol.* **2018**, *13*, 696–701.
- (124) Ward, C. W.; Lawrence, M. C. Ligand-Induced Activation of the Insulin Receptor: A Multi-Step Process Involving Structural Changes in Both the Ligand and the Receptor. *BioEssays* **2009**, *31*, 422–434.
- (125) Popa, I.; Kosuri, P.; Alegre-Cebollada, J.; Garcia-Manyes, S.; Fernandez, J. M. Force Dependency of Biochemical Reactions Measured by Single-Molecule Force-Clamp Spectroscopy. *Nat. Protoc.* **2013**, *8*, 1261–1276.
- (126) Fernandez, J. M.; Li, H. Force-Clamp Spectroscopy Monitors the Folding Trajectory of a Single Protein. *Science* **2004**, *303*, 1674–1678.
- (127) Oberhauser, A. F.; Hansma, P. K.; Carrion-Vazquez, M.; Fernandez, J. M. Stepwise Unfolding of Titin under Force-Clamp Atomic Force Microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 468–472.
- (128) Schlierf, M.; Li, H.; Fernandez, J. M. The Unfolding Kinetics of Ubiquitin Captured with Single-Molecule Force-Clamp Techniques. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 7299–7304.
- (129) Garcia-Manyes, S.; Dougan, L.; Badilla, C. L.; Brujić, J.; Fernández, J. M. Direct Observation of an Ensemble of Stable Collapsed States in the Mechanical Folding of Ubiquitin. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 10534–10539.
- (130) Kosuri, P.; Alegre-Cebollada, J.; Feng, J.; Kaplan, A.; Ingles-Prieto, A.; Badilla, C. L.; Stockwell, B. R.; Sanchez-Ruiz, J. M.; Holmgren, A.; Fernandez, J. M. Protein Folding Drives Disulfide Formation. *Cell* **2012**, *151*, 794–806.
- (131) Wiita, A. P.; Perez-Jimenez, R.; Walther, K. A.; Gräter, F.; Berne, B. J.; Holmgren, A.; Sanchez-Ruiz, J. M.; Fernandez, J. M. Probing the Chemistry of Thioredoxin Catalysis with Force. *Nature* **2007**, *450*, 124–127.
- (132) Le Trong, I.; Aprikian, P.; Kidd, B. A.; Forero-Shelton, M.; Tchesnokova, V.; Rajagopal, P.; Rodriguez, V.; Interlandi, G.; Klevit, R.; Vogel, V.; et al. Structural Basis for Mechanical Force Regulation of the Adhesin Fimh Via Finger Trap-Like Sheet Twisting. *Cell* **2010**, *141*, 645–655.
- (133) Redondo-Morata, L.; Giannotti, M. I.; Sanz, F. AFM-Based Force-Clamp Monitors Lipid Bilayer Failure Kinetics. *Langmuir* **2012**, *28*, 6403–6410.
- (134) Krieg, M.; Helenius, J.; Heisenberg, C.-P.; Muller, D. J. A Bond for a Lifetime: Employing Membrane Nanotubes from Living Cells to Determine Receptor-Ligand Kinetics. *Angew. Chem., Int. Ed.* **2008**, *47*, 9775–9777.
- (135) White, A. D.; Nowinski, A. K.; Huang, W.; Keefe, A. J.; Sun, F.; Jiang, S. Decoding Nonspecific Interactions from Nature. *Chem. Sci.* **2012**, *3*, 3488–3494.
- (136) Meyer, E. E.; Rosenberg, K. J.; Israelachvili, J. Recent Progress in Understanding Hydrophobic Interactions. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 15739–15476.
- (137) Ricci, M.; Quinlan, R. A.; Voitchovsky, K. Sub-Nanometre Mapping of the Aquaporin-Water Interface Using Multifrequency Atomic Force Microscopy. *Soft Matter* **2017**, *13*, 187–195.
- (138) Butt, H. J. Measuring Electrostatic, Van Der Waals, and Hydration Forces in Electrolyte Solutions with an Atomic Force Microscope. *Biophys. J.* **1991**, *60*, 1438–1444.
- (139) Kim, D. T.; Blanch, H. W.; Radke, C. J. Direct Imaging of Lysozyme Adsorption onto Mica by Atomic Force Microscopy. *Langmuir* **2002**, *18*, 5841–5850.
- (140) Medalsy, I.; Hensen, U.; Muller, D. J. Imaging and Quantifying Chemical and Physical Properties of Native Proteins at Molecular Resolution by Force-Volume AFM. *Angew. Chem., Int. Ed.* **2011**, *50*, 12103–12108.
- (141) Müller, D. J.; Engel, A. The Height of Biomolecules Measured with the Atomic Force Microscope Depends on Electrostatic Interactions. *Biophys. J.* **1997**, *73*, 1633–1644.
- (142) Pfreundschuh, M.; Hensen, U.; Müller, D. J. Quantitative Imaging of the Electrostatic Field and Potential Generated by a Transmembrane Protein Pore at Subnanometer Resolution. *Nano Lett.* **2013**, *13*, 5585–5593.
- (143) Meckes, B.; Arce, F. T.; Connelly, L. S.; Lal, R. Insulated Conducting Cantilevered Nanotips and Two-Chamber Recording System for High Resolution Ion Sensing AFM. *Sci. Rep.* **2015**, *4*, 4454.
- (144) Aramesh, M.; Forró, C.; Dorwling-Carter, L.; Lüchtfeld, I.; Schlotter, T.; Ihle, S. J.; Shorubalko, I.; Hosseini, V.; Momotenko, D.; Zambelli, T.; et al. Localized Detection of Ions and Biomolecules with a Force-Controlled Scanning Nanopore Microscope. *Nat. Nanotechnol.* **2019**, *14*, 791–798.
- (145) Merkel, R.; Nassoy, P.; Leung, A.; Ritchie, K.; Evans, E. Energy Landscapes of Receptor-Ligand Bonds Explored with Dynamic Force Spectroscopy. *Nature* **1999**, *397*, 50–53.
- (146) Hinterdorfer, P.; Dufrêne, Y. F. Detection and Localization of Single Molecular Recognition Events Using Atomic Force Microscopy. *Nat. Methods* **2006**, *3*, 347–355.
- (147) Ott, W.; Jobst, M. A.; Schoeler, C.; Gaub, H. E.; Nash, M. A. Single-Molecule Force Spectroscopy on Polypeptides And Receptor-

Ligand Complexes: The Current Toolbox. *J. Struct. Biol.* **2017**, *197*, 3–12.

(148) Rankl, C.; Kienberger, F.; Wildling, L.; Wruss, J.; Gruber, H. J.; Blaas, D.; Hinterdorfer, P. Multiple Receptors Involved in Human Rhinovirus Attachment to Live Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 17778–17783.

(149) Zimmermann, J. L.; Nicolaus, T.; Neuert, G.; Blank, K. Thiol-Based, Site-Specific and Covalent Immobilization of Biomolecules for Single-Molecule Experiments. *Nat. Protoc.* **2010**, *5*, 975–985.

(150) Yang, B.; Liu, Z.; Liu, H.; Nash, M. A. Next Generation Methods for Single-Molecule Force Spectroscopy on Polyproteins and Receptor-Ligand Complexes. *Front. Mol. Biosci.* **2020**, *7*, 85.

(151) Koehler, M.; Lo Giudice, C.; Vogl, P.; Ebner, A.; Hinterdorfer, P.; Gruber, H. J.; Alsteens, D. Control of Ligand-Binding Specificity Using Photocleavable Linkers in AFM Force Spectroscopy. *Nano Lett.* **2020**, *20*, 4038–4042.

(152) Lo Giudice, C.; Zhang, H.; Wu, B.; Alsteens, D. Mechanochemical Activation of Class-B G-Protein-Coupled Receptor upon Peptide-Ligand Binding. *Nano Lett.* **2020**, *20*, 55757–5582.

(153) Schmitt, L.; Ludwig, M.; Gaub, H. E.; Tampé, R. A Metal-Chelating Microscopy Tip as a New Toolbox For Single-Molecule Experiments by Atomic Force Microscopy. *Biophys. J.* **2000**, *78*, 3275–3285.

(154) Pfreundschuh, M.; Alsteens, D.; Wieneke, R.; Zhang, C.; Coughlin, S. R.; Tampé, R.; Kobilka, B. K.; Müller, D. J. Identifying and Quantifying Two Ligand-Binding Sites while Imaging Native Human Membrane Receptors by AFM. *Nat. Commun.* **2015**, *6*, 8857.

(155) Theile, C. S.; Witte, M. D.; Blom, A. E. M.; Kundrat, L.; Ploegh, H. L.; Guimaraes, C. P. Site-Specific N-Terminal Labeling of Proteins Using Sortase-Mediated Reactions. *Nat. Protoc.* **2013**, *8*, 1800–1807.

(156) Walder, R.; Van Patten, W. J.; Adhikari, A.; Perkins, T. T. Going Vertical to Improve the Accuracy of Atomic Force Microscopy Based Single-Molecule Force Spectroscopy. *ACS Nano* **2018**, *12*, 198–207.

(157) Chen, G.; Ning, X.; Park, B.; Boons, G.-J.; Xu, B. Simple, Clickable Protocol for Atomic Force Microscopy Tip Modification and its Application for Trace Ricin Detection by Recognition Imaging. *Langmuir* **2009**, *25*, 2860–2864.

(158) Zhu, R.; Sinwel, D.; Hasenhuettl, P. S.; Saha, K.; Kumar, V.; Zhang, P.; Rankl, C.; Holy, M.; Sucic, S.; Kudlacek, O.; et al. Nanopharmacological Force Sensing to Reveal Allosteric Coupling in Transporter Binding Sites. *Angew. Chem., Int. Ed.* **2016**, *55*, 1719–1722.

(159) Schoeler, C.; Bernardi, R. C.; Malinowska, K. H.; Durner, E.; Ott, W.; Bayer, E. A.; Schulten, K.; Nash, M. A.; Gaub, H. E. Mapping Mechanical Force Propagation Through Biomolecular Complexes. *Nano Lett.* **2015**, *15*, 7370–7376.

(160) Sedlak, S. M.; Schendel, L. C.; Gaub, H. E.; Bernardi, R. C. Streptavidin/Biotin: Tethering Geometry Defines Unbinding Mechanisms. *Sci. Adv.* **2020**, *6* (13), eaay5999.

(161) Linke, W. A.; Kulke, M.; Li, H.; Fujita-Becker, S.; Neagoe, C.; Manstein, D. J.; Gautel, M.; Fernandez, J. M. PEVK Domain of Titin: An Entropic Spring with Actin-Binding Properties. *J. Struct. Biol.* **2002**, *137*, 194–205.

(162) Schwaiger, I.; Schleicher, M.; Noegel, A. A.; Rief, M. The Folding Pathway of a Fast-Folding Immunoglobulin Domain Revealed by Single-Molecule Mechanical Experiments. *EMBO Rep.* **2005**, *6*, 46–51.

(163) Schillers, H.; Rianna, C.; Schäpe, J.; Luque, T.; Doschke, H.; Wälte, M.; Uriarte, J. J.; Campillo, N.; Michanetzis, G. P. A.; Bobrowska, J.; et al. Standardized Nanomechanical Atomic Force Microscopy Procedure (SNAP) for Measuring Soft and Biological Samples. *Sci. Rep.* **2017**, *7*, 5117.

(164) Te Riet, J.; Katan, A. J.; Rankl, C.; Stahl, S. W.; Van Buul, A. M.; Phang, I. Y.; Gomez-Casado, A.; Schön, P.; Gerritsen, J. W.; Cambi, A.; et al. Interlaboratory Round Robin on Cantilever Calibration for AFM Force Spectroscopy. *Ultramicroscopy* **2011**, *111*, 1659–1669.

(165) Sader, J. E.; Borgani, R.; Gibson, C. T.; Haviland, D. B.; Higgins, M. J.; Kilpatrick, J. I.; Lu, J.; Mulvaney, P.; Shearer, C. J.; Slattery, A. D.; et al. A Virtual Instrument to Standardise the Calibration of Atomic Force Microscope Cantilevers. *Rev. Sci. Instrum.* **2016**, *87*, 093711.

(166) Sumbul, F.; Hassanpour, N.; Rodriguez-Ramos, J.; Rico, F. One-Step Calibration of AFM in Liquid. *Front. Phys.* **2020**, *8*.

(167) Neuman, K. C.; Nagy, A. Single-Molecule Force Spectroscopy: Optical Tweezers, Magnetic Tweezers and Atomic Force Microscopy. *Nat. Methods* **2008**, *5*, 491–505.

(168) Haupts, U.; Tittor, J.; Oesterhelt, D. Closing in On Bacterial Rhodopsin: Progress in Understanding the Molecule. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 367–399.

(169) Müller, D. J.; Heymann, J. B.; Oesterhelt, F.; Möller, C.; Gaub, H.; Büldt, G.; Engel, A. Atomic Force Microscopy of Native Purple Membrane. *Biochim. Biophys. Acta, Bioenerg.* **2000**, *1460*, 27–38.

(170) Pfreundschuh, M.; Alsteens, D.; Hilbert, M.; Steinmetz, M. O.; Müller, D. J. Localizing Chemical Groups While Imaging Single Native Proteins by High-Resolution Atomic Force Microscopy. *Nano Lett.* **2014**, *14*, 2957–2964.

(171) Evans, E.; Williams, P. Dynamic Force Spectroscopy. In *Physics of Bio-molecules and Cells*; Flyvbjerg, F., Jülicher, F., Ormos, P., David, F., Eds.; Springer, 2002.

(172) Bell, G. Models for the Specific Adhesion of Cells to Cells. *Science* **1978**, *200*, 618–627.

(173) Evans, E.; Ritchie, K. Dynamic Strength of Molecular Adhesion Bonds. *Biophys. J.* **1997**, *72*, 1541–1555.

(174) Friddle, R. W.; Noy, A.; De Yoreo, J. J. Interpreting The Widespread Nonlinear Force Spectra of Intermolecular Bonds. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 13573–13578.

(175) Ratto, T. V.; Rudd, R. E.; Langry, K. C.; Balhorn, R. L.; Mcelfresh, M. W. Nonlinearly Additive Forces in Multivalent Ligand Binding to a Single Protein Revealed with Force Spectroscopy. *Langmuir* **2006**, *22*, 1749–1757.

(176) Bullerjahn, J. T.; Sturm, S.; Kroy, K. Theory of Rapid Force Spectroscopy. *Nat. Commun.* **2014**, *5*, 4463.

(177) Amo, C. A.; Garcia, R. Fundamental High-Speed Limits in Single-Molecule, Single-Cell, and Nanoscale Force Spectroscopies. *ACS Nano* **2016**, *10*, 7117–7124.

(178) Kramers, H. A. Brownian Motion in a Field of Force and the Diffusion Model of Chemical Reactions. *Physica* **1940**, *7*, 284–304.

(179) Evans, E.; Ritchie, K.; Merkel, R. Sensitive Force Technique to Probe Molecular Adhesion and Structural Linkages at Biological Interfaces. *Biophys. J.* **1995**, *68*, 2580–2587.

(180) Claudia, F.; Angelika, K. W.; Ferdinand, K.; Hermann, E. G. Dynamic Single-Molecule Force Spectroscopy: Bond Rupture Analysis with Variable Spacer Length. *J. Phys.: Condens. Matter* **2003**, *15*, S1709–S1723.

(181) Ray, C.; Brown, J. R.; Akhremitchev, B. B. Correction of Systematic Errors in Single-Molecule Force Spectroscopy with Polymeric Tethers by Atomic Force Microscopy. *J. Phys. Chem. B* **2007**, *111*, 1963–1974.

(182) Löf, A.; Walker, P. U.; Sedlak, S. M.; Gruber, S.; Obser, T.; Brehm, M. A.; Benoit, M.; Lipfert, J. Multiplexed Protein Force Spectroscopy Reveals Equilibrium Protein Folding Dynamics and the Low-Force Response of Von Willebrand Factor. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 18798–18807.

(183) Koehler, M.; Aravamudhan, P.; Guzman-Cardozo, C.; Dumitru, A. C.; Yang, J.; Gargiulo, S.; Soumillion, P.; Dermody, T. S.; Alsteens, D. Glycan-Mediated Enhancement of Reovirus Receptor Binding. *Nat. Commun.* **2019**, *10*, 4460.

(184) Delguste, M.; Zeippen, C.; Machiels, B.; Mast, J.; Gillet, L.; Alsteens, D. Multivalent Binding of Herpesvirus to Living Cells is Tightly Regulated During Infection. *Sci. Adv.* **2018**, *4*, eaat1273.

(185) Rico, F.; Moy, V. T. Energy Landscape Roughness of the Streptavidin-Biotin Interaction. *J. Mol. Recognit.* **2007**, *20*, 495–501.

(186) Dudko, O. K.; Hummer, G.; Szabo, A. Intrinsic Rates and Activation Free Energies From Single-Molecule Pulling Experiments. *Phys. Rev. Lett.* **2006**, *96*, 108101.

(187) Cossio, P.; Hummer, G.; Szabo, A. Kinetic Ductility and Force-Spike Resistance of Proteins From Single-Molecule Force Spectroscopy. *Biophys. J.* **2016**, *111*, 832–840.

- (188) Makarov, D. E. Communication: Does Force Spectroscopy of Biomolecules Probe Their Intrinsic Dynamic Properties? *J. Chem. Phys.* **2014**, *141*, 241103.
- (189) Dudko, O. K.; Hummer, G.; Szabo, A. Theory, Analysis, and Interpretation of Single-Molecule Force Spectroscopy Experiments. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 15755–15760.
- (190) Rief, M.; Oesterhelt, F.; Heymann, B.; Gaub, H. E. Single Molecule Force Spectroscopy on Polysaccharides by Atomic Force Microscopy. *Science* **1997**, *275*, 1295–1297.
- (191) Alsteens, D.; Pfeundschuh, M.; Zhang, C.; Spoerri, P. M.; Coughlin, S. R.; Kobilka, B. K.; Müller, D. J. Imaging G Protein-Coupled Receptors while Quantifying their Ligand-Binding Free-Energy Landscape. *Nat. Methods* **2015**, *12*, 845–851.
- (192) Jarzynski, C. Nonequilibrium Equality for Free Energy Differences. *Phys. Rev. Lett.* **1997**, *78*, 2690–2693.
- (193) Crooks, G. E. Entropy production fluctuation theorem and the nonequilibrium work relation for free energy differences. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.* **1999**, *60*, 2721–2726.
- (194) Hummer, G.; Szabo, A. Free energy reconstruction from nonequilibrium single-molecule pulling experiments. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 3658–3661.
- (195) Liphardt, J.; Onoa, B.; Smith, S. B.; Tinoco, I.; Bustamante, C. Reversible Unfolding of Single RNA Molecules by Mechanical Force. *Science* **2001**, *292*, 733–737.
- (196) Collin, D.; Ritort, F.; Jarzynski, C.; Smith, S. B.; Tinoco, I.; Bustamante, C. Verification of the Crooks Fluctuation Theorem and Recovery of RNA Folding Free Energies. *Nature* **2005**, *437*, 231–234.
- (197) Oesterhelt, F.; Rief, M.; Gaub, H. E. Single Molecule Force Spectroscopy by AFM Indicates Helical Structure of Poly(Ethylene-Glycol) in Water. *New J. Phys.* **1999**, *1*, 6.
- (198) Sulchek, T.; Friddle, R. W.; Noy, A. Strength of Multiple Parallel Biological Bonds. *Biophys. J.* **2006**, *90*, 4686–4691.
- (199) Mulvihill, E.; Pfeundschuh, M.; Thoma, J.; Ritzmann, N.; Müller, D. J. High-Resolution Imaging of Maltoporin Lamb while Quantifying the Free-Energy Landscape and Asymmetry of Sugar Binding. *Nano Lett.* **2019**, *19*, 6442–6453.
- (200) Williams, P. M. Analytical Descriptions of Dynamic Force Spectroscopy: Behaviour of Multiple Connections. *Anal. Chim. Acta* **2003**, *479*, 107–115.
- (201) Hummer, G.; Szabo, A. Kinetics from Nonequilibrium Single-Molecule Pulling Experiments. *Biophys. J.* **2003**, *85*, 5–15.
- (202) Li, Y.; Xu, Y.; Kurths, J. First-Passage-Time Distribution in a Moving Parabolic Potential with Spatial Roughness. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.* **2019**, *99*, 052203.
- (203) Cossio, P.; Hummer, G.; Szabo, A. On Artifacts in Single-Molecule Force Spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 14248–14253.
- (204) Grandbois, M.; Beyer, M.; Rief, M.; Clausen-Schaumann, H.; Gaub, H. E. How Strong is a Covalent Bond? *Science* **1999**, *283*, 1727–1730.
- (205) Knoops, B.; Becker, S.; Poncin, M. A.; Glibert, J.; Derclaye, S.; Clippe, A.; Alsteens, D. Specific Interactions Measured by AFM on Living Cells Between Peroxiredoxin-5 and TLR4: Relevance for Mechanisms of Innate Immunity. *Cell. Chem. Biol.* **2018**, *25*, 550–559.
- (206) Formosa-Dague, C.; Fu, Z.-H.; Feuille, C.; Derclaye, S.; Foster, T. J.; Geoghegan, J. A.; Dufrene, Y. F. Forces Between *Staphylococcus Aureus* and Human Skin. *Nanoscale Horiz.* **2016**, *1*, 298–303.
- (207) Dague, E.; Alsteens, D.; Latgé, J.-P.; Verbelen, C.; Raze, D.; Baulard, A. R.; Dufrene, Y. F. Chemical Force Microscopy on Single Live Cells. *Nano Lett.* **2007**, *7*, 3026–3030.
- (208) Alsteens, D.; Trabelsi, H.; Soumillion, P.; Dufrene, Y. F. Multiparametric Atomic Force Microscopy Imaging of Single Bacteriophages Extruding from Living Bacteria. *Nat. Commun.* **2013**, *4*, 2926.
- (209) Roduit, C.; Van Der Goot, F. G.; De Los Rios, P.; Yersin, A.; Steiner, P.; Dietler, G.; Catsicas, S.; Lafont, F.; Kasas, S. Elastic Membrane Heterogeneity of Living Cells Revealed by Stiff Nanoscale Membrane Domains. *Biophys. J.* **2008**, *94*, 1521–1532.
- (210) Zhang, J.; Chtcheglova, L. A.; Zhu, R.; Hinterdorfer, P.; Zhang, B.; Tang, J. Nanoscale Organization of Human GnRH-R on Human Bladder Cancer Cells. *Anal. Chem.* **2014**, *86*, 2458–2464.
- (211) Li, M.; Liu, L.; Xi, N.; Wang, Y.; Xiao, X.; Zhang, W. Imaging and Measuring the Biophysical Properties of Fc Gamma Receptors on Single Macrophages Using Atomic Force Microscopy. *Biochem. Biophys. Res. Commun.* **2013**, *438*, 709–714.
- (212) Helenius, J.; Heisenberg, C.-P.; Gaub, H. E.; Müller, D. J. Single-Cell Force Spectroscopy. *J. Cell Sci.* **2008**, *121*, 1785–1791.
- (213) Selhuber-Unkel, C.; López-García, M.; Kessler, H.; Spatz, J. P. Cooperativity in Adhesion Cluster Formation During Initial Cell Adhesion. *Biophys. J.* **2008**, *95*, 5424–5231.
- (214) Panorchan, P.; Thompson, M. S.; Davis, K. J.; Tseng, Y.; Konstantopoulos, K.; Wirtz, D. Single-Molecule Analysis of Cadherin-Mediated Cell-Cell Adhesion. *J. Cell Sci.* **2006**, *119*, 66–74.
- (215) Friedrichs, J.; Helenius, J.; Müller, D. J. Stimulated Single-Cell Force Spectroscopy to Quantify Cell Adhesion Receptor Crosstalk. *Proteomics* **2010**, *10*, 1455–1462.
- (216) Oh, Y. J.; Hubauer-Brenner, M.; Gruber, H. J.; Cui, Y.; Traxler, L.; Siligan, C.; Park, S.; Hinterdorfer, P. Curli Mediate Bacterial Adhesion to Fibronectin via Tensile Multiple Bonds. *Sci. Rep.* **2016**, *6*, 33909.
- (217) Ng, G.; Sharma, K.; Ward, S. M.; Desrosiers, M. D.; Stephens, L. A.; Schoel, W. M.; Li, T.; Lowell, C. A.; Ling, C.-C.; Amrein, M. W.; et al. Receptor-Independent, Direct Membrane Binding Leads to Cell-Surface Lipid Sorting and Syk Kinase Activation in Dendritic Cells. *Immunity* **2008**, *29*, 807–818.
- (218) Meister, A.; Gabi, M.; Behr, P.; Studer, P.; Vörös, J.; Niedermann, P.; Bitterli, J.; Polesel-Maris, J.; Liley, M.; Heinzelmann, H.; et al. Fluid FM: Combining Atomic Force Microscopy and Nanofluidics on a Universal Liquid Delivery System for Single Cell Applications and Beyond. *Nano Lett.* **2009**, *9*, 2501–2507.
- (219) Bershadsky, A.; Kozlov, M.; Geiger, B. Adhesion-Mediated Mechanosensitivity: a Time to Experiment, and a Time to Theorize. *Curr. Opin. Cell Biol.* **2006**, *18*, 472–481.
- (220) Evans, E. A.; Calderwood, D. A. Forces and Bond Dynamics in Cell Adhesion. *Science* **2007**, *316*, 1148–1153.
- (221) Sahl, S. J.; Hell, S. W.; Jakobs, S. Fluorescence Nanoscopy in Cell Biology. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 685–701.
- (222) Vicidomini, G.; Bianchini, P.; Diaspro, A. STED Super-Resolved Microscopy. *Nat. Methods* **2018**, *15*, 173–182.
- (223) Hell, S. W. Microscopy and its Focal Switch. *Nat. Methods* **2009**, *6*, 24–32.
- (224) Odermatt, P. D.; Shivanandan, A.; Deschout, H.; Jankele, R.; Nievergelt, A. P.; Feletti, L.; Davidson, M. W.; Radenovic, A.; Fantner, G. E. High-Resolution Correlative Microscopy: Bridging the Gap Between Single Molecule Localization Microscopy and Atomic Force Microscopy. *Nano Lett.* **2015**, *15*, 4896–4904.
- (225) Eilers, Y.; Ta, H.; Wosch, K. C.; Balzarotti, F.; Hell, S. W. MINIFLUX Monitors Rapid Molecular Jumps with Superior Spatiotemporal Resolution. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 6117–6122.
- (226) Van Zanten, T. S.; Gómez, J.; Manzo, C.; Cambi, A.; Buceta, J.; Reigada, R.; Garcia-Parajo, M. F. Direct Mapping of Nanoscale Compositional Connectivity on Intact Cell Membranes. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 15437–15442.
- (227) Hinterdorfer, P.; Garcia-Parajo, M. F.; Dufrene, Y. F. Single-Molecule Imaging of Cell Surfaces Using Near-Field Nanoscopy. *Acc. Chem. Res.* **2012**, *45*, 327–336.
- (228) Hayazawa, N.; Inouye, Y.; Sekkat, Z.; Kawata, S. Metallized Tip Amplification of Near-Field Raman Scattering. *Opt. Commun.* **2000**, *183*, 333–336.
- (229) Mehtani, D.; Lee, N.; Hartschuh, R. D.; Kisliuk, A.; Foster, M. D.; Sokolov, A. P.; Maguire, J. F. Nano-Raman Spectroscopy with Side-Illumination Optics. *J. Raman Spectrosc.* **2005**, *36*, 1068–1075.
- (230) Stadler, J.; Schmid, T.; Zenobi, R. Nanoscale Chemical Imaging Using Top-Illumination Tip-Enhanced Raman Spectroscopy. *Nano Lett.* **2010**, *10*, 4514–4520.

- (231) Bustamante, C.; Macosko, J. C.; Wuite, G. J. L. Grabbing the Cat by the Tail: Manipulating Molecules one by one. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 130–136.
- (232) Sotomayor, M.; Schulten, K. Single-Molecule Experiments *In Vitro* And *In Silico*. *Science* **2007**, *316*, 1144–1148.
- (233) Urbanska, M.; Muñoz, H. E.; Shaw Bagnall, J.; Otto, O.; Manalis, S. R.; Di Carlo, D.; Guck, J. A Comparison of Microfluidic Methods For High-Throughput Cell Deformability Measurements. *Nat. Methods* **2020**, *17*, 587–593.
- (234) Wu, P.-H.; Aroush, D. R.-B.; Asnacios, A.; Chen, W.-C.; Dokukin, M. E.; Doss, B. L.; Durand-Smet, P.; Ekpenyong, A.; Guck, J.; Guz, N. V.; et al. A Comparison of Methods to Assess Cell Mechanical Properties. *Nat. Methods* **2018**, *15*, 491–498.
- (235) Rosendahl, P.; Plak, K.; Jacobi, A.; Kraeter, M.; Toepfner, N.; Otto, O.; Herold, C.; Winzi, M.; Herbig, M.; Ge, Y.; et al. Real-Time Fluorescence and Deformability Cytometry. *Nat. Methods* **2018**, *15*, 355–358.
- (236) Kuroski, D.; Dazzi, A.; Zenobi, R.; Centrone, A. Infrared and Raman Chemical Imaging and Spectroscopy at the Nanoscale. *Chem. Soc. Rev.* **2020**, *49*, 3315–3347.
- (237) Zheng, L.-Q.; Wang, X.; Shao, F.; Hegner, M.; Zenobi, R. Nanoscale Chemical Imaging of Reversible Photoisomerization of an Azobenzene-Thiol Self-Assembled Monolayer by Tip-Enhanced Raman Spectroscopy. *Angew. Chem., Int. Ed.* **2018**, *57*, 1025–1029.
- (238) Gwosch, K. C.; Pape, J. K.; Balzarotti, F.; Hoess, P.; Ellenberg, J.; Ries, J.; Hell, S. W. MINFLUX Nanoscopy Delivers 3D Multicolor Nanometer Resolution in Cells. *Nat. Meth.* **2020**, *17*, 217–224.
- (239) Hell, S. W. Far-Field Optical Nanoscopy. *Science* **2007**, *316*, 1153–1158.
- (240) Weiss, S. Fluorescence Spectroscopy of Single Biomolecules. *Science* **1999**, *283*, 1676–1683.