Multiparametric Atomic Force Microscopy Imaging of Biomolecular and Cellular Systems

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CONSPECTUS: There is a need in biochemical research for new tools that can image and manipulate biomolecular and cellular systems at the nanoscale. During the past decades, there has been tremendous progress in developing atomic force microscopy (AFM) techniques to analyze biosystems, down to the single-molecule level. Force–distance (FD) curve-based AFM in particular has enabled researchers to map and quantify biophysical properties and biomolecular interactions on a wide variety of specimens. Despite its great potential, this AFM method has long been limited by its low spatial and temporal resolutions. Recently, novel FD-based multiparametric imaging modalities have been developed, allowing us to simultaneously image the structure, elasticity and interactions of biological samples at high spatiotemporal resolution. By oscillating the AFM tip, spatially resolved FD curves are obtained at much higher frequency than before, and as a result, samples are mapped at a speed similar to that of conventional topographic imaging.

In this Account, we discuss the general principle of multiparametric AFM imaging and we provide a snapshot of recent studies showing how this new technology has been applied to biological specimens, from soluble proteins to membranes and cells. We emphasize novel methodologies that we recently developed, in which multiparametric imaging is combined with probes functionalized with chemical groups, ligands, or even live cells, in order to image and quantify receptor interaction forces and free-energy landscapes in a way not possible before. Key breakthroughs include observing the mechanical and chemical properties of single proteins in purple membranes, measuring the electrostatic potential of transmembrane pore forming proteins, structurally localizing chemical groups of water-soluble proteins, mapping and nanomechanical analysis of single sensors on yeast cells, imaging the sites of assembly and extrusion of single filamentous bacteriophages in living bacteria, unravelling the adhesive properties of biofilm-forming microbial pathogens, mapping the ligand-binding free energy landscape of human membrane receptors in proteoliposomes, and finally, the nanomechanical mapping of the first binding events of viruses to animal cells. In the coming years, it is anticipated that multiparametric AFM imaging will be increasingly used by chemists from broad horizons, enabling them to shed light into the sophisticated functions of biomolecular and cellular systems.

1. INTRODUCTION

Atomic force microscopy (AFM) techniques have offered a wealth of novel applications for characterizing biomolecular systems at the nanoscale.1–3 While AFM topographic imaging provides three-dimensional views of biological structures with unprecedented resolution, in real time, and under physiological conditions,4 force–distance (FD)-based AFM (also called force spectroscopy) enables one to quantify and map physical properties and molecular interactions. Force curves are obtained at a given (x,y) location by monitoring the cantilever deflection as a function of the vertical displacement of the piezoelectric scanner (Figure 1a).5,6 Raw data are presented as a "voltage–displacement" curve. Using the slope of the retraction force curve in the region where probe and sample are in contact, the photodiode voltage can be converted into a cantilever deflection (d). The cantilever deflection is then converted into a force (F) using Hooke’s law: F = −kd, where k is the cantilever spring constant.

The resulting FD curves (Figure 1b) provide information on mechanical properties, surface forces, and molecular binding strengths. At large tip–sample separation, there is no interaction and the deflection of the free cantilever defines the zero-force baseline. At shorter distance, surface forces cause the cantilever to bend and a jump to contact is sometimes observed. Upon contact between the tip and the sample, a deformation of the cantilever is observed, which may provide direct information on the mechanical properties of the sample. When the tip is withdrawn from the sample, the curve often shows a hysteresis referred to as the adhesion force, which depends on the area, time of contact, and on the surface energy between the tip and the sample. This adhesive interaction can be related to the surface energy or the binding forces between rigid surfaces or small molecules. In the case of long, flexible
molecules, an attractive force (or elongation force) develops up to large distances until rupture of the tip-molecule bond (Figure 1b). Functionalizing the AFM tip with specific antibodies or ligands makes it possible to quantify the strength of single receptor–ligands bonds. 7,8

In addition, FD curves can be recorded at multiple well-defined locations of the (x, y) plane (for more details, see ref 9) yielding spatially resolved FD maps of sample properties and molecular interactions (Figure 1c,d). In particular, in adhesion force mapping, the adhesion (unbinding) force value is estimated for each curve and then displayed as gray or colored pixels, where the brightness reflects the magnitude of the force. The use of biofunctionalized tips enables to localize and force probe specific receptors across the sample surface, a method also known as affinity or molecular recognition imaging. 10 Until recently, FD-based imaging had been strongly limited by poor temporal and lateral resolutions. For instance, recording an image at 30 nm resolution (32 × 32 pixels) usually takes 20 min, while increasing the resolution dramatically lowers the recording speed. This drawback has severely hampered the widespread use of the method in biochemical research. Solving this problem would increase the throughput of single-molecule analyses and enable statistically solid data sets to be obtained in short periods of time. Furthermore, this would permit studying dynamic processes in a way not accessible before, revealing for instance novel bond rupture mechanisms or unravelling single molecular bonds with short lifetimes.

2. MULTIPARAMETRIC AFM IMAGING

In the past years, there has been much progress in developing novel FD-based imaging methods with increased spatiotemporal resolution. 11 The speed of conventional FD-based AFM is limited by the linear movement of the piezo scanner. Using an oscillating movement has enabled to reach higher velocities, where the cantilever oscillates back and forth against the sample surface at high frequency. The feasibility of such dynamic force mapping was first provided by TREC imaging, a method developed by the Hinterdorfer team where topography and recognition images are recorded at the same speed as that used for conventional topographic imaging (1 frame/min). 12 Using magnetized cantilevers driven by a small solenoid, the tip is oscillated in the kHz range with small amplitude (nm range). The feedback loop is set up on the amplitude and the tip is scanned across the sample. Simultaneously to the topography, the recognition signal is obtained by extracting the rapid changes in the value of the positive-going peak of a filtered signal. This approach was successfully applied to map specific antibody–antigen interactions 12,13 in vitro and to map receptors directly on cells. 14 Yet a drawback is the indirect extraction of the interactions, meaning true FD curves are not provided along with the recognition image.

Several years later, the Sahin group introduced a second dynamic method based on tapping mode, in which the cantilever is oscillated at its resonance frequency. Using newly designed T-shaped cantilevers, the torsional harmonic is used to record tip–sample interactions. 15,16 This mode enables to monitor forces at every single oscillation cycle with sufficient signal-to-noise ratio. As a proof-of-principle, this so-called HarmoniX mode was use to investigate the interaction of biotin with streptavidin adsorbed onto mica. Variation of the loading rate was achieved by changing the vibration amplitude at a constant oscillation frequency and allowed exploration of the energy landscape of receptor–ligand interactions. Once the tip–sample–force waveform is determined, it is possible to reconstruct FD curves using the distance information in the vertical deflection signal. Then the curves are analyzed using adequate physical models to extract sample properties. Using this approach fiber networks used for tissue scaffolds were analyzed in term of topography, elastic modulus and stiffness at high-resolution. 17 Despite the power of this approach, its widespread use is limited by the sophisticated and time-consuming calibration of the T-shaped cantilevers.

Based on a similar principle, a new mode was introduced few years ago, which has the advantage of much simpler calibration procedure and analysis. As in the previous mode, the cantilever is oscillated in the z-axis in the kHz frequency range using either a sinusoidal 18 or rectangular 19 driving oscillation. Using standard AFM tips, this mode is based on the analysis of the z-deflection of the cantilever over time. Since the movement occurs at a frequency well below the resonance frequency, physical interactions between the tip and the sample will influence its deflection. By computing the background (due to the high frequency movements, drag force...) and executing a real time subtraction of measured force and background by a high-speed field-programmable gate array, the true tip surface interaction can be extracted and displayed as conventional FD curves. Then all usual parameters are directly extracted from the individual FD curves, simultaneously to the topography, and displayed as maps. In early studies, the technique was successfully applied to investigate complex biological samples such as membrane proteins 20 and cells. 21 Complementary to FD-based AFM, another approach emerged, where the cantilever is excited using simultaneous multifrequency modes. 22 Here, the cantilever is at the same

Figure 1. Force–distance curve-based AFM. (a) FD curves are obtained by moving the tip up and down at a given location of the sample surface. (b) Typical FD curve obtained for a single receptor–ligand bond. The approach curve is in blue and the retraction in red. The tip is initially away from the surface (right part) and comes into contact with the surface (left part). During retraction, a specific binding event (Fbind) is observed. (c, d) In FD-based imaging, spatially resolved curves are recorded in the x, y plane. A given parameter such as the adhesion force value is then displayed as gray pixels (the brighter the pixel, the stronger the adhesion).
time excited close to its resonance frequency with a small amplitude (nm range) and at a lower frequency with a larger amplitude (ten to hundreds of nm). The first excitation mode is used to image the topography of the surface while the other mode is used to measure changes in the mechanical or adhesion properties of the surface. While this technology has promise for imaging the multiple properties of materials, it is rather limited for quantitative affinity imaging as it basically lacks FD curves.

Hereafter we discuss recent studies from our teams where FD-based multiparametric imaging was used to probe the structure, properties, and interactions of biomolecular and material systems.
As extraction of the physicomechanical properties, thus revealing the existence of a two-layered polyelectrolyte brush, the fuzzy coat, surrounding the fibril core. The polyelectrolyte brushes consist of charged polypeptide chains with a dense inner and mechanically more rigid brush that protrude 8 nm from the fibril core and a second less dense and mechanically more flexible polymer brush layer formed by the remaining part of the polypeptide. This study thus demonstrates that human protein samples can be characterized at high-resolution in physiological conditions, and their polymer conformation modeled at the single-molecule level.

To bring specific information on protein assembly, functionalized tips may be used. By means of AFM tips bearing Ni\(^{2+}\)-nitrilotriacetate groups, specific interaction sites could be detected on the self-assembling soluble spindle assembly abnormal protein 6 homologue (SAS-6), a protein implicated in centriole duplication (Figure 3).\(^{24}\) After adsorption on mica, AFM showed a surface densely covered with a network of cartwheels like structures. Each spike corresponded to the C-terminal of the SAS-6 end and was engineered to bear at its end a His\(^{6}\)-tag. Maps recorded using functionalized tips showed sparsely adhesive events located close to the spike’s C-terminal end. The shape of individual FD-curves demonstrated the specificity of the interaction, i.e. adhesive forces of \(\approx\)120 pN and rupture distances from 4 to 22 nm. Correlation of AFM height images and adhesion maps showed that only a small proportion of the tagged C-terminal ends were detected by the tip. This observation can be due to several factors: (i) poor accessibility of the His-tag to the tip, (ii) too short contact time, or (iii) not ideal transient conformation of the NTA on the tip. However, repeated imaging allowed detection of accumulating binding events in most of the expected locations.

3. APPLICATIONS

Mapping the Structural, Mechanical, and Chemical Properties of Membrane Proteins

Already more than 20 years ago AFM has been applied to image and to characterize the structural, mechanical and electrostatic properties of native membrane proteins.\(^{22}\) To measure these properties by AFM was not straightforward and rather complex procedures had to be developed to be able to “map” these parameters to the membrane protein topography. Since the invention of FD-based AFM, the multiparametric imaging of membrane proteins to simultaneously probe their structure and biophysical properties has become much easier. Also FD-based AFM approaches have allowed us to become much more quantitative. The method has been applied to measure and to structurally map the deformation, adhesion, energy dissipation and elasticity of native membrane proteins at a resolution approaching one nanometre.\(^{23,24}\) It has also been possible to structurally map the electrostatic field and potential generated by transmembrane protein pores at subnanometer resolution.\(^{25}\) This field plays roles in guiding ions from or to the pore for translocation.

Imaging the Structure and Assembly of Soluble Proteins

AFM is widely used to observe soluble proteins in physiological solution. However, polypeptide chains can sometimes form fuzzy coats that are invisible in the conventional AFM imaging mode. FD-based AFM allows to image such proteins and to map their physicochemical properties at nanometer resolution. A good illustration of this is the investigation of Tau fibrils by AFM in physiological conditions (Figure 2).\(^{23}\) Tau can assemble into amyloid-like fibrillar aggregates that spread in hierarchical patterns in the Alzheimer’s disease brain during disease progression. Hexapeptide motifs facilitate the aggregation of Tau repeat domains in the fibril core, the largely unstructured and flexible N- and C-terminal parts protrude from the fibril core. High-resolution imaging of these fibrils revealed a structure with thickness of \(\approx\)16 nm corresponding to the size of the repeat domains while the largely unstructured terminal parts were invisible to the AFM tip. FD-based AFM was used to investigate the hTau40, the largest isoform of Tau protein in the human central nervous system (441 residues). Maps were recorded with bare silicon oxide tips at different pH, ion concentrations and ion compositions and allowed the extraction of the physicochemical properties, thus revealing the existence of a two-layered polyelectrolyte brush, the fuzzy coat, surrounding the fibril core. The polyelectrolyte brushes consist of charged polypeptide chains with a dense inner and mechanically more rigid brush that protrude 8 nm from the fibril core and a second less dense and mechanically more flexible polymer brush layer formed by the remaining part of the polypeptide. This study thus demonstrates that human protein samples can be characterized at high-resolution in physiological conditions, and their polymer conformation modeled at the single-molecule level.

Figure 4. Studying the localization, kinetics and thermodynamics of membrane receptor–ligand bonds. (a) Pixel-for-pixel FD-based AFM approaches and retracts the tip of an AFM cantilever from the sample to record interaction forces F over the tip–sample distance in FD curves. Therefore, the cantilever is oscillated in the kilohertz range (green curve). (b) Height image of individual PAR-1 receptors reconstituted into a lipid bilayer adsorbed on mica and corresponding adhesion image (c). (d) Dynamic force spectroscopy plot plotting the force required to separate the SFLLRN ligand from PAR-1 against the loading rate. Fitting the data using the Friddle–Noy–de Yoreo model\(^{29}\) provides kinetic and thermodynamic parameters of the receptor-ligand bond. Adapted with permission from ref 28. Copyright 2015 Springer.
Probing the Kinetics and Thermodynamics of Receptor–Ligand Interactions

Over the year, AFM has become a tool of choice to investigate interactions at the single molecule level and to extract the kinetic and thermodynamic properties of receptor–ligand interactions. Using biophysical methods, Evans et al. showed that the kinetics of a ligand receptor can be extracted by measuring the force required to rupture the ligand–receptor bond at different retraction speeds of the tip.26 By plotting the rupture force versus the loading rate (the increase of force over time), one can get access to the kinetic off rate and the distance to the transition state. However, in FD-based AFM, the driving oscillation of the tip confers a nonlinear movement to the tip and therefore a nonconstant speed and loading rate. Thus, depending at which time point of the retraction movement of the tip the ligand–receptor bond dissociates, the loading rate can vary by several orders of magnitude. FD-based AFM can detect these rupture forces of ligand-bonds over a wide range of loading-rates while imaging the receptors. Yet, to approach the detect these rupture forces of ligand-bonds over a wide range of can vary by several orders of magnitude. FD-based AFM can

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Probing Assembly and Binding Events of Viruses

New insights into the mechanisms of viral assembly and infection have also been gained. In a first study, single bacteriophages were detected and localized on the surface of living bacteria.26 Filamentous bacteriophages are capable to infect a variety of Gram-negative bacteria such as *Escherichia coli*. Currently, it is not yet fully understood how exactly single phages assemble in and escaping from the bacterial cell wall. *E. coli* bacteria infected by His-tagged bacteriophages were imaged with tips functionalized with Ni2+–NTA groups. Directly correlated multiparametric images of the structure, adhesion and elasticity of the bacteria revealed that the sites of the assembly machinery localize at the bacterial septum in the form of soft nanodomains surrounded by stiff cell wall material. A model was suggested where the extrusion nanodomains may interfere with peptidoglycan assembly, leading to the accumulation of newly formed cell wall material around these sites.

Recently, AFM tips functionalized with single-viruses were used to force probe interaction binding sites directly on living mammalian cells.31 EnvA-pseudotyped rabies viruses were grafted to the apex of the tips and brought in contact with a confluent layer of Madin-Darby canine kidney (MDCK) cells expressing TVA receptors coupled to a ds-red fluorophore. As an internal control, those cells were mixed with nonexpressing cells. FD-based AFM showed specific adhesion events only on the fluorescent cells, assessing the specificity of the cells. Furthermore, analysis of the adhesion events allowed determining the binding-free energy of the interactions.

Mapping the Adhesion and Elasticity of Microbes

The nanobiophysical properties of microbial cells have also been investigated, with the aim to better understand cellular function.32 An interesting example of such measurements is the imaging of the bacterial pathogen *Staphylococcus aureus*. *S. aureus* forms biofilms on indwelling medical devices using a variety of surface proteins, among which SasG which promotes cell–cell adhesion. Nanoscale images of living bacteria showed that Zn2+ ions induce a major remodelling of the cell surface; that is, the surface becomes smoother, stiffer, and stickier in the presence of zinc (Figure 5).33 Force measurements demonstrated that SasG mediates Zn2+-dependent homophilic bonds between cells, and that the individual proteins were mechanically strong, a behavior expected to help adhering

Figure 5. Imaging the nanoscale adhesion and elasticity of living *S. aureus* bacteria. (a, b) Height images of two dividing *S. aureus* cells expressing SasG proteins in TBS buffer in the absence (a) or presence (b) of zinc ions, and simultaneous elasticity (c, d) and adhesion (e, f) images. Scale bars: 1 μm. Adapted with permission from ref 33.

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bacteria to resist physiological shear forces. Collectively, the results strongly suggest that zinc plays a dual role in activating cell–cell adhesion during biofilm formation.

The cationic polysaccharide intercellular adhesin (PIA) forms an extracellular matrix that connects the cells together in S. aureus biofilms but the molecular forces involved are poorly understood. Multiparametric imaging revealed that PIA-producing bacteria are surrounded by a soft and adhesive matrix of extracellular polymers, while mutant cells deficient for the synthesis of PIA showed much lower softness and adhesion.34 Using force spectroscopy, it was found that PIA promotes cell–cell adhesion via the multivalent electrostatic interaction with polyanionic teichoic acids on the S. aureus cell surface.

To provide chemical and biological specificity on microbial cell surfaces, FD-based AFM has been combined with the use of chemically modified tips. For instance, this enabled to map hydrophobic forces on the human opportunistic pathogen Aspergillus fumigatus.35 Adhesion images recorded simultaneously to the topography showed strongly heterogeneous contrast, in the form of well-defined hydrophobic patches displaying strong adhesion forces, surrounded by hydrophilic material. Hydrophobic forces were shown to originate from regularly arranged rodlets composed of hydrophobins, a family of hydrophobic proteins that favor spore dispersion by air currents and mediate adherence to host cells. A similar approach was used to detect single sensor proteins on yeast cells, and to probe their elasticity in relation to mechanosensing.35 Imaging can also be performed using an AFM cantilever functionalized with a living cell, an approach that enabled to localize and quantify the nanoscale interaction forces between single S. aureus bacteria and human skin.36 Adhesion images recorded on skin corneocytes using a single bacterial probe featured strong adhesion forces (∼500 pN; Figure 6). Analysis of a bacterial mutant lacking all adhesion proteins strongly suggested that the S. aureus–corneocyte adhesion forces are due to the specific interaction between adhesins on the bacterial cell surface and target ligands on the corneocyte surface. This new method offers a means to quantitatively map interaction forces of host surfaces using a single bacterium as a probe.

In summary, the studies surveyed here demonstrate that multiparametric imaging, using either bare tips or tips functionalized with chemical groups, ligands, viruses, or cells, has recently offered exciting new possibilities to quantitatively map the biophysical properties of complex biomolecular and cellular systems in physiological conditions.

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**Notes**

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**ACKNOWLEDGMENTS**

D.A. was supported by the Université catholique de Louvain (Fonds spéciaux de Recherche) and the National Fund for Scientific Research (FNRS). D.J.M. was supported by the Swiss National Science Foundation (SNF; Grants 205320_160199 and 310030B_160225 to D.J.M.), the NCCR Molecular Systems Engineering and the Swiss Commission for Technology and Innovation (CTI, Grant 17970.1). Y.F.D. was supported by the Université catholique de Louvain, the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant Agreement No [693630]), the WELBIO (Grant n°WELBIO-CR-2015A-05), the FNRS, the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the Research Department of the Communauté française de Belgique (Concerted Research Action). D.A. and Y.F.D. are Research Associate and Research Director at the FNRS, respectively.

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