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Review

High-speed atomic force microscopy: Imaging and force spectroscopy

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ABSTRACT

Atomic force microscopy (AFM) is the type of scanning probe microscopy that is probably best adapted for imaging biological samples in physiological conditions with submolecular lateral and vertical resolution. In addition, AFM is a method of choice to study the mechanical unfolding of proteins or for cellular force spectroscopy. In spite of 28 years of successful use in biological sciences, AFM is far from enjoying the same popularity as electron and fluorescence microscopy. The advent of high-speed atomic force microscopy (HS-AFM), about 10 years ago, has provided unprecedented insights into the dynamics of membrane proteins and molecular machines from the single-molecule to the cellular level. HS-AFM imaging at nanometer-resolution and sub-second frame rate may open novel research fields depicting dynamic events at the single bio-molecule level. As such, HS-AFM is complementary to other structural and cellular biology techniques, and hopefully will gain acceptance from researchers from various fields. In this review we describe some of the most recent reports of dynamic bio-molecular imaging by HS-AFM, as well as the advent of high-speed force spectroscopy (HS-FS) for single protein unfolding.

1. Introduction

Atomic force microscopy (AFM) was invented in 1986 [1], and probably is the best-known offspring in the family of scanning probe microscopies, at least in biological sciences. AFM frequently draws comparisons with another revolutionary invention that predates it by at least 100 years, the gramophone: Indeed, in its primary operation mode (also known as 'contact mode'), AFM measures the topography of a surface by scanning it horizontally with a sharp tip placed at the extremity of a cantilever. Deflections of the cantilever arising from interactions of the tip with bumps and clefts of the surface are detected using the 'optical lever' system [2], in which a laser beam is reflected on the backside of the cantilever towards a segmented photodiode. Any angle change as a result of cantilever deflection results in a signal change on the photodiode: To preserve both the tip and sample integrity, the cantilever deflection (in other words the tip-sample interaction force) is maintained to a constant setpoint value. Cantilever deflection therefore translates into a vertical motion of the tip (or the sample) to restore the setpoint force using feedback control. When utilized on solid surfaces (for example graphite), AFM is able to resolve single atoms [3,4], for review see [5]. This astounding vertical and lateral resolution capability – still a hallmark of AFM to this day – is determined not only by the detection system, but first and foremost by the dimensions of the apex of the scanning tip and the ability of piezoelectric actuators to apply sub-nanometer displacements to the cantilever in response to small deviations of the detected signal from the setpoint. An important step of development, enabling biological AFM, was the development of a liquid cell in which cantilever, tip and sample are permanently immersed in buffer solution [6]. However, in the case of biological samples, softer than graphite by orders of magnitude, non-destructive imaging at nanometer-resolution in physiological conditions (i.e. in aqueous buffer, at ambient temperature and pressure) requires the ability to control forces $\leqslant$ 100 pN. With a high optical lever sensitivity, one is able to control normal forces applied by the cantilever down to about 50 pN in liquid, enabling contact mode imaging of biological samples. Thus, although lateral scanning forces impose a restriction on the sample type to be imaged in contact mode, it has yielded fascinating insights into the organization of proteins in biological membranes [7–13].

In addition, AFM has opened novel research avenues in 'force spectroscopy' mode, where the tip indents and retracts from a sample with controlled force and velocity. This mode of operation allowed mechanical properties of cells to be determined [14,15], and the study of receptor/ligand unbinding and protein unfolding at the single molecule level [16–19].

Abbreviations: HS-AFM, high-speed atomic force microscopy; HS-FS, high-speed force spectroscopy

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Concomitant to these achievements, the advent of 'intermittent contact mode' (also known as 'AC mode' or 'tapping mode') extended the capacities of bio-imaging by AFM. Operated in this mode, AFM can scan fragile and loosely attached biological samples by reducing the tip-sample interaction time and eliminating friction forces. In theory, an AFM cantilever may be considered as a harmonic oscillator that can be brought into resonance by acoustic excitation. In AC mode, the oscillation amplitude may be used as feedback parameter, as it decreases upon contact of the tip with the sample. If the amplitude of the cantilever in contact is the setpoint value, then normal and lateral scanning forces are largely decreased if the setpoint is close to the free amplitude (i.e. when the tip is out of contact). In such operational conditions the sample is contacted by the tip only very briefly, during about 10% of the time interval at the bottom of each oscillation cycle. With that in hand, AFM operators could image a variety of soft biomolecules in their physiological environment, label-free, at a topography resolution close to that of cryo-electron microscopy.

However, AFM was severely limited by its image acquisition rate in AC mode (also in contact mode, though this is slightly faster), at least an order of magnitude slower than most biological processes. In intermittent contact modes the cantilever oscillates at resonance and height detection is based on the oscillation amplitude measurement, as a consequence the operation speed is primarily limited by the resonance frequency of the cantilever. That limit is in turn dictated by the cantilever dimensions, following the relation (for rectangular cantilevers only):

$$f_0 = \frac{1}{2\pi}\sqrt{\frac{k}{m}}, \qquad \text{and} \qquad k = E \cdot t^3 \cdot w/4L^3$$

where k is the spring constant, m the mass of the cantilever, and t, w, and L, the thickness, width and length of the cantilever and E the Young's modulus of the cantilever material [20].

Most commercially available AFMs are built to accommodate 50–500 μm long cantilevers, and as a consequence bio-imaging at nanometric resolution is generally achieved within a timescale of one to several minutes, thereby limiting bio-AFM imaging to structural applications of stable samples. Fortunately, the speed limitations of AFM was possible to be overcome: With cantilevers of the smallest possible size, yet with soft enough spring constant, high resolution scanning of biological samples at video rate, termed high-speed atomic force microscopy (HS-AFM), in physiological conditions is now possible [21].

First efforts in this direction pushed the image acquisition rate to 1 Hz [22], but further developments to increase the feedback response speed, improve the piezo scanner speed and stability, miniaturize the cantilever and optimize the optical detection system were required in order to push the scan rate below 1 Hz [23–25]. More than 10 years of technical development were required to achieve this and perform high-speed scanning with fast feedback response (the bandwidth of feedback control now reaches 100 kHz), controlling displacements in horizontal and vertical directions with nanometer accuracy, and to engineer soft ($k \sim 100$ pN/nm) and small ($\sim 2 \times 6$ μm) cantilevers with ~1 MHz resonance frequency in liquid. Ultimately, video-speed AFM imaging of biomolecular dynamics of single molecules at nanometric resolution became reality [26]: This remarkable feat was illustrated by direct imaging of Myosin-V walking on actin filaments [27]. Importantly, whereas the displacement of a fluorescent spot is observed in optical microscopy, or noise changes in an optical trapping experiment, in dynamic HS-AFM the molecule itself is visualized while working and moving on its biological track, providing concomitant structural and dynamic data. Besides the visual power of those movies, the insights that they have provided into the mechanics of myosin-V is unmatched by any other biophysical method: Not only did the observation confirm the hand-over-hand walking mechanism of myosin-V, it did reveal that the power stroke of this motor is driven by intramolecular mechanical tension [28]. Another nice example of direct observation of processive enzymatic action was cellulose processing by the enzyme cellulase, showing that pre-processing of cellulose may increase its conversion efficiency by reducing the occurrence of crowding-induced enzymatic 'halts', with important implications in biofuel engineering [29]. HS-AFM also enabled direct imaging of single molecule diffusion on and in membranes [30–32], amyloid fibril assembly [33], and the rotary catalysis of ATPase [34]. In spite of those breakthroughs, in which HS-AFM provided novel insights into fundamental biological problems probably inaccessible to any other technique that does not analyze structure and dynamics concomitantly, the technique remains little known to many biologists, and has gained only little recognition outside of biophysics. This is on the one hand due to the fact that the technique is young and complex, and only experts are able to take full advantage of its capabilities. On the other hand, the technique is still lacking standards and established sample preparation procedures. Today still, each bio-sample to be studied represents a full novel project for which preparation and analysis conditions must be established from scratch. These are major obstacles for a more generalized use in biology. For other techniques, like electron microscopy, researchers have invested considerable efforts to overcome such bottlenecks. Clearly, while the novel capabilities that HS-AFM offers – concomitant nanometer resolution real-space information and dynamics – makes the technique unique and complementary to other structural biology techniques, in order for AFM to be more widely used and accepted, these bottlenecks must be overcome.

In this review, we will show that in the last couple of years HS-AFM has demonstrated its power for combined dynamic structural and functional analysis on biological systems of increasing complexity from purified membranes to live cells, with tremendous potential to enhance its present capabilities through further technological development and improved amenability. We hope that this short review will motivate researchers from various fields to contribute to the efforts undertaken to make HS-AFM a widely used tool in biological sciences.

2. Main text

2.1. HS-AFM imaging: from isolated membranes to cells

AFM being a surface imaging technique, its usefulness in the characterization of membrane proteins in flat lipid bilayers comes naturally. Fundamental processes in the biology of prokaryotes and eukaryotes start at the cell membrane: those encompass passive and active transport of molecules, generation of a transmembrane potential and energy, signal transduction cascades, to name a few.

Membrane proteins have been – and are still to some extent – the orphans of structural and molecular biology. It took 25 years from the first soluble protein (myoglobin) X-ray structure [35] to the first membrane protein (reaction center) atomic structure determination [36]. At the date this review is written, scientists dispose of 1454 membrane protein structures of 464 unique proteins [37], while a total of 99293 structures were deposited in the protein data bank (PDB, [38]). Hence the structural information on membrane proteins of ~1% of all structures is still in unfavorable relation to the ~25% of all genes coding for membrane proteins [39].

Beyond structural analysis, the lateral organization of membrane proteins in the cell membrane, as well as the diffusion properties of individual proteins and/or the formation/dissociation of protein clusters have been recognized of crucial importance and