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High-speed atomic force microscopy: Structure and dynamics of single proteins

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For surface analysis of biological molecules, atomic force microscopy (AFM) is an appealing technique combining data acquisition under physiological conditions, for example buffer solution, room temperature and ambient pressure, and high resolution. However, a key feature of life, dynamics, could not be assessed until recently because of the slowness of conventional AFM setups. Thus, for observing bio-molecular processes, the gain of image acquisition speed signifies a key progress. Here, we review the development and recent achievements using high-speed atomic force microscopy (HS-AFM). The HS-AFM is now the only technique to assess structure and dynamics of single molecules, revealing molecular motor action and diffusion dynamics. From this imaging data, watching molecules at work, novel and direct insights could be gained concerning the structure, dynamics and function relationship at the single bio-molecule level.

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Introduction

The atomic force microscope (AFM; [1]) is able to resolve atoms on solid surfaces [2]. Since the development of a fluid cell [3], AFM has become a powerful structural analysis tool in biology [4]. It has been extensively used for imaging protein surface structure under close-to-native conditions and also as a force probe tool [5]. Therefore, it has become a complementary technique to X-ray crystallography, NMR and electron microscopy [6].

As main advantages, (i) the high signal-to-noise ratio allowing single molecule imaging [7,8] and (ii) the physiological environment, ambient temperature and pressure in liquid [6], have been recognized. These two advantages are trump cards compared to electron microscopy, which works under vacuum conditions and low temperatures and needs to make use of extensive image processing for structure assessment. These two advantages of the AFM would in principle also allow watching single molecules at work, but unfortunately conventional AFM setups with image acquisition rates of several minutes (Figure 1, left) impeded the analysis of relevant biological dynamic processes.

Around the year 2000, first reports have been made about AFMs with increased scan rate [9,10••,11,12••]. Key adaptations for scan speed increase comprise the miniaturization of the moving parts of the AFM setup, these are the scanner and the cantilever, because the speed at which elements can be moved with accuracy is proportional to the inverse of the square root of their mass.

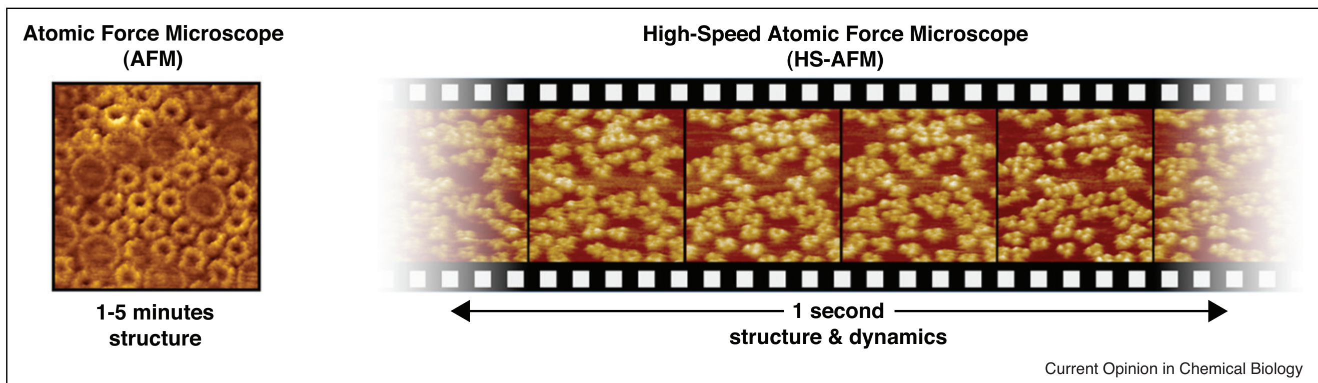
First, small and light cantilevers have been developed that have typically resonance frequencies (ω_0) between 500 kHz and 1 MHz in liquid [9]. Indeed, the resonance frequency depends on the spring constant (k) and the mass (m) of the cantilever, following:

$$\omega_0 = \sqrt{\frac{k}{m}} \text{ with, } k = \frac{Ewt^3}{4L^3} \quad (1)$$

where the spring constant depends on the elastic properties (E), the width (w), and strongly on the thickness (t) and length (L) of the cantilever. Given that softness, low spring constant, is a prerequisite for bio-molecular imaging, the length and mass had to be significantly reduced. HS-AFM cantilevers have typical lengths of 5–10 μm (conventional cantilevers of 100–200 μm [13]). Another advantage of such short levers is the sensitivity of the optical beam detection [14] system, consisting of a laser focused on and reflected by the backside of the cantilever and detected by a split photodiode.

Second, scanners composed of individual X, Y, and Z piezos have been designed. These scanners must fulfil several criteria, such as high resonance frequency, few resonant peaks at similar frequency, small quality factor, small cross-talk, while assuring a reasonable scan range sufficient for bio-molecular surface analysis [15,16]. For stability, HS-AFM scanners contain dummy piezo elements (these are piezos that counterbalance the movement of the essential piezos by moving into the opposite direction) and a dummy sample stage [12••]. Crucial for fast surface contouring is a small Z-piezo with high resonance frequency around 400 kHz [16,17]. The reaction response of the Z-piezo is accelerated using a dynamic feedback-controller that alters automatically

Figure 1



High-speed atomic force microscopes (HS-AFM) acquire topographic data with an approximate three orders of magnitude increased speed compared to conventional atomic force microscope (AFM) setups. Hence while taking individual image frames with conventional AFMs (frame acquisition time: 1–5 min; image shows the photosynthetic apparatus of *Rhodospirillum photometricum* adapted from Refs. [8,13]), now movies can be acquired using HS-AFMs (frame acquisition time: 50–500 ms; image series shows OmpF trimers adapted from Ref. [20]). Thus, using HS-AFM, single molecule dynamics can be captured.

the gain parameters to follow steep surface features and with a bandwidth of about 100 kHz on faint surface corrugation in the nanometer range, for example protein size [18]. All the developments have been described in detail in Ref. [19].

Featuring these key elements and some more adaptations it is now possible to acquire movies at rates of about 10 frames/s and about 200 data points squared per frame. For bio-molecular observations where a scan area of about 100 nm is requested, the achievable resolution is hence 1 nm, allowing the visualization of single molecules processes at submolecular resolution (Figure 1, right). These features allow for the first time the concomitant observation of single molecules at work under close to physiological conditions, providing a fantastic opportunity for researchers to establish structure-function relationships and to determine biophysical parameters of single molecules previously inaccessible by other techniques.

Life is dynamic

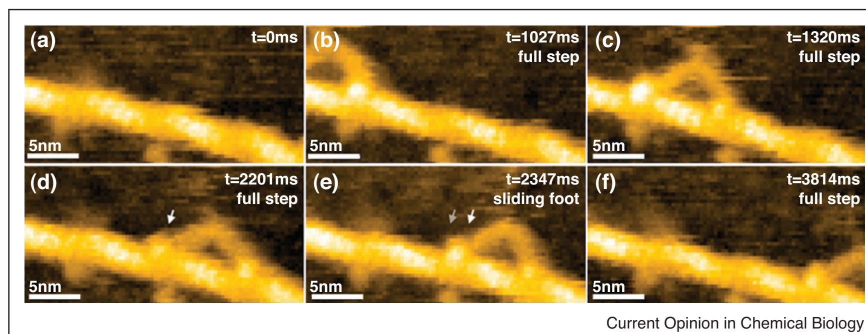
Three qualities define life: reproduction, self-sustainable metabolism and mutation. These processes imply dynamic action, such as enzyme catalysis and transport function by molecular machines. These processes are often driven by cellular energy, while other processes essential for life, like diffusion, are thermally driven. Studying the dynamics is therefore essential for the comprehension of biology.

Biological structure can be studied by light microscopy, electron microscopy, NMR and X-ray diffraction from cells to molecules. In contrast, dynamics remained only accessible at low resolution using video light microscopy of labelled molecules. The HS-AFM with its capability of observing molecules at work opens therefore a new research area that will have an important impact in structural biology, biophysics and biochemistry.

Molecular motors at work

Molecular motors use cellular energy stored in the form of ATP or GTP to perform work or transform potential energies such as membrane gradients into transport or storable energy forms [21]. Paradigm molecular motors are proteins of the Myosin family [22]. They interact with elements of the cytoskeleton (actin), mediate contraction and transport cargo along these filaments inside cells. Researchers have early been attracted by these molecules because of their structure with two 'legs' and 'feet', as visualized early by electron microscopy [23], suggesting a walking movement not so different from humans walking. Single molecule tracking experiments confirmed that myosins walk in discrete steps. However, the detailed mechanism of the movement of the molecules eluded investigators for a while and several walking hypotheses have been discussed [24]. Now, HS-AFM has been used to study the walking mechanism of myosin-V along actin filaments at the single molecule level with unprecedented resolution, confirming indirectly measured mechanisms and providing novel insights to myosin-V function (Figure 2). It has been shown that the myosin-V takes ~36 nm steps at an ATP-dependent velocity of maximally ~400 nm/s. The walking mechanism is hand-over-hand and implies the leg of the leading head acting like a swinging lever (Figure 2b) and a rotation of the leading head [25••]. Foot 'stomping', detachment and reattachment of a foot from and to the actin filament were captured. In some cases the 'stomping' was accompanied by a slide of the foot, where it occupies a slightly shifted

Figure 2



Molecular motor motion dynamics of myosin-V walking along an actin filament. Movie parameters are: 147 ms/frame (6.8 frames/s), 130 nm × 65 nm scan size at 80 × 40 pixels. **(a)**–**(d)** and **(f)** Full step of myosin-V motor. **(e)** Sliding of trailing head (arrows in **(d)** and **(e)**).
Figure adapted from Ref. [25••].

position on the actin filament after 'stomping' (Figure 2e). Occasionally, unwinding of the coiled–coiled domain at the connection of the two legs of myosin-V has been observed [25••].

Unlabelled membrane protein diffusion in the molecular environment

Membrane diffusion phenomena have been early and largely studied by fluorescence recovery after photobleaching (FRAP) studies [26]. This technique requires fluorescently labelled membrane molecules that are distributed and bleached over large areas, and the recovery of fluorescence is observed, indirectly documenting that non-bleached molecules diffuse into the prior bleached area. This is an ensemble technique and provided early valuable data about differences of molecular diffusion behaviour, however, today we know that this ensemble technique could not assess non-linear diffusion behaviour or differences of diffusion of single molecules [27].

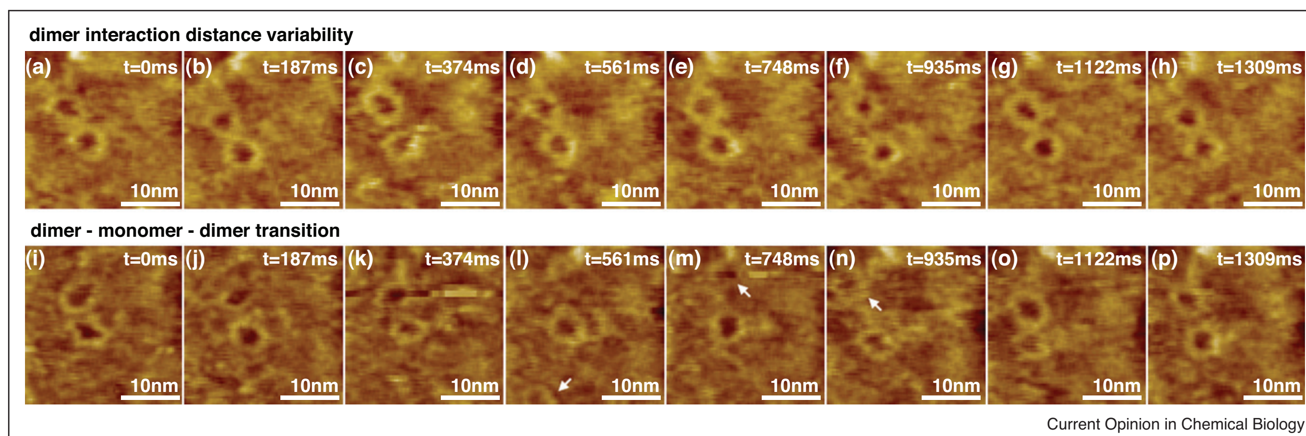
Single molecule fluorescence labelling and single particle tracking have shaped during the past ten years our view of the membrane structure [28•,29•]. Single membrane components are labelled via an antibody carrying a fluorescence dye or a quantum dot. Given only few molecules are labelled they can be individually tracked fitting the fluorescence signal that has a size about 50 times larger than a molecule with nanometer precision [30]. Such experiments have considerably advanced our knowledge about diffusion behaviour of membrane components, and have been interpreted in terms of membrane structure [28•,29•]. However, single fluorescence molecule tracking experiments do not 'see' the membrane, and do not reveal the molecular environment of the molecule that is tracked. Now, HS-AFM offers new perspectives for membrane protein diffusion analysis, as the molecule itself, unlabelled, and its molecular environment can be directly visualized.

The proof of concept and potential of HS-AFM imaging of moving unlabelled membrane proteins has been illustrated on ATP-ase c-rings in the purple membrane [31•]. The purple membrane consists of large amounts of crystalline bacteriorhodopsin (bR), a light-driven proton-pump that works together with the ATP-ase, using the created proton gradient, for ATP synthesis. bR has been studied by conventional AFM in hundreds of studies for over fifteen years [32], but the ATP-ase remained undetected throughout the years. Recently, HS-AFM was used for studying bR and provided novel insights into its function and dynamics such as (i) the dynamic vacancy motion in the bR lattice [33] and at the edges [34], and (ii) the cooperative structural change of bR in response to a light illumination [35]. Along with the studies of bR, ATP-ase c-rings have been visualized for the first time in the purple membrane (Figure 3). At 187 ms frame rate c-rings dimers have been observed in their dynamic ensemble behaviour (Figure 3a–h), and during a dimer–monomer–dimer transition (Figure 3i–p). These direct observations of interactions and transitions of unlabelled membrane protein movement, represent unprecedented experimental data on membrane-mediated protein–protein interaction. The data provided experimental basis for the 'hydrophobic mismatch' contributing to the interaction between membrane proteins, and allowed the description of an interaction energy landscape that has a strength of $3.5k_{\mathrm{B}}T$ and reaches out ~5 nm into the membrane [31•].

Current limitations and perspectives

Often the question is raised if scanning so fast does not imply more important and eventually destructive tip–sample interactions. The answer is clearly no. Indeed, the current HS-AFM setup controls better the applied forces and scan-related interactions than conventional AFMs. The fast and slow scan axes, and change of the scanning frequency, do not influence protein movement direction and occurrence. The maximal applied force

Figure 3



ATP-synthase c-ring diffusion movement in native purple membrane. Movie parameters are: 187 ms/frame (5.3 frames/s), 30 nm × 30 nm scan size at 76 × 76 pixels. **(a)–(h)** Dimer association fluctuations. **(i)–(p)** Dimer–monomer–dimer transition (arrows in **(l)**, **(m)**, **(n)** depict dissociating and associating monomers).
Figure adapted from Ref. [31•].

during tip-sample contact in oscillating mode can be estimated as $F = k\Delta z$, where the spring constant k is ~0.1 N/m and the cantilever bending Δz is ~0.1 nm (the setpoint is defined as 0.9 times the free amplitude A_0 that is 1 nm) giving an applied force of ~10 pN. The response time (τ) of HS-AFM cantilevers, can be expressed as $\tau = Q/\pi\omega_0$, where Q is the quality factor of the cantilever and ω_0 is the resonance frequency. The Q factor is typically around 2 (in liquid) and ω_0 is about 1 MHz, hence the response time is less than 1 μs, less than the delay of one full oscillation cycle. Given that the cantilever oscillates at about 1 MHz (1 μs/oscillation cycle) and the contact is applied only during about 10% of the cantilever oscillation, hence about 100 ns, the impulse can be estimated as ~1 attoN s. However, in oscillating mode it is more appropriate to approximate the energy injected into the system that is $\Delta E = kA_0^2/2 - kA_D^2/2$, where A_0 is the free amplitude and A_D is the 'damped' amplitude in tip–sample contact ($A_D \sim 0.9A_0$), giving ~9.5 pN nm, hence $\Delta E \approx 9.5 \times 10^{-21}$ J = $2.3k_BT$. However, this energy is applied mainly perpendicular to the observation plane and will furthermore distribute over a relatively large (some nm^2) surface area.

What are the current limitations of the HS-AFM? At the moment, we feel that three major obstacles must be overcome:

(i) HS-AFM technology must still get *faster* for studying more biologically relevant dynamic phenomena. At the moment, the speed is indeed limited by the cantilever oscillation. Given, the current cantilevers have resonance frequencies of ~1 MHz in liquid, the tip probes the sample about a million times per second. Hence at best, we can acquire one million data points $dp_{(s)}$ per second, distributable into defined numbers of pixels p along scan lines l at a number of frames per second $f_{(s)}$ in trace and retrace, $dp_{(s)} = plf_{(s)}2 = 1,000,000$. Hence, wanting to well resolve the molecules at an image size of 200p and 200l, the maximum frame rate is readily limited to 12.5 images/s. It is therefore obvious that further developments must be performed to allow faster HS-AFM scanning.

(ii) HS-AFM technology shall be developed for *cellular studies*, thus moving forward from the molecular to the cellular scale, which means adopting the scanner stage for larger scan areas and/or coupling the HS-AFM with optical microscopy. Many dynamic phenomena can be studied on a purely molecular level, such as enzyme activity, motor action and diffusion, described above. Nevertheless, biologists will want to study these features on life cells, assuring nativeness of the system and providing an insight to the interplay with other molecules. For this, HS-AFM must be applied on cells. However, in order to achieve this, technical and conceptual obstacles must be overcome. Cells are large and less flat than a solid support, indicating the need for large X, Y scan ranges as well as a larger dynamic range in Z-dimension without loss of scan speed. This is technically difficult as larger piezo elements are slower. A further challenge is that working on cells implies the precise localization of the tip and scan range on a zone of interest on a cell. This implies coupling of the HS-AFM with optical microscopy, which is again a difficult task, because best AFM performance is provided when the sample is scanned, and the tip and optical cantilever detection system are fixed. Possible solutions imply hence

either a fast tip scanning table top HS-AFM or the implementation of a powerful optical path into a sample scanning HS-AFM. Doubtlessly, these developments will take another few years, but the gain is evident – it is to date impossible to directly visualize a single molecule on a life cell.

(iii) HS-AFM technology shall be developed for *single molecule force spectroscopy* in both dynamic and force clamp modes. Since the first AFM measurements of the forces required for breaking molecular bonds and unfolding proteins, the time resolution of AFM force spectroscopy measurements has been limited by the resonance frequency of conventional cantilevers (few kHz in liquid, thus, ms) [36,37]. However, the internal relaxation time of proteins lies in the ns to μs range, there is thus a demand for enhancing the temporal resolution in force measurements to access this timescale [38]. Recent works using torsional cantilevers or photothermal actuation have allowed force spectroscopy measurements at high loading rates, bridging the gap between molecular dynamic simulations and experiments [39,40]. The high resonance frequency and relatively low spring constant of HS-AFM cantilevers would enhance the time resolution to, at least, the μs regime, few orders of magnitude higher than with conventional AFM systems. This resolution may allow us to detect hidden intermediate states during protein unfolding and/or molecular unbinding events. In addition, force clamp experiments, although an elegant and valuable approach, are still limited to a timescale of few ms [41•]. The high bandwidth of the dynamic feedback-controller of HS-AFM would provide, in principle, responses of ~20 μs, at least two orders of magnitude faster than conventional force-clamp systems. Thus, the application of HS-AFM technology to well-known systems such as the already classic streptavidin/biotin complex or the I27 domain of titin, may provide new insights in the mechanism of unbinding and unfolding processes at the relevant molecular timescales.

The recent progress in HS-AFM technology [12••,42] and application has readily provided novel so far inaccessible data as documented here and will doubtlessly open novel avenues for biological studies at the molecular level in the near future. The concomitant assessment of molecule structure, dynamics and function under physiological conditions is unique in structural biology at date. This appealing combination will inspire biologists, chemists and physicists to tackle their specific molecular questions. Furthermore, HS-AFM data will stimulate large-scale molecular dynamics simulations and theory.

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