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High-Speed AFM Reveals the Dynamics of Single Biomolecules at the Nanometer Scale

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Atomic force microscopy allows visualization of biomolecules with nanometer resolution under physiological conditions. Recent advances have improved the time resolution of the technique from minutes to tens of milliseconds, meaning that it is now possible to watch single biomolecules in action in real time. Here, we review this development.

The atomic force microscope is a mechanical microscope that can visualize the three-dimensional topography of surfaces with $(nm)^3$ resolution (Box 1). In addition to label-free high-resolution imaging under physiological conditions, AFM can also provide access to mechanical properties of cells and molecules, which are powerful capabilities that lead to novel insights into the working of biological processes. Conventional AFM is only used to image static snapshots of biomolecules because each image takes minutes to acquire. However, this acquisition time has improved 1,000-fold in the past decade, and it is now possible to take more than ten images per second.

Toshio Ando and his coworkers at Kanazawa University have been leading innovators in this high-speed AFM technology (Kodera et al., 2010; Igarashi et al., 2011; Shibata et al., 2011; Uchihashi et al., 2011). For example, their AFM movies of myosin V at seven frames/s show the progressive hand-over-hand motion of a single motor protein as it moves along an actin filament (some stills are depicted in Figure 1A). They follow several steps that the protein takes and even demonstrate small details of the motion, including a "foot stomp." Such movies existed before only as animations but have now become reality thanks to high-speed AFM. This Minireview highlights a number of recent results obtained with high-speed AFM, recapitulates some of the technical innovations that enabled them, and looks ahead to the prospects and challenges of the technique.

The Pros and Cons of AFM

AFM offers several advantages over other visualization techniques. True atomic resolution can, in principle, be obtained with AFM even in liquid (Fukuma et al., 2006), but a more routine number for resolution on biomolecules is about 0.1 nm in z and 5 nm in the xy plane mostly limited by tip sharpness. The AFM requires no fixing, staining, or labeling of samples and can be operated in many different environments, including aqueous solutions of various salt concentrations. However, a fundamental limitation of AFM is that it is a surface technique: it can only be applied to image surfaces that are exposed to the tip and are connected to a support. So neither molecules freely floating in solution nor the inside of cells can be imaged.

Recent High-Speed Highlights

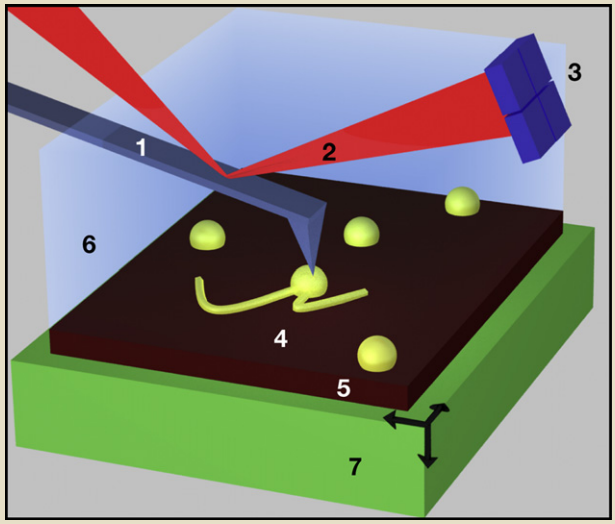
Let us first illustrate the power of high-speed AFM by quickly surveying some examples from the past 2 years. The Ando group showed another striking example of molecular motor action (Figure 1B) in their AFM movies of the isolated stator subcomplex of the rotary motor protein F_1-ATPase (Uchihashi et al., 2011). Previous single-molecule experiments on parts of this enzyme had measured rotation, but they could only be done if at least one subunit of the rotor was attached. The AFM, however, could visualize the conformational change that the β subunits of the stator undergo when they bind ATP. By imaging at 12.5 frames/s, the authors followed the time dependence of these conformational changes, leading to the surprising conclusion that, contrary to what was widely assumed before, the catalysis on the enzyme maintains its sequential rotary order even in absence of the rotor subunits.

Membrane surfaces are traditionally well resolved in AFM studies. Applying time-resolved AFM provides insights into the dynamics of movement within them. Native membranes of photosynthetic membranes visualized previously with AFM (Bahatyrova et al., 2004) showed a static pattern of proteins. By imaging a similar system at five frames/s, Simon Scheuring and coworkers (Casuso et al., 2010) showed that the membrane organization is highly dynamic (Figure 1C), with ATP-synthase rings diffusing through the membrane and intermittently forming dimers, which are kept together through interactions with the membrane lipids.

Moving from molecules to cells and nanometers to micrometers, Fantner and coworkers (Fantner et al., 2010) investigated the effect of an antimicrobial peptide on *E. coli* cells (Figure 1D). Although the tip scanning velocity in this work was about twice that used by Uchihashi et al. on F_1-ATPase, the large scan size constrained their image time to 13 s/frame. The pore-forming peptide induces roughness on the cell surface, which can be detected in the phase signal of the AFM. The increased time resolution compared to conventional AFM allowed the authors to show that the onset of roughening varies between otherwise identical bacteria from a few seconds to several minutes and that this variation is the cause of variation in cell survival times.

Box 1. The Basic Principle of AFM

Biological samples are largely assayed using the amplitude modulation mode of AFM. In this technique, a sharp tip at the end of a flexible cantilever (1) is brought into proximity of a sample surface (5) covered with cells or proteins of interest (4) in a liquid medium (6). The cantilever is oscillated near its resonance frequency. Force exerted on the tip as it touches the sample decreases the oscillation amplitude, which is detected by means of a laser beam (2) that is reflected from the cantilever onto a photo diode (3). A feedback system keeps the tip sample force constant by adjusting the separation (z) between tip and sample. A topographical image of the sample surface is obtained by raster scanning (7) the tip over the in-plane coordinates (x and y) of the sample and recording the feedback output.



There are also several recent papers in which high-speed AFM was used to track the dynamics of DNA and DNA-binding proteins, such as histones (Miyagi et al., 2011) and the DNA repair protein RAD54 (Sanchez et al., 2011). These studies uncovered new effects in chromatin dynamics such as spontaneous nucleosome sliding and hopping of proteins between DNA segments. Though promising, it is at the moment not entirely clear what role the surface attachment of the DNA plays in these early studies.

Overall, the progress in applying high-speed AFM to biological problems has been impressive in recent years. High-speed AFM clearly has moved beyond the proof-of-principle stage to providing real new insights in molecular biology.

How Fast Is Fast Scanning?

When discussing the imaging speed of AFM, it is important to realize that there are several possible definitions of the speed. If the AFM is used to follow a dynamic process, the image acquisition time is the most meaningful measure of speed. But at a given image time, a larger scan size necessitates that the tip moves across the surface at higher velocity, which makes it more difficult to exactly follow the surface without exerting high forces. Therefore, one cannot expect to achieve the same frame rate when imaging a whole cell at the tens of microns scale as when imaging single molecules in a 100 nm field of view. Likewise, operating the AFM with parameters that give more gentle contact between tip and sample slows down the scanning considerably, so processes that are more easily perturbed by the AFM tip require lower frame rates. Paradoxically, dynamic processes, which require a high image rate to follow them, are often also easily perturbed and therefore more difficult to image at high speeds.

Conventional AFMs operate with a typical image acquisition time of several minutes. Even when imaging active biomolecules in solution, this can be pushed to a few tens of seconds per frame (Moreno-Herrero et al., 2005). Indeed, there is no black and white distinction between high-speed and conventional AFM, but the transition can be set at approximately one frame/s. The current speed record for AFM is more than 1,000 frames/s (Picco et al., 2007), but this was achieved in air, with severely limited control over the tip sample interaction force.

High-speed AFM imaging has been around for more than a decade (Viani et al., 2000), and at first sight, it seems that the speed of imaging has not increased much in the past 10 years. When comparing early (Ando et al., 2001) and recent (Kodera et al., 2010) work on myosin V, for example, the image rate has even decreased from 12 to 7 frames/s. However, the early work merely showed a protein loosely adhered to a mica support moving, but not performing a function. To go beyond this, several technologies had to be invented, which now allow the high-speed scanning to be performed with much lower forces exerted by the tip on the proteins. In the recent work (Figure 1A), these increased capabilities have transformed a mere technical demonstration or proof of principle into direct informative visualizations of biological processes.

Enabling Technology for High-Speed AFM

The nearly 1,000-fold increase in AFM imaging speed that enables dynamic measurements of biomolecules relies on several advances. A key breakthrough was the use of smaller cantilevers (Box 1) (Walters et al., 1996). Though typical AFM cantilevers are tens of μm wide and hundreds of μm long, most high-speed AFMs now use cantilevers 2 by 10 μm in size. This choice has two advantages: first, the resonance frequency of smaller cantilevers is higher, which means that higher oscillation frequencies can be used, leading to smaller acquisition times per pixel. Second, the force noise decreases with the size of the cantilever, yielding a lower noise in a given bandwidth.

Smaller cantilevers, however, present engineering challenges related to fabrication and signal detection. The detection optics were adapted with smaller laser spot sizes (Walters et al., 1996; Ando et al., 2001), and novel methods for amplitude detection (Ando et al., 2008) have enabled the use of the full bandwidth offered by the cantilevers. Nonlinear feedback was developed to ensure stable imaging in a low-force regime (Ando et al., 2008). The current state of the art closely approximates the thermal limit, with peak forces during imaging of around 20 pN (Kodera et al., 2010). Though this number may seem high compared to, say, the ~3 pN stall force of myosin V, it is important to realize that this force is only applied in submicrosecond pulses, and the transfer of momentum is very small. The translocation process of myosin V is not affected by the tip,

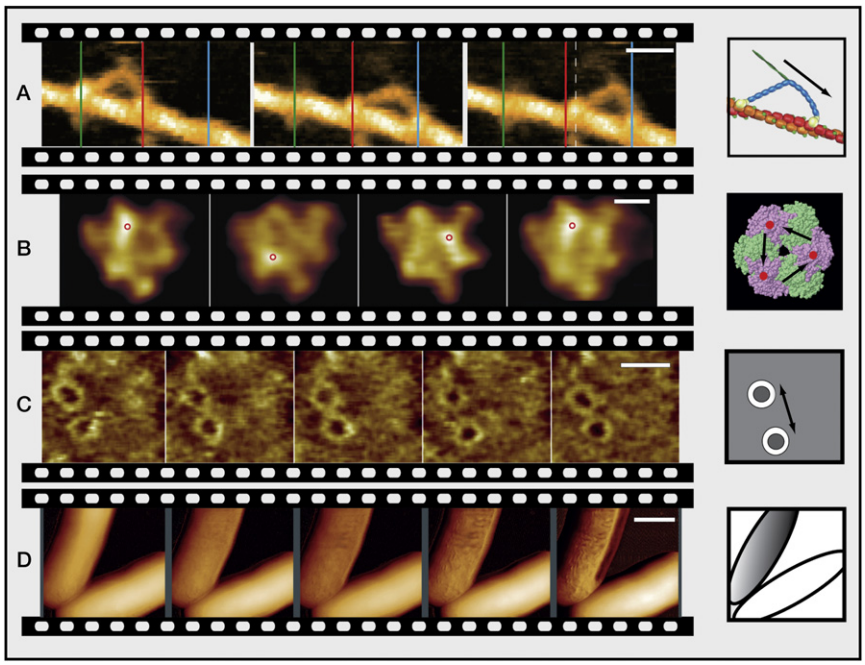


Figure 1. High-Speed AFM Captures Movies of Biomolecules

(A) Tail-truncated myosin V walking along an actin filament (Kodera et al., 2010). Selected frames recorded at 147 ms/frame. Scale bar, 30 nm. Vertical lines denote the same positions on the actin filament across frames. The steps of the molecule can be seen, and the third frame shows that the trailing head has moved its position by 8 nm, the so-called "foot stomp." Reprinted by permission from Macmillan Publishers Ltd: *Nature* 468, 72–76, copyright 2010.

(B) A single $\alpha_3\beta_3$ stator subcomplex of F_0F_1 ATP synthase undergoing conformational changes driven by ATP (Uchihashi et al., 2011). Selected frames recorded at 80 ms/frame. Scale bar, 5 nm. As ATP is hydrolyzed by one of the subunits, it undergoes a conformational change and sticks out slightly higher from the surface, which the AFM can detect (indicated by red circles). Reprinted from *Science*.

(C) Diffusion of two ATP-synthase C-rings in a native photosynthetic membrane of *Halobacterium Salinarium* (Casuso et al., 2010). Consecutive frames recorded at 187 ms/frame. Scale bar, 10 nm. The proteins form dimers that transiently dissociate. Reprinted from *Biophysical Journal*.

(D) Roughening of *E.coli* cell surface due to exposure to the antimicrobial peptide CM15 (Fantner et al., 2010). Consecutive frames (phase signal) recorded at 13 s/frame. Scale bar, 1 μm. The bacterium on the left shows surface roughening starting in the second image, visible through the appearance of dark inhomogeneities, whereas that on the right is unchanged for the duration shown here. Reprinted by permission from Macmillan Publishers Ltd: *Nature Nanotech* 5, 280–285, copyright 2010.

which demonstrates that AFM imaging induces only a minimal perturbation of the system.

Although small cantilevers are now available commercially from several sources, tip sharpness is still an issue. The authors of many of the papers discussed here used electron beam-induced deposition to create sharper tips than available out of the box. The mass fabrication of small cantilevers with sharp tips of < 5 nm apex radius is a key challenge for widespread use of high-speed AFM.

The mechanical properties of the scanner (Box 1), which limit the maximum frequency for scanning, present the most difficult engineering challenge in high-speed AFM design. AFM always involves moving a massive object (either the cantilever tip or the sample, including their respective holders), so inertia is an important factor. The design of stiff and compact piezo-scanners, combined with sophisticated methods to control their motion (Schitter et al., 2007; Ando et al., 2008), have vastly improved the speeds at which imaging can be performed. Unfortunately, high scanner resonance frequencies are often attained at the cost of total scan range. The highest-speed scanners therefore have only submicron maximum image sizes, which precludes the study of larger subjects such as whole cells and makes finding the right region of interest cumbersome.

Future Prospects and Challenges

As witnessed by the recent stream of publications, high-speed AFM has passed the stage of technology demonstration and is now actively used to obtain previously inaccessible information on biological systems. Although the number of labs involved has been small so far, the technology has recently become commercially available, which greatly lowers the barrier for access to the technique. The instrument developed by the Ando group is now available from RIBM and offers up to 20 frames/s imaging speed with submicron scan sizes. Both Bruker AXS and Asylum Research offer instruments with imaging speeds up to approximately one frame/s and scan sizes of several tens of microns.

With the technological barriers out of the way, the biological systems that high-speed AFM can be applied to seem countless. There are already several techniques that can study subsecond dynamics of single proteins, like fluorescence microscopy or optical tweezers, but the AFM offers better spatial resolution. An important advantage of the AFM is also that it can image complete proteins and their environment simultaneously, as opposed to just the labeled parts of a molecule. Moreover, we expect the AFM to open up new opportunities for studying those systems where production of functional labeled proteins is hard to achieve or where the labeling interferes with the dynamics. For example, as initially discussed above, molecular motors, from cytoskeletal transport proteins to polymerases and chromatin remodelers, are obvious targets for high-speed AFM studies because of their small size and the subsecond timescale of their dynamics. The (sub)molecular details of conformational changes involved in motor action can be resolved by AFM. As mentioned earlier, the two-dimensional nature of membranes makes them particularly amenable to a surface-sensitive technique like AFM. Possible subjects for study that would capitalize on protein imaging in concert with the surrounding environment would be inhomogeneous diffusion of membrane proteins (the lipid raft hypothesis) and the dynamics of membrane organization, e.g., the clustering of membrane receptors in response to stimuli.

Given the new availability of the instrumentation, the most immediate challenge for obtaining biologically relevant results from single-molecule studies will be in sample preparation. Static AFM imaging merely requires that the subject is attached to a surface. The imaging of a dynamic process requires the researcher to tune conditions to the subtle boundary between an attachment that is too restrictive for molecules to execute their biological function and one that is so loose that no clear images can be obtained. Several successful strategies based on supported lipid bilayers or protein crystals have been developed (Ando et al., 2008), but new topics will undoubtedly require additional methods.

For some systems, it will be desirable to increase the time resolution even further. One promising direction that may alleviate restrictions imposed by the scanner is the development of microelectromechanical systems (MEMS) for scanning probe microscopy (Disseldorp et al., 2010) and the development of self-sensing ultra-high-frequency cantilevers (Li et al., 2007). Combined, these innovations have the potential to increase the imaging rates by another factor of 10 compared to current high-speed AFMs. As an example, this may allow single-step resolution imaging of bacterial RNA polymerase in in vivo conditions, where it has been measured to perform more than 50 steps/s.

For larger systems like bacteria or even eukaryotic cells, there are many opportunities for high-speed AFM. Several groups have shown preliminary data suggesting that they can resolve single molecules on the outside of living bacteria. They did this by scanning very small areas of the cell surface with image rates of the order of one frame/s. Nevertheless, some further instrument optimization is desirable. For example, increasing the scan size for surveying, without loss of ultimate speed capabilities at small scan size, will alleviate practical problems in locating regions of interest. The combination of improved scanning with simultaneous high-quality optical imaging will allow monitoring the global state of the cell and/or intracellular processes during high-resolution AFM imaging. These developments will likely become available in the near future. On the other hand, video rate imaging of whole mammalian cells with the kind of spatial resolution and force control that is currently attained for single molecules will require another 100-fold speed increase. We do not expect such a development to be available within the coming decade.

As the systems under study get more complex, one of the AFM's strengths becomes a weakness: the absence of labeling. Many proteins do not show distinctive morphological features and are indistinguishable from each other in an AFM image. Further development of techniques that allow specific recognition of molecules in AFM (Stroh et al., 2004) will be vital for dynamic imaging of specific proteins in complex environments.

Prospects

In summary, the development of high-speed AFM has now matured to a level that allows imaging of dynamic biological processes with nanometer precision at sub-100 ms timescales and with minimal invasiveness. This new tool provides biologists and biophysicists with unprecedented amounts of detail through a direct visualization of functional proteins and cellular structures and has the potential to take a lot of the guesswork out of modeling dynamic processes by simply visualizing the dynamics.

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