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Review

High-speed atomic force microscopy for observing dynamic biomolecular processes

Toshio Ando[1,2]*, Takayuki Uchihashi[1,2], Noriyuki Kodera[1,2], Daisuke Yamamoto[1,2], Masaaki Taniguchi[1], Atsushi Miyagi[1] and Hayato Yamashita[1]

[1]Department of Physics, Kanazawa University, Kanazawa 920-1192, Japan
[2]CREST, JST, Sanban-cho, Chiyoda-ku, Tokyo 102-0075, Japan

The atomic force microscope (AFM) is unique in its capability to capture high-resolution images of biological samples in liquids. This capability will become more valuable to biological sciences if AFM additionally acquires an ability of high-speed imaging, because 'direct and real-time visualization' is a straightforward and powerful means to understand biomolecular processes. With conventional AFMs, it takes more than a minute to capture an image, while biomolecular processes generally occur on a millisecond timescale or less. In order to fill this large gap, various efforts have been carried out in the past decade. Here, we review these past efforts, describe the current state of the capability and limitations of high-speed AFM, and discuss possibilities that may break the limitations and lead to the development of a truly useful high-speed AFM for biological sciences. Copyright © 2007 John Wiley & Sons, Ltd.

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INTRODUCTION

The atomic force microscope (AFM) was invented in 1986 (Binnig *et al*., 1986), four years after the invention of the scanning tunneling microscope (STM) (Binnig *et al.*, 1982). Unlike STM or electron microscopy, AFM is unique in its ability to observe insulating objects in liquids. Although it was examined not in water but in paraffin oil, this in-liquid observation ability was first demonstrated at Hansma's laboratory by the observation of atomic lattice structures of graphite and sodium chloride surfaces (Marti *et al*., 1987). This unique feature of AFM was of considerable advantage to biological sciences because biological samples show vital activities only in aqueous solutions. Around 1988, cantilevers manufactured by using microfabrication techniques became available (Binnig *et al.*, 1987), and the optical lever method for cantilever deflection detection was introduced (Meyer and Amer, 1988); this promoted AFM imaging of biological samples (Gould *et al*., 1988; Marti *et al*., 1988a,b; Drake *et al*., 1989; Lin *et al*., 1990). Remarkably, at this very early stage, Paul Hansma and his colleagues already observed the dynamic behavior of biological samples in action. For example, they observed at ∼1 min intervals the clotting process by fibrin molecules that was initiated by the digestion of fibrinogen with thrombin (Drake *et al*., 1989). After this experiment, some trial observations of biological processes had been actively made to explore the potential of AFM as a novel tool in biological sciences (Häberle *et al*., 1992; Ohnesorge *et al.*, 1992). However, this enthusiastic research activity seemed to have declined gradually, in comparison with the increase in the AFM-user population due to the availability of commercial AFMs. This is understandable because at this stage only contact mode was used (tapping mode was invented in 1993 (Zhong *et al*., 1993)), and therefore, biomolecules weakly attached to surface were easily swept away by the scanning cantilever tip. At this stage, greater efforts were directed toward attaining appropriate conditions under which high spatial-resolution images could be obtained (Butt *et al*., 1990a,b; Edstrom *et al*., 1990; Egger *et al*., 1990; Gould *et al*., 1990; Weisenhorn *et al*., 1990; Hoh *et al*., 1991; Appel *et al*., 1992; Karrasch *et al*., 1993; Schabert and Engel, 1994). After the tapping mode was introduced, the research activity on exploring biological processes was revived moderately, although the imaging rate was as low as before. For example, in 1994, Bustamante and his colleagues imaged DNA diffusion on mica surface (Bustamante *et al*., 1994) and DNA bending on binding to λ Cro protein (Erie *et al*., 1994), and Hansma and his colleagues imaged DNA digestion with DNase (Bezanilla *et al*., 1994) and DNA–RNA polymerase binding process (Guthold *et al*., 1994). These two groups continued these studies and obtained time-lapse images (∼30 s intervals) on the RNA replication reaction by DNA and RNA polymerase (Kasas *et al*., 1997) and on the one-dimensional diffusion of RNA polymerase along a DNA strand (Guthold *et al*., 1999).

*Correspondence to: T. Ando, Department of Physics, Kanazawa University, Kanazawa 920-1192, Japan. E-mail: tando@kenroku.kanazawa-u.ac.jp

Abbreviations used: AFM, Atomic Force Microscope; STM, Scanning Tunneling Microscope; PM-AFM, Phase-modulation Atomic Force Microscopy; FM-AFM, Frequency-modulation Atomic Force Microscopy; AM-AFM, Amplitude-modulation Atomic Force Microscopy; Q, uality Factor; PID, Proportional-Integral-Differential; nc-AFM, Noncontact Atomic Force Microscopy; SNFUH, Scanning Near-field Ultrasound Holography; ICSPM, Ion-conductance Scanning Probe Microscopy; SPM, Scanning Probe Microscopy.

In 1993, the scan speed limit of AFM was described (Butt *et al*., 1993). Although we found no publications, some studies aiming at increasing AFM scan speed must have been initiated at least before 1995. In fact, we started to develop high-speed scanners in 1994 and small cantilevers in 1997. Hansma's group also started to develop devices for high-speed AFM around 1995. They presented the first report on short cantilevers (23 μm by 12 μm) in 1996 (Walters *et al*., 1993), and subsequently a report on fast imaging in 1999, in which small cantilevers and an optical deflection detector (Schäffer *et al*., 1996) designed for the small cantilevers were used to take a DNA image in 1.7 s (Viani *et al*.). Next year, they imaged the formation and dissociation of the GroES–GroEL complexes (Viani *et al.*, 2000). However, due to the limited feedback bandwidth, this molecular process was traced by scanning the sample stage only in the x and z directions. A more complete high-speed AFM system was reported by us in 2001 and 2002 (Ando *et al.*, 2001, 2002), in which a high-speed scanner and fast electronics were introduced in addition to small cantilevers (resonant frequency ∼600 kHz in water) and an optical deflection detector for the small cantilevers. An imaging rate of 12.5 frames/s was achieved, and therefore, the swinging lever-arm motion of myosin V molecules was filmed as successive images with a scan range of 240 nm. This study inspired the study on high-speed AFM and seemed to bring about a groundswell toward the full-scale development of high-speed AFM and its application to biological issues that were difficult to solve by other techniques.

In this article, we review studies on high-speed AFM that were performed in the past decade. After a brief description of the high-speed AFM capabilities required by biological sciences, we give a quantitative description of the imaging rate and the feedback bandwidth; this is followed by a description of the devices developed so far and a few examples of high-speed biological imaging. In the last part, after summarizing the current state of our high-speed AFM and its limitations, we discuss the future prospects of possible techniques that may break the limitations and thereby can fulfill all the requirements of biological sciences.

DEMANDS ON HIGH-SPEED AFM

It is quite understandable that during the early stage immediately after the invention of AFM, researchers were enthusiastic about the possibilities of observing biomolecular processes. Before the AFM era, it was absolutely impossible to directly observe biological macromolecules (DNA, protein) in water, and therefore, the dynamic behavior of biomolecules had to be deduced from indirect data. From such inferences without a clincher, it was quite difficult to reach a complete consensus on a model of a particular biomolecular process. Biological functions proceed through dynamic processes that occur on a millisecond timescale or less with biomolecules, biosupramolecules, organelles, and cells. Therefore, direct- and real-time visualization is a powerful means of understanding biological functions. Thus, biological sciences demand AFM (or any microscope) to have the ability to film dynamic behavior of proteins that are purified and weakly attached to a substratum as well as those naturally embedded in living cell membranes. Furthermore, AFM must capture the dynamic behavior of intracellular organelles. Although the last demand appears to be an impossible one, a recent study (Shekhawat and Dravid, 2005) is probably making it possible.

Biomolecules are generally fragile and dynamic protein–protein interactions are more delicate. The force acting on protein–protein interactions ranges approximately from 1 pN to 100 pN. Even with the single 'rigor' complex of a muscle–myosin head and an actin filament that hardly dissociates in equilibrium, it is ruptured quickly by a pulling force of ∼15 pN (Nakajima *et al*., 1997). The force produced by motor proteins during ATP hydrolysis is generally a few piconewtons (e.g., see Schmidt and Montemagno, 2004). Moreover, living cell membranes are extremely soft. Therefore, biological science further demands that AFM maintains the tip–sample interaction force at a very (or negligibly) small level.

FEEDBACK BANDWIDTH AND IMAGING RATE

Suppose that an image is taken in a time T for a scan range $W \times W$ with scan lines N, the scan velocity V_s is then given by $V_s = 2WN/T$. For $W = 240$ nm, $N = 100$, and $T = 30$ ms, V_s becomes 1.6 mm/s. Suppose that a sample has a sinusoidal shape with a periodicity λ, the scan velocity V_s requires a feedback operation at a frequency $f = V_s/\lambda$ to maintain the tip–sample distance. When λ is 10 nm and V_s is 1.6 mm/s, f becomes 160 kHz. The feedback bandwidth f_B should be equal to f or higher and therefore can be expressed as

$$f_B \geq 2WN/\lambda T \tag{1}$$

The feedback bandwidth is a function of various factors and difficult to express explicitly. Qualitative descriptions were previously made for its dependence on various factors (Sulchek *et al*., 2002). Numerical simulations were also made for this purpose, including the dynamics of tip–sample interaction (Kokavecz *et al*., 2006a). However, neither gives a quantitative and practical guideline for developing a high-speed AFM. As shown below, we employed a simple way to derive an analytical expression for the feedback bandwidth, slightly sacrificing exactness (Kodera *et al*., 2006). Because of the 'chasing-after nature' of feedback control, the sample topography is always traced with a finite phase delay φ. The phase delay φ is given by $2\pi f\Delta\tau$, where $\Delta\tau$ is the time delay of the feedback control. The time delay depends on the imaging mode and is a function of various factors. Here, we consider only tapping mode that is suitable for imaging fragile samples. The main delays are the reading time of the cantilever's oscillation amplitude, the cantilever's response time, the z-scanner's response time, the integral time (τ_I) of error signals in the feedback controller, and the parachuting time (τ_p). 'Parachuting' means that the cantilever tip completely detaches from the sample surface

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at a steep down-hill region of the sample and thereafter takes time until it lands on the surface again. It takes at least $1/2f_c$ to measure the amplitude of a cantilever that is oscillating at its resonant frequency f_c. The response time of second-order resonant systems such as cantilevers and piezoactuators is expressed as $Q/\pi f_0$, where Q and f_0 are the quality factor and the resonant frequency, respectively. The feedback bandwidth is usually defined by the feedback frequency that results in a phase delay of $\pi/4$. With this definition, the feedback bandwidth f_B is approximately expressed as

$$f_B = \frac{f_c}{8} \Big/ \left(\frac{1}{2} + \frac{Q_c}{\pi} + \frac{Q_s f_c}{\pi f_s} + f_c(\tau_p + \tau_I) \right) \tag{2}$$

where f_s is the z-scanner's resonant frequency; Q_c and Q_s the quality factors of the cantilever and z-scanner, respectively. The parachuting time is a function of various parameters such as the sample height h_0, the free oscillation amplitude A_0 of the cantilever, the amplitude set point r, the phase delay φ, and the cantilever's resonant frequency. Its approximate analytical expression has been derived (Kodera *et al.*, 2006) as

$$\tau_p \approx [(\tan\beta)/\beta - 1]/f_c \tag{3}$$

where β is $\cos^{-1}[2A_0(1-r)/3h_0 \sin(\varphi/2)]$. Equations (2) and (3) give us a quantitative guideline for the development of high-speed tapping mode AFM.

DEVICES FOR HIGH-SPEED AFM

Small cantilevers

The resonant frequency f_c and the spring constant k_c of a rectangular cantilever with thickness d, width w, and length L are expressed as

$$f_c = 0.56 \frac{d}{L^2} \sqrt{\frac{E}{12\rho}} \tag{4}$$

$$k_c = \frac{wd^3}{4L^3} E \tag{5}$$

where E and ρ are the Young's modulus and the density of the material used, respectively. The Young's modulus and the density of silicon nitride (Si_3N_4), which is often used as a material for soft cantilevers, are $E = 1.46 \times 10^{11}\,\mathrm{N/m^2}$ and $\rho = 3{,}087\,\mathrm{kg/m^3}$, respectively. For a given spring constant, the resonant frequency increases with decreasing mass of the cantilever. The total thermal noise depends only on the spring constant and the temperature. Therefore, a cantilever with a higher resonant frequency has a lower noise density. In tapping mode, the frequency region used for imaging is the imaging bandwidth (its maximum is the feedback bandwidth) centered on the resonant frequency. Thus, a cantilever with a higher resonant frequency is less affected by thermal noise. In addition, shorter cantilevers give a higher optical deflection sensitivity, because the sensitivity follows $\Delta\theta/\Delta z = 3/2L$, where Δz is the end displacement and $\Delta\theta$ is the end angle change of a cantilever of length L. A high resonant frequency and a small spring constant result in a large ratio (f_c/k_c), which affords the cantilever a high sensitivity to the gradient (k) of force exerted between the tip and sample. The force gradient shifts the cantilever's resonant frequency by approximately $-0.5kf_c/k_c$. Therefore, small cantilevers with large values of f_c/k_c are very useful for phase-modulation AFM (PM-AFM) and frequency-modulation AFM (FM-AFM). With respect to the feedback bandwidth, the most important aspect is that the amplitude detection time and the cantilever's response time decrease in inverse proportion to the resonant frequency. To realize a small spring constant and a high resonant frequency simultaneously, cantilevers with small dimensions must be fabricated. The small cantilevers recently developed by Olympus are manufactured using silicon nitride and coated with gold of thickness ~20 nm. They have dimensions of length ~6 μm, width ~2 μm, and thickness ~90 nm, which result in the resonant frequency of ~3.0 MHz in air and ~1.2 MHz in water, spring constant ~0.2 N/m, and Q ~2.5 in water. However, these cantilevers are not commercially available yet. Cantilevers integrated with piezoelectric films have advantages, because they can self-sense their deflection and even self-actuate (Manalis *et al.*, 1996). However, more complicated fabrication processes are required, and hence, at present it is very difficult to manufacture small cantilevers with such capabilities.

The tip radius of microfabricated small cantilevers developed by Olympus is ~17 nm (Kitazawa *et al.*, 2003), which is not sufficiently small for high-resolution imaging of biological samples. We usually attach an additional tip using electron beam deposition and sharpen it (~4 nm in radius) by plasma etching in argon gas. However, this piece-by-piece attachment is time consuming. Batch procedures for attaching a sharp tip to each cantilever have been attempted either by using direct growth of a single carbon nanofiber (Tanemura *et al.*, 2006) or a carbon nanotube at the cantilever tip (Cheung *et al.*, 2000).

Optical beam deflection detector

To focus an incident laser beam onto a small cantilever, a lens with a high numerical aperture (resulting in a short working distance) has to be used. With such a lens, the beam reflected back from the cantilever has to be collected with the same lens. The incident and reflected laser beams can be separated using a quarter wavelength plate and a polarization splitter. Two groups reported similar optical deflection detection systems with this basic design (Schäffer *et al.*, 1996; Viani *et al.*, 1999; Ando *et al.*, 2001).

Amplitude detection

Conventional rms-to-dc converters use a rectifier circuit and a low-pass filter, which requires at least several oscillation cycles to give an accurate rms value as output . To detect the

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cantilever's oscillation amplitude from a half oscillation cycle, a method to capture the peak and bottom voltages and then produce their difference as the amplitude was developed (Ando *et al*., 2001). A different method (the Fourier method) to output the amplitude from a single oscillation was proposed (Kokavecz *et al*., 2006b). The Fourier method calculates the Fourier sine and cosine coefficients for the fundamental frequency from the deflection signal and then produces the square root of the sum of their second powers as the amplitude. This method is probably less susceptible to noise but requires analogue or digital calculation systems with bandwidth at least two orders of magnitude higher than the cantilever's resonant frequency.

High-speed scanner

The scanner is the device that is most difficult to optimize for high-speed scanning. High-speed scan of mechanical devices with macroscopic dimensions tends to produce unwanted vibrations. Three techniques are required to minimize unwanted vibrations: (a) a technique to suppress the impulsive forces that are produced by quick displacement of the actuators, (b) a technique to increase the resonant frequencies, and (c) an active damping technique to reduce the quality factor.

The first issue was solved by a counterbalancing technique (Ando *et al*., 2001, 2002). For example, for the z-scanner that moves at much higher frequencies than the x- and y-scanners, two identical piezoactuators are placed at their supporting base in the counter directions and displaced simultaneously with the same length. An alternative way is to support a piezoactuator at both the ends with flexures. This method was applied to the x-scanner and z-scanner and worked very well (unpublished data). Recently, we tried a different method. The z-piezoactuator is held only at the four corners of its surface perpendicular to the displacement direction. The piezoactuator can be displaced almost freely in both the counter directions, and therefore impulsive forces are barely exerted on the holder; thus, only a very low mechanical excitation is produced to the scanner mechanics. This holding method has an additional advantage that the resonant frequency is not lowered by the holding, although the maximum displacement decreases by half.

The resonant frequency of a piezoactuator is determined almost solely by its maximum displacement (in other words, by its length). However, it can be effectively extended by an inverse compensation method as described later. The structural resonant frequency is enhanced by the use of a compact structure and a material that has a large ratio of the Young's modulus to the density. However, a compact structure tends to produce interferences between the three-scan axes. A ball-guide stage (Ando *et al*., 2001) is one choice for avoiding the interferences. An alternative way is to use flexures (blade springs) that are flexible enough to be displaced but stiff enough in the directions perpendicular to the displacement axis (Kindt *et al*., 2004; Ando *et al*., 2005). It should be noted that the scanner mechanics except for piezoacutators has to be produced by monolithic processing in order to minimize the number of resonant elements. An asymmetrical *x–y* configuration has been employed to gain a high resonant frequency for the x-scanner (the fast scan direction) (Ando *et al*., 2005). However, a symmetrical *x–y* configuration has an advantage of being capable of rotating the scan direction (Kindt *et al*., 2004). As a material for the scanner, aluminum or duralumin is often used. However, magnesium and magnesium alloys seem better candidates for it because of their larger mechanical damping coefficients and larger ratios of the Young's modulus to the density.

The active *Q*-control is well known as an active damping technique and has been often used to control the quality factor of cantilevers (Anczykowski *et al*., 1998; Sulchek *et al*., 2000; Tamayo *et al*., 2000, 2002). When this control is applied to the z-scanner, its displacements have to be detected. However, it is difficult to do so. Kodera *et al*. (2005) developed a new method in which instead of detecting the displacements, output signals from an electric circuit characterized by the same transfer function as the z-scanner were used to damp the z-scanner. With this technique, they achieved a bandwidth of 150 kHz and a quality factor of 0.5, which resulted in a response time of 1.1 μs. This method worked well for the z-scanner with a simple transfer function but not for one with a complicated one having multiple resonant peaks. An alternative method can be used for the active damping. The z-scanner is driven through a circuit with a transfer function 1/G(s), where G(s) is the transfer function of the z-scanner. However, for a complicated G(s), it is very difficult to design an electric circuit with 1/G(s). We invented a circuit that can automatically produce a transfer function that is approximately the same as the inverse transfer function of a given transfer function (Morita *et al*., 2007; Yamashita *et al*., 2007). This approximation becomes better with the use of operational amplifiers with higher bandwidths. In addition, this approximation becomes better with nested circuits even when the amplifiers' bandwidth is not high enough. This method works not only for reducing the quality factor but also for enhancing the apparent resonant frequency, so long as the driver for the piezoactuators has sufficient gain at high frequencies. Using this method and holding a piezoactuator (resonant frequency = 370 kHz) at its corners, we achieved a z-scanner bandwidth of 500 kHz with a maximum displacement of 1 μm (unpublished data).

The active damping of the x- and y-scanners is easy because their scan speed is not high and their scan waves are already known, and therefore, a feedforward controller for damping can be implemented in a digital mode. For example, the x-scanner is moved in isosceles triangles X(t) with a periodicity T_x. Its Fourier transform $F_x(\omega)$ is given by

$$F_x(\omega) = 2\pi X_0 \left[\frac{1}{2}\delta(\omega) - \frac{2}{\pi^2} \sum_{k=-\infty}^{+\infty} \frac{1}{k^2}\delta(\omega - k\omega_0) \right] \quad (k : \text{odd}), \tag{6}$$

where X_0 is the maximum displacement and $\omega_0 = 2\pi/T_x$. Suppose that the transfer function $G_x(i\omega)$ $(= |G_x(i\omega)| \times exp[i\Phi(\omega)])$ of the x-scanner is experimentally measured, the inverse Fourier transform of $F_x(\omega)/G_x(i\omega)$ gives the driving signal X'(t) for moving the x-scanner exactly in isosceles triangle waves. However, in practice, we need only

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the first ~15 terms of $F_x(\omega)$. Therefore, the driving signal is expressed as

$$X'(t) = \frac{X_0}{2G_x(0)} - \frac{4X_0}{\pi^2}\sum_{k=1}^{29}\frac{1}{k^2}\frac{1}{|G_x(ik\omega_0)|}\cos[k\omega_0 t - \Phi(k\omega_0)] \quad (7)$$

$(k : \text{odd}),$

To achieve a very fast scan in the *x*-direction, a resonant scan system with a tuning fork was used by Miles' group (Humphris *et al*., 2005; Picco *et al*., 2007). The scanner uses mechanical resonances instead of avoiding them. Due to the large quality factor of the tuning fork, the scanner oscillates sinusoidally with high stability. However, this method has two disadvantages. The scan speed cannot be changed easily, and the scan speed and the pixel size vary depending on the position in the *x*-direction.

Cantilever actuation

The tip–sample distance can be controlled not only by the z-scanner but also by actuating the cantilever. The latter has the potential of affording a higher feedback bandwidth because small cantilevers can have higher resonant frequencies than those of the z-piezoactuators. In addition, driving only a small cantilever beam produces much less hydraulic pressure on the sample, compared with driving the sample stage or the whole cantilever chip. However, we have to note that direct driving of a cantilever with any methods for controlling the tip–sample distance has the drawback of reducing the deflection detection sensitivity because a larger dynamic range is required for the sensing. Magnetic actuation of a cantilever coated with a magnetic material has been used for exciting the cantilever at its resonant frequency (Han *et al*., 1996). It can also be used for controlling the tip–sample distance (Jayanth *et al*., 2006). However, magnetic coating often reduces the cantilever's resonant frequency and makes the cantilever stiffer. Optical actuation has also been used for exciting a cantilever at its resonant frequency (Umeda *et al.*, 1991; Marti *et al.*, 1992; Ramos *et al*., 2006; Verbridge *et al*., 2006). In liquids, optical actuation derives from a thermal expansion effect rather than optical pressure (Ramos *et al*., 2006). Because heat transmission is slow, photothermal driving of a cantilever cannot be made quickly. To solve this problem, we again applied the inverse transfer function compensation as mentioned above. This compensation resulted in an apparent transfer function very close to the pure mechanical transfer function (Yamashita *et al*., 2007). With the natural Q (~2.5) of our small cantilevers (resonant frequency = 1.2 MHz in water, spring constant ~0.2 N/m), the cantilever responds to the power-modulated laser in 0.66 μs. By this control of the tip–sample distance together with other devices optimized for high-speed scanning, myosin V was imaged at video rate (30 ms/frame) for the scan range of 240 nm with 100 scan lines (Yamashita *et al*., 2007). A drawback of this photothermal driving is its small deflection efficiency. With a laser of 980 nm, it was ~1 nm/mW when applied to our small cantilevers (the laser power measured was not the one irradiated onto the cantilever but that at the outlet of the focusing lens). A laser of 405 nm gave a higher efficiency of 10 nm/mW. However, this wavelength cannot be used because biological samples are damaged by the irradiation. Wavelengths between these two have not been tested yet.

Dynamic PID control

Various efforts have been carried out to increase the AFM scan speed. However, little attention has been directed toward the reduction in the tip–sample interaction force. This reduction is quite important for biological AFM imaging as mentioned in 'Demands on High-speed AFM.' The most ideal scheme is the use of noncontact AFM (nc-AFM); however, the potential of high-speed nc-AFM has not been exploited at all so far. We still do not know if the high-speed and noncontact conditions can be achieved together in principle. We will discuss this issue later. There may be several methods to reduce the force in tapping mode: (a) using softer cantilevers, (b) enhancement of the quality factor of small cantilevers, (c) using a shallower amplitude set point (i.e., r is close to 1). None of these methods seem compatible with high-speed scanning. Softer cantilevers can be obtained only by sacrificing the resonant frequency. The most advanced small cantilevers developed by Olympus seem to have almost reached the limitation in appropriately balancing the high resonant frequency and the small spring constant. The tapping force decreases with increasing Q of the cantilever. However, its response speed decreases with increasing Q. A shallower amplitude set point promotes 'parachuting' during which the error signal is saturated at $2A_0(1 - r)$, and therefore, the parachuting time increases with increasing r, resulting in decrease in the feedback bandwidth. This difficult issue was solved by the invention of a new PID controller called 'dynamic PID controller' whose gains were automatically changed depending on the cantilever's oscillation amplitude (Kodera *et al*., 2006). Briefly, a threshold level is set between the peak-to-peak free oscillation amplitude $2A_0$ and the set point amplitude $2A_0r$. When the cantilever oscillation amplitude exceeds this threshold level, a false error signal is added to the true error signal, which shortens the parachuting time or avoids parachuting. In fact, the dynamic PID controller can avoid parachuting even when r is increased up to ~0.9, and therefore, the feedback bandwidth becomes independent of r so long as r is set less than ~0.9. Thus, high-speed scanning and gentleness with the sample became compatible with each other to some extent.

Drift compensation

With a given cantilever, the tapping force can be reduced by using small free-oscillation amplitude and a shallow set point. However, under this setting, the amplitude attenuation due to the tip–sample contact becomes very small. For example, with $2A_0 = 5$ nm and $r = 0.9$, the attenuation of the peak-to-peak amplitude becomes 0.5 nm. When the cantilever's free oscillation amplitude changes due to drift in the

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cantilever excitation efficiency, the oscillation amplitude during scanning also changes. The AFM feedback system misunderstands this change. For example, when the excitation efficiency is lowered, the feedback system interprets the resulting decrease in the oscillation amplitude as the tip interacting with the sample too strongly. Therefore, the feedback responds by withdrawing the sample stage from the tip, which is an incorrect direction. Without stability in the excitation efficiency or A_0, successive imaging under a small tapping force cannot be realized. It is difficult to eliminate causes for the drift. In addition, we cannot detect the free oscillation amplitude A_0 while imaging. This problem was first challenged by Kindt *et al*. (2002). They calculated feature richness from sample images obtained in tracing and retracing with slightly different set points. The richness was maintained by regulating the set point in the tracing regime. This method is effective but may not be applicable to high-speed imaging because the calculation is time consuming. A different method for drift compensation was invented by Schiener *et al*. (2004). The second harmonic amplitude is sensitive to tip–sample interaction, and hence drift in A_0 is reflected in the second harmonic amplitude averaged over a period longer than the image-acquisition time. They regulated r to maintain the constant difference $2A_0(1-r)$. However, this control varies the tapping force and feedback bandwidth. We also used the second harmonic amplitude but regulated the power of exciting the cantilever in order to maintain the constant $A_0(1-r)$. By using this control together with the dynamic PID control, stable high-speed imaging with maintained weaker tip–sample interaction became possible (Kodera *et al*., 2006).

Electronics

In high-speed AFM, large amounts of data have to be handled in real time to display image data while saving the data onto the computer hard disk. Recent commercial-data-acquisition systems and personal computers are fast enough to do this handling. Quantitative descriptions of this issue were given by Fantner *et al*. (2006). More problematic devices are the feedback controller, active damping systems, and the drivers for z-piezoactuators. Although analog feedback controllers are faster than digital ones, implementation of analog mode feedback-control algorithms that are more complicated than those of the dynamic PID control is difficult. Various advanced feedback or feedforward-control algorithms have recently been proposed for high-speed AFM (Schitter *et al*., 2001; Schitter *et al*., 2004a,b; Salapaka *et al*., 2005). However, by using digital systems, the efficiency of these algorithms has been demonstrated only in the case of relatively slow AFMs. At present, it is not certain whether proposed control algorithms can be implemented in the digital mode for a real high-speed AFM. The driver for z-piezoactuators (the capacitive load is a few nanofarads (nF) or less) should have a large current capacity, a high slew rate, ~100 V output, high bandwidth (>1 MHz), a small output resistance, and low noises. It is not easy to fulfill all these requirements. Rost *et al*. (2005) developed a z-driver with a bandwidth of 400 kHz and a slew rate of 75 V/μs for capacitive loads up to 1 nF. We sacrificed the output voltage (50 V) and thereby gained rms noises <2 mV, a high slew rate of 1000 V/μs, and a bandwidth of 3 MHz for capacitive loads up to 2 nF.

High-speed phase imaging

Tapping mode AFM (AM-AFM) has the capability to image compositional variations in heterogeneous surfaces in addition to surface topography (Tamayo and Garciá, 1996; Bar *et al*., 1997; Magonov *et al*., 1997). The phase difference between the excitation signal and the cantilever oscillation is affected by several surface properties. With energy-conservative tip–sample interaction, as mentioned before, the resonant frequency shifts by approximately $-0.5kf_c/k_c$. The frequency shift results in a phase shift because the excitation frequency is fixed. For a given frequency shift, the phase shift increases with Q. With conventional cantilevers, the frequency shift is generally around 50 Hz. Therefore, phase-contrast imaging had been possible only with a large Q (hence only at a small imaging rate). Because the ratios f_c/k_c with the most advanced small cantilevers are ~1000 times larger than that with conventional cantilevers, we can expect a shift of ~50 kHz. Therefore, even with a small Q, a relatively large phase shift occurs, which suggests a possibility of detecting the phase shift without using a very sensitive—yet very slow—phase detector such as lock-in amplifiers; hence, there is a possibility of high-speed phase-contrast imaging. Even with non-conservative interaction (Cleveland *et al*., 1998; Martínez and Garciá, 2006), we can expect the occurrence of a relatively large phase shift with small cantilevers, because their oscillation is damped weakly in water compared with conventional cantilevers; therefore, energy dissipation by tip–sample interaction can be significantly reflected on the cantilever oscillation. In order to explore the possibility of high-speed phase-contrast imaging, a fast phase detector was developed by Uchihashi *et al.* (2006) based on a previous design (Stark & Guckeberger, 1999). This can detect phase shifts within a single oscillation cycle and importantly at any timing within a cycle. This flexibility of detection timing is of great importance. Firstly, we can choose the timing when the largest phase shift occurs in the cycle, and therefore unlike conventional phase-contrast imaging, we do not have to use small amplitudes. In conventional phase-contrast imaging, the phase signal is averaged over many oscillation cycles, and therefore the cantilever oscillation amplitude has to be reduced to ~1 nm so that the tip is almost always oscillating within a region where the force field exists. Due to the small amplitude, phase-contrast imaging is difficult for samples with large roughness. The second importance of the flexibility of detection timing is that it can be distinguished whether the interactions conserve energy or dissipate it. The phase shift due to an energy-conservative interaction decreases with time very fast, while the phase shift caused by energy–dissipative interaction is maintained over the oscillation cycle. In fact, images of styrene–butadiene–styren block copolymer films having different contrasts were obtained in liquids, depending on

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the detection timing within the oscillation cycle. Remarkably, this phase imaging was carried out at ~80 ms/frame (Uchihashi *et al.*, 2006). This new technique for phase-contrast imaging will allow us to study dynamic changes in the physicochemical nature of protein molecules in action.

BIOIMAGING

Full-scale high-speed imaging studies on biological processes have not been carried out in the past decade because more efforts have been directed to develop high-speed AFM apparatuses. Various attempts to capture biological processes have been made mainly for testing the ability of high-speed AFM, which were feedbacked to the development of high-speed AFM. For example, in 2003, we imaged at 0.5 s/frame, the unidirectional movement of a chimera kinesin along a microtubule in an ATP-containing solution (Ando *et al*. 2003). In this kinesin, the C-terminal tail ends were replaced with gelosolin in order to avoid strong binding of its intrinsic tail ends to the mica surface. The observed kinesin moved unidirectionally along a microtubule, while being attached weakly to the mica surface. We could not observe kinesin that was moving along microtubules without touching the mica surface. This is because tip–sample interaction was too strong even with assistance from a prototype dynamic PID controller for keeping a constant shallow set point. The tip removed kinesin that had been attached only to the microtubules. Without assistance by the dynamic PID controller, the microtubules were destructed. Under conventional PID control, actin filaments on myosin V-coated surfaces could also not be clearly imaged without destruction. It was more difficult to observe actin filaments gliding over the myosin V-coated surface in the presence of ATP. In this case, actin filaments could not appear in the scanning region because actin filaments were easily removed from myosin V by too strong tip–sample interaction. With assistance by dynamic PID control, actin filaments gliding on the mica surface that was densely coated with myosin V were captured on video (Ando *et al*., 2005, 2006). However, when the myosin V density was lowered, the gliding movement was hardly observed. With an improved dynamic PID controller together with an optical deflection sensor with low noises, the set point could be set at >0.9; thus, actin filaments gliding on a surface sparsely coated with myosin V were successfully imaged (Ando *et al*., 2005, 2006). Accidentally, a short actin filament entered the observed region. Its entire length was within the region and therefore, all the myosin V molecules interacting with this filament were identified. All the myosin V heads interacting with the filament were oriented in one direction, which was similar to a well-known arrow-head structure in muscles. From this oriented structure, the polarity of the actin filament was identified. The filament moved toward the minus (pointed) end, which was the natural direction. However, conformational changes in the interacting myosin V heads were not evident during the unidirectional movement of the filament. Some AFM movies can be seen at http://www.s.kanazawa-u.ac.jp/phys/biophys/roadmap.htm.

Recently, a prototype high-speed AFM, which is an improved version of our first design (Ando *et al.*, 2001, 2002), became commercially available (Nano Live Vision™ manufactured by Olympus and distributed by RIBM). Its users recently filmed dynamic processes of GroEL–GroES interaction that were regulated by the ATPase reaction of GroEL immobilized onto the mica surface in an end-up orientation (Yokokawa *et al*., 2006a). Moreover, they observed the formation and dissociation of a streptavidin–biotinylated DNA complex (Kobayashi *et al*., 2007) and one-dimensional diffusion of a restriction enzyme along a DNA strand which was followed by the cleavage reaction (Yokokawa *et al*., 2006b).

The dynamic processes mentioned above are already known or expected from a series of biochemical and biophysical studies, and therefore, the filmed images may not give new insights to the molecular mechanisms. However, the new imaging tool has not matured yet, and hence, is not recognized yet as an established one. Therefore, in the present stage, biological processes that have been expected or known to occur have to be demonstrated by high-speed AFM imaging. In addition, techniques for preparing samples and substrates for their attachment have to be developed, which are often different from those for still imaging. Along with the gradual accumulation of successful imaging of known molecular processes, high-speed AFM will be realized as a reliable tool while newly filmed data on unexplored biological processes will be accepted widely.

We have been seeking to image single myosin V molecules walking along actin filaments, using the most recent version of high-speed AFM. Single molecules myosin V moves processively along actin tracks (Sakamoto *et al*., 2000). The hand-over-hand walking of myosin V is already established (Yildiz *et al.*, 2003; Forkey *et al.*, 2003; Warshaw *et al*., 2005; Syed *et al*., 2006) but its detailed behavior is still unknown. The tail part of myosin V was removed by digestion (Koide *et al.*, 2006) because it tended to attach to the mica surface. However, in a low ionic solution, the truncated myosin V (HMM) still tended to attach to the mica surface. Therefore, we elevated the ionic strength, although this lowered the affinity of myosin V heads for actin. Because of the weak affinity, the oscillating cantilever tip with the usual free amplitude (~5 nm) disturbed actin–myosin V interaction. Therefore, we reduced the free amplitude down to ~1 nm, sacrificing the feedback bandwidth. Under these conditions, we could successfully capture walking myosin V on video at 0.1 s/frame, in which the leading and trailing heads altered their positions with a walking stride of ~72 nm (Kodera *et al*., manuscript in preparation). In addition, the lead lever-arm bent just before the rear head detached from the actin filament. The detached rear head rotated around the junction between the two lever arms and then attached to a frontward actin. Just after the attachment, the new lead head moved farther by ~5 nm along the actin filament. We have been also seeking to image GroEL–GroES interaction dynamics in an ATP-containing solution, in which biotinylated GroEL is immobilized to streptavidin 2D-crystal sheets in a side-on orientation (Taguchi *et al*., 2001). Due to this orientation, both the GroEL rings were accessible to GroES that were floating in the solution. Because floating GroES did not

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interfere with imaging, a high-concentration of GroES could be used, unlike single-molecule fluorescence microscopy. The negative cooperativity between the two GroEL rings was confirmed; GroEL alternated its rings between the GroES associated and dissociated states. However, interestingly, releasing one GroES associated complex and forming another did not necessarily occur simultaneously. Two controversial intermediates, bare GroEL and GroEL-$(GroES)_2$, were detected just prior to the switching. The latter intermediate appeared with a large probability (Yamamoto *et al*., manuscript in preparation). The ternary GroEL–$(GroES)_2$ complex could be distinguished from the GroEL–GroES complex; the former looked like a football while the latter looked like a bullet.

THE PRESENT STATE AND PROSPECTS OF HIGH-SPEED AFM

Our current high-speed AFM is characterized by delay times contained in the feedback loop: the time of $\sim$0.4 μs for reading the amplitude of a cantilever oscillating at its resonant frequency of $\sim$1.2 MHz and with $Q \sim 2.5$ in water, the cantilevers' response time of $\sim$0.66 μs, the response time of $\sim$0.32 μs of the z-scanner with the effective resonant frequency of 500 kHz and $Q \sim 0.5$, no parachuting with the amplitude set point $\sim$0.9, and fast electronics with negligible delays as compared with the other delays. Together with the phase-compensation effect ($\sim$40%) of the differential operation of the dynamic PID controller, the sum of these delay times results in a feedback bandwidth of 125 kHz (see Eq. 2). Here, we neglected the integral time of error signals because it is hard to estimate. An experimental estimate of the feedback bandwidth also resulted in $\sim$125 kHz, when the ratio $2A_0/h_0 = 5$ was used. Therefore, from Eq. 1, this bandwidth corresponds to an imaging rate of 25 frames/sec (40 ms/frame) for $W = 250$ nm, $N = 100$, and $\lambda = 10$ nm. With contact mode or no electronic feedback operation, the imaging rate becomes much higher. However, both tapping mode and electronic feedback operation are certainly indispensable for imaging biological processes. Therefore, for faster imaging, the scan size or the number of scan lines has to be reduced at present.

How can we further increase the speed without reducing the scan size or the number of scan lines? In the current state, as seen above, the cantilever's resonant frequency has a dominant effect on the limited feedback bandwidth. It is rather easy to enhance the resonant frequency by the use of thicker cantilevers. However, they become stiffer and hence exert larger tapping forces on the sample. A unique solution that fulfills all desired conditions seems to be realization of high-speed nc-AFM. Noncontact imaging has been realized only with AM-AFM or FM-AFM in vacuum, where cantilevers with large quality factors are used. However, the large quality factor significantly reduces the cantilever's response speed. We must seek a noncontact condition that is compatible with cantilevers having a small quality factor, if this is possible in principle. Although it has not been explored theoretically and experimentally and hence not evidenced yet, it may be materialized by using ultrasonic interference between the cantilever tip and the sample that are ultrasonically excited at different frequencies f_1 and f_2 (f_1, $f_2 \gg f_c$). The difference $|f_1 - f_2|$ is set close to the cantilever's resonant frequency. This configuration is the same as that employed for scanning near-field ultrasound holography (SNFUH) that has recently been developed for high-resolution sub-surface imaging (Shekhawat and Dravid, 2005). A high frequency acoustic wave is launched from the bottom of the sample and propagates through the sample. Materials with different elastic moduli embedded in the sample modulate the phase and amplitude of the propagating acoustic waves. These modulations are reflected on the acoustic interference that occurs at the cantilever tip and therefore reflected on the cantilever oscillation around its resonant frequency. When the sample in a solution is only protein molecules attached to a uniform substratum, the acoustic wave front in the solution may trace the sample topography. This wave front may be detected by the cantilever tip that is not in contact with but is close to the sample surface.

The second possibility of high-speed noncontact imaging may derive from ion-conductance scanning probe microscopy (ICSPM) that has already satisfied the noncontact condition (Hansma *et al*., 1989). Due to the progress of fabrication techniques to produce very sharp glass capillaries with a small pore at the end, the spatial resolution has reached a few nanometers (Ying *et al*., 2005). Immobile protein molecules of $\sim$14 nm on living cell membranes have been successfully imaged (Schevchuk *et al*., 2006). However, in order to materialize high-speed ICSPM, we have to find a method to increase the bandwidth of ion-conductance detection because ionic currents through a small pore are very low.

High-speed nc-SPM in liquids will extensively expand the range of biological systems to be visualized, because the noncontact condition has the potential of enhancing the imaging rate. So far high-speed AFM imaging has been possible only for biological samples on a relatively hard substratum. Noncontact imaging will make it possible to visualize the molecular processes that occur on very soft living cells. Membrane proteins unanchored to membrane cytoskeletons diffuse very fast within the membranes when they are not surrounded by protein clusters. High-speed nc-SPM will be able to capture such proteins clearly. This capability is very valuable and applicable to a wider range of biological issues. Extracellular stimuli (such as drugs, endogenous ligands, or electric one) are first received by membranes' proteins (receptors or ionic channels) and then transmitted to the interiors to produce various cellular actions. Therefore, high-speed nc-SPM will also be used for drug assessment and discovery.

Although not for high-speed nc-AFM, control algorithms to reconcile a large quality factor of the cantilever with high-speed imaging have been proposed (Sahoo *et al*., 2003; Sahoo *et al*., 2005; Jeong *et al*., 2006). The position and velocity of the oscillating cantilever are continuously monitored (or discretely monitored with small time-bins). From these quantities measured, an estimator calculates the tip–sample interaction force of each tapping cycle. A model-based predictor uses the estimated force to control the tip–sample distance in the next tapping cycle. Their experiments with conventional AFMs implemented with

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the new controllers demonstrated regulation of the tip–sample interaction force at each tapping cycle, irrespective of the time delay of the cantilever's response. However, in order to apply this method to a real high-speed AFM, extremely fast digitization and calculations are required.

It seems quite difficult to enhance the bandwidth of piezoactuator-based scanners further. For a piezoactuator with a maximum displacement of 1 μm, the resonant frequency is approximately 500 kHz at most. Therefore, we require different types of actuators for further enhancement of the bandwidth. Microfabrication techniques can probably produce small actuators with both a large displacement and a high resonant frequency. Recently, an aluminum membrane actuator/sensor with a diameter of 150 μm was developed by Degertekin and his colleagues (Degertekin *et al*., 2005; Onaran *et al*., 2006). This membrane is driven by an electrostatic force exerted between the membrane and the counter electrode with a separation of 2 μm. This design was not created with the intention of producing a high-speed z-scanner but it has such a potential.

At present, we have no technology that allows us to study the structural dynamics of intracellular organelles at high spatial and temporal resolution. Recently demonstrated intracellular imaging by SNFUH (Shekhawat and Dravid, 2005) may afford such technology. It seems easy to combine SNFUH with the high-speed scanning techniques developed so far. It is still unclear whether this new imaging mode has resolution in the *z*-direction. However, the images obtained with ultrasonic waves launched at different angles should contain information along the *z*-axis. Therefore, there may be a way to reconstitute a 3D image from multiple images obtained at different launching angles.

SUMMARY

By focusing on the research activities on high-speed AFM in the past decade, we reviewed various studies on the instrumentation and imaging of biomolecular processes. Various devices have been created in these years based on the efforts of pioneers in this field, which led to high-speed AFM that can capture successive images at ∼video rate without disturbing the biological processes to some extent. This ability was demonstrated, for example, by filming myosin V molecules walking along actin filaments in a hand-over-hand fashion. One of the dreams of biological sciences had finally materialized. However, the force exerted between the oscillating tip and sample is in a level that is just small enough not to break weak protein–protein association. For high-speed AFM to become truly useful to studies on a wide variety of biological systems, reduction in the force is essential. We proposed possible methods for achieving this end. If the noncontact condition is realized in high-speed imaging, small cantilevers that have higher stiffness and hence higher resonant frequencies can be used for bioimaging, which leads to higher imaging rates. In addition, it allows us to study biomolecular processes that occur on living cell membranes, which tremendously expands the usefulness of high-speed AFM in biological sciences. We need to carry out more efforts to exploit this difficult challenge toward achieving our dream.

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