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HIGH-RESOLUTION IMAGING OF MYOSIN MOTOR IN ACTION BY A HIGH-SPEED ATOMIC FORCE MICROSCOPE

Noriyuki Kodera, Tatsuya Kinoshita, Takahiro Ito, and Toshio Ando
Department of Physics, Kanazawa University, Kanazawa 920-1192, Japan

ABSTRACT

The atomic force microscope (AFM) is a powerful tool for imaging biological molecules on a substrate, in solution. However, there is no effective time axis with AFM; commercially available AFMs require minutes to capture an image, but many interesting biological processes occur at much higher rate. Hence, what we can observe using the AFM is limited to stationary molecules, or those moving very slowly. We sought to increase markedly the scan speed of the AFM, so that in the future it can be used to study the dynamic behaviour of biomolecules. For this purpose, we have developed various devices optimised for high-speed scanning. Combining these devices has produced an AFM that can capture a 100 x 100 pixel image within 80 ms, thus generating a movie consisting of many successive images of a sample in aqueous solution. This is demonstrated by imaging myosin V molecules moving on mica, in solution.

1. INTRODUCTION

Motor proteins are very sophisticated nano-machines. They produce force to pull cytoskeletal fibers, or move along these fibers. This mechanical function is coupled to ATP hydrolysis, and the chemical energy released by the hydrolysis is transduced to mechanical energy. To understand the mechanism by which motor proteins operate, we have to know (a) their physiological action, (b) their fine structures at atomic resolution, (c) the kinetics of ATP hydrolysis, (d) the structural dynamics in action, and we further have to know the relationship between these different aspects. The first aspect has been studied by optical microscopy, the second by x-ray crystallography, and the third has been studied by various transient techniques. The fourth aspect is the most difficult to study. In general, in life science it has been a dream to view the nanometer-scale dynamic behavior of individual biopolymers in solution. The structure of protein changes with time, and the function of protein appears during the structural changes. Hence, a technique, which can reveal

temporal changes in the protein structure, has earnestly been desired in life science. Especially researchers who study biological molecular motors are very enthusiastic about viewing the nanometer-scale dynamic behavior of motor prtoteins. An atomic force microscope (AFM), which was invented 16 years ago (Binnig *et al*., 1986), allows highly resolved imaging of protein in solution. Yet, it cannot be a candidate for the desired technique, because its scan speed is too slow to capture protein in motion. A high-speed AFM seems to be only the device to fulfill the capacity that life science has longed to achieve. This has been our motivation for developing a high-speed AFM. We have optimized various devices involved in AFM for high-speed scanning, and have succeeded in producing an AFM that can capture a 100 x 100 pixel^2 image within 80 ms and therefore can generate a movie consisting of many successive images (80-ms intervals) of a sample in aqueous solution (Ando et al, 2001, 2002). This is demonstrated by imaging myosin V molecules moving on mica, in solution. The neck and head portions appear to move rigidly while the head/neck region and the neck/coiled-coil region are bending dramatically. At present, we are improving the performance of our AFM, and introducing a UV-irradiation system for producing ATP from caged-ATP. We hope that in the near future we can view nanometer-scale dynamic attitude of myosin V during it is moving along its track.

2. FACTORS LIMITING THE SCAN SPEED OF AFM

What factors of the AFM limit the scan speed? Here, we consider only the tapping mode of operation (Digital Instruments, Santa Barbara, CA). This is the mode most suitable for observing soft samples weakly attached to a substrate, in solution. In this mode the cantilever is oscillated at (or near) its resonance frequency. The oscillating tip briefly taps the surface of the sample at the bottom of each swing, resulting in a decrease in the oscillation amplitude. This decrease gives information of the sample height. The cantilever, therefore, has to oscillate for at least one cycle for each pixel of the image. To obtain an image consisting of NxN pixels using a cantilever having the resonance frequency of F_c, we require an imaging time (T), given by

$$T \geq 2nN^2 / F_c \qquad (1),$$

where n is the number of waves of oscillation required for measuring the oscillation amplitude. An imaging time of 80 ms for 100x100 pixels requires a resonance frequency higher than n x 250 kHz in water. Cantilevers with a higher resonance frequency tend to have a larger spring constant, undesirable for imaging soft samples. Therefore, to minimize the resonance frequency required for high-speed imaging, we need an RMS-DC converter that can output the amplitude voltage of the input sinusoidal signals as quickly as possible.

In addition to the resonance frequency of cantilevers, we have to consider another factor that limits the scan speed. Suppose that a sample on a substrate has a periodicity of λ, and the sample stage is moved horizontally with a velocity of Vs, the spatial frequency of $1/\lambda$ is converted to a temporal frequency of Vs/λ. The feedback system, that keeps the cantilever's oscillation amplitude constant, moves the sample stage up and down. The feedback bandwidth (F_b) should be wider than Vs/λ. Therefore, the scan speed is limited by the bandwidth as $Vs < \lambda F_b$. This scan speed determines the imaging time as

$$T = 2pN^2 / Vs \geq 2pN^2 / \lambda F_b \qquad (2),$$

where p is the pixel size. For example, if T=80 ms, N=100, p=2 nm, and λ =10 nm, then there is a required feedback bandwidth larger than 50 kHz. Various devices are involved in the feedback loop, as shown in Fig.1. It is not very difficult to achieve a high bandwidth for electronic devices. However, the scanner is the mechanical device most difficult to optimize for high-speed scanning. A well-known guiding principle for fabricating a mechanical device with a high resonance frequency is to make it with a small, compact, and

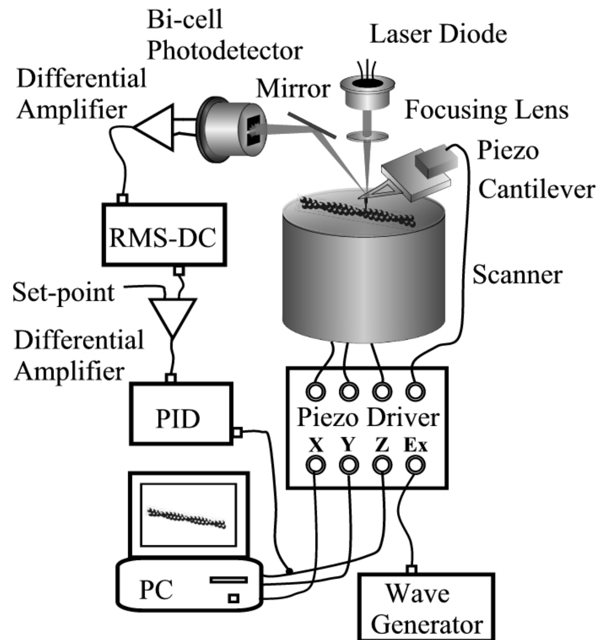

Fig.1 A schematic of a conventional AFM system for the tapping mode of operation.

light body. The sketches (Fig.2) show the conventional designs for the scanners that have been employed for the AFM. As long as the dimensions of these scanners are sufficiently large, the movements along the three axes do not interfere with each other. However, such large dimensions result in a low resonance frequency. We, therefore, require a different design for the high-speed scanner. Also required of the high-speed scanner is high rigidity

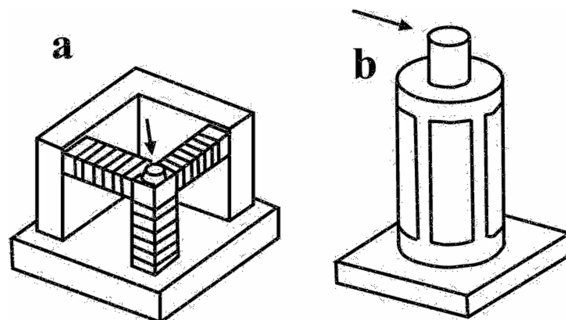

Fig.2 Sketches of AFM scanners with conventional designs. (a) a tripod type, (b) a cylindrical type. The arrows indicate the sample stages.

against the impulsive forces produced by rapid movements of the piezo actuators. When an object having the mass of 1 g is moved at 50 kHz with an amplitude of 10 nm, a peak impulsive force of 1.0 N is produced. The AFM scanner should not generate unwanted vibrations of even 1 nm against this impulsive force. Therefore, the required rigidity becomes 100 kg/μm. From simple calculations, it is evident that it is quite difficult to fabricate a mechanical device having such a high resonance frequency as well as high rigidity. Therefore, we need alternative guidelines for fabricating a high-speed scanner. Several laboratories throughout the world have been trying to develop a high-speed AFM (Sulcheck *et al*., 2000; Viani *et al*, 1999). They must also have encountered the greatest difficulty when making a high-speed scanner. An alternative means of achieving a high-speed scanner is to use a cantilever with an integrated piezoelectric actuator such as zinc oxide. However, such integration inevitably results in a large spring constant of the cantilever.

3. NEW DEVICES

3.1. Small cantilevers

A high resonance frequency and a small spring constant are conflicting requirements for any mechanical device. This is evident from the following equations for a strip type cantilever.

$$F_c = 0.56\frac{d}{L^2}\sqrt{\frac{E}{12\rho}} \quad , \tag{3}$$

$$k = \frac{wd^3}{4L^3}E \quad , \tag{4}$$

where k is the spring constant, d, L, and w are the thickness, the length and the width of the cantilever, and E and ρ are the Young's modulus and the density of material used for the cantilever, respectively. These conflicting requirements can be met only by using small dimensions. We fabricated small cantilevers from silicon nitride using micromachining techniques (Fig.3). They are 140 nm thick, 2 μm wide, and 9-11 μm long. The rear side of each cantilever is coated with gold of 20 nm thickness. The tips were grown by electron-beam deposition, with a growth rate of about 5 nm/s. The tip length was adjusted to about 1 μm. The radius of the tip end is 5-8 nm. The mechanical properties of the cantilevers were

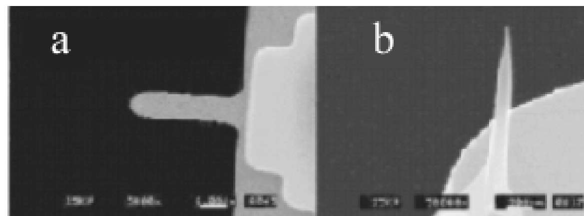

Fig.3 Electron micrographs of the small cantilever developed for our high-speed AFM. (a) the cantilever made from silicon nitride has no tip. (b) a tip was grown on the cantilever end by electron beam deposition.

tested by measuring the spectra of their thermal motions. The resonance frequencies are 1.3-1.8 MHz in air, and 450-650 kHz in water, and the spring constants are estimated to be 150-280 pN/nm. The highest resonance frequency in water, i.e., 650 kHz, can reduce the imaging time to 30 ms for 100x100 pixels.

3.2 RMS-DC converter

Conventional RMS-DC converters require at least 5~6 waves for conversion. This requirement arises because the converter has to use a low-pass filter in order to separate the carrier (basic) wave from the amplitude-modulation wave. We designed a new converter that requires only a half wave for conversion (Fig.3 in Ando *et al*., 2001). This converter is a type of peak-hold circuit. Two S/H circuits hold the peak and bottom voltages separately. The timing signals for this holding are made by the input sinusoidal signal itself. This guarantees stable and precise conversion even when the cantilever oscillation changes its frequency and phase. The difference between the two voltages held with the two S/Hs is output as the amplitude (not the RMS value) of the input sinusoidal signal. This new converter is satisfactory for an input sinusoidal signal of up to 1 MHz.

3.3 Optical deflection detection system

Since the cantilevers are very small, we required that the laser beam be focused onto the small cantilever as a small spot. Therefore, we could not use the optical deflection detection system that has widely been used in commercial AFMs. We designed an objective-lens type of deflection detection system (Fig.4). The incident laser beam is focused onto a small cantilever using an objective lens (CFI Plan Fluor ELWD 20xC, Nikon). The reflected beam is collected and collimated with the same objective lens. The incident and reflected beams are separated by a polarization beam splitter and a quarter-wavelength plate. The incident beam is entered into the objective lens at a slightly off-centered position to make the outgoing beam axis normal to the plane of the cantilever. The focused spot is 2-3 μm in diameter, sufficiently small for our small cantilevers. The optical lever magnification is about 2,000. This large magnification results from the short length of the cantilevers.

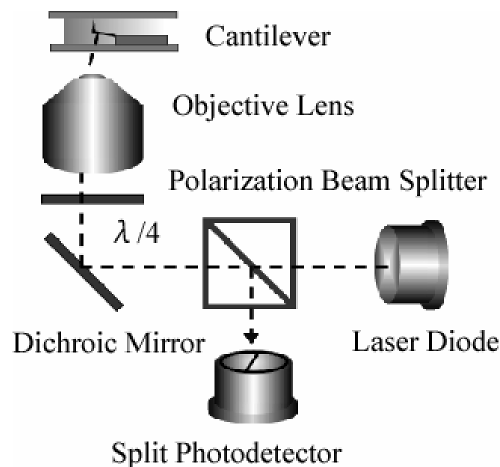

Fig.4 A schematic of the objective-lens type of optical deflection detection system.

3.4 Scanner

As mentioned above, it is quite difficult to fabricate a mechanical device with the high resonance frequency (>50 kHz) and high rigidity (> 100 kg/μm) required of the high-speed scanner. We considered the following possibility: (1) We might somehow reduce the resonance amplitude even when the resonance occurs at low frequencies; (2) We might somehow counteract the impulsive forces produced by the quick movement of the piezo actuators. After making and testing a number of scanners with different designs, we reached the design illustrated in Fig.5. Stack-type piezoelectric actuators (AE0203D04, Tokin, Tokyo) are used in this scanner. They have a resonance frequency of 260 kHz in free oscillation, and their maximum displacement is 4.5 μm. This scanner has a two-layered structure. One layer is for scanning in the y-direction, and the other layer is for scanning in the x- and z-directions. This structure guarantees little interference between the movements along the three axes. The z-scanner has two z-piezo actuators placed in opposite directions to one another. A sample stage is attached to one of the z-piezo actuators via a thin layer of vacuum grease. These actuators are displaced simultaneously in the same distance, but in counter directions, so that any impulsive forces produced are canceled out. The base plate (Base-2), to which the two z-actuators as well as an x-actuator are attached, is clamped in the z-direction by two flat surfaces (Base-1 and Plate-2) via steel ball bearings. This design allows smooth movement of the base (and hence, the sample stage) in the x-direction, and minimizes the vibrations of the base in the z-direction. When the z-piezo is displaced quickly, hydrodynamic force is generated as a reaction from the sample solution to the sample stage. To minimize this reactive force, a glass of circular-trapezoid shape with a small top surface of 1 mm diameter is used as the sample stage. The performance test of the z-scanner indicated that it was able to be driven stably up to about 60 kHz. Because the devices involved in the feedback loop, other than the scanner, have bandwidths much wider than 60 kHz, the feedback bandwidth is thus determined to be about 60 kHz.

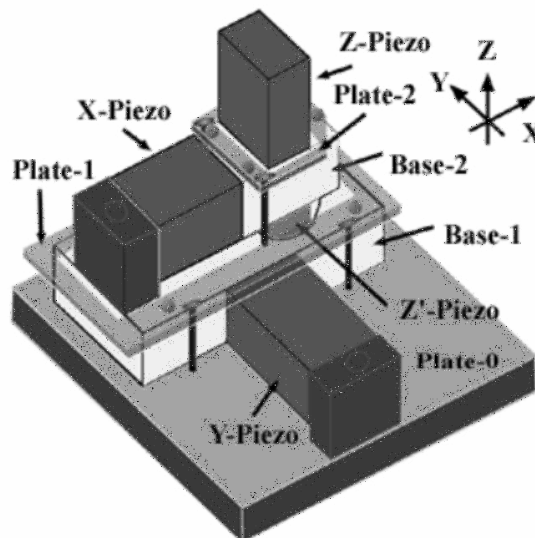

Fig.5 Scanner assembly. The piezo actuators are 5 mm long, 4 mm wide, and 2.7 mm thick. A sample stage is attached to the top of the z-piezo.

4. IMAGING

According to eqs. (1) and (2), the feedback bandwidth of 60 kHz as well as the high resonance frequencies of the small cantilevers can reduce the time for capturing an image with 100^2 2 nm pixels to 70 ms, as long as the apparent width of the sample is not too small. We examined whether imaging can really be carried out at (or near) the maximum rate predicted here. We chose a motor protein, myosin V as the first sample to be examined by our high-speed AFM, since it is the first unconventional myosin identified as a processive motor ; it travels a great distance along an actin filament without detaching from actin (Sakamoto et al., 2000). We are aiming at viewing its nanometer-scale dynamic process in real time. We started with imaging myosin V alone directly attached to mica, in solution. In Fig.6 the images we first obtained are shown (270 nm scan range; 100^2 pixels; for 1.6 s (20 frames)). The scan rate was 1.25 kHz, corresponding to a tip speed of 0.68 mm/s, and the frame rate was 12.5/s. The myosin V molecule is attached to mica through one of the two heads, and the other moieties are free and moving. The typical Y shape is clearly seen. ATP is absent in this solution. Next, we show, in Fig.7, AFM images of myosin V in a solution containing ATP. The angle of the long tail relative to the head/neck regions changed between the eighth and ninth frames among the successive 50 frames (see the second and third images in Fig.7). This quick change took place within 30 ms. After this change, the tail and the tail end are slowly moving. This marked contrast suggests that this quick orientational change may be driven by ATP.

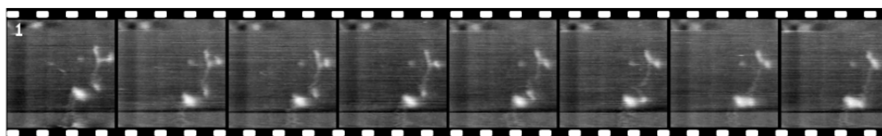

Fig.6 Successive images, at 80 ms intervals, of myosin V weakly attached to mica surface, in buffer solution.

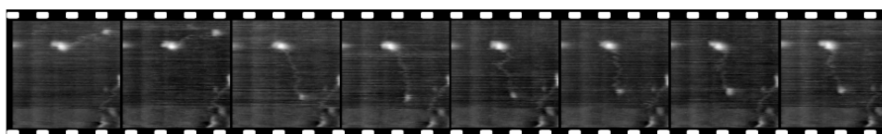

Fig.7 Successive images, at 80 ms intervals, of myosin V on mica in a solution containing ATP. The reconstructed movie and the other movies can be viewed at the web site (http://www.s.kanazawa-u.ac.jp/phys/biophys/bmv_movie.htm).

5. DISCUSSION

In life science, it has been a dream to view the nanometer-scale dynamic behaviour of individual biopolymers in solution. The capacity to acquire successive images every 80 ms will allow a large expansion in the scope of biological processes that can be examined in real time. In the near future, we should be able to observe the behavior of processive motors such as kinesin and myosin V moving along their tracks, of molecular chaperones assisting a polypeptide chain to fold, or of a ribosome synthesizing a polypeptide. Such direct observations will provide insight into the mechanisms by which biomolecular machines operate. We think that the high-speed AFM has further potential in life science. If we can link dynamic images of a protein

acquired by the high-speed AFM to its known atomic structure, we may be able to construct dynamic atomic models not obtainable by other techniques. How can we make such a link? Cryo-EM imaging may be a candidate that can mediate this linking process. Suppose cryo-EM images are obtained for protein molecules that were performing a function immediately before freezing; these molecules will be found with different conformations among these images. These conformations must occur on a single molecule of protein that dynamically changes its structure along the time axis. Therefore, the conformations found in the cryo-EM images can be aligned along the time axis, reflecting the dynamic AFM images. Then, we must deform the atomic structure so as to fit it to the conformations found in the cryo-EM images. In this way, we can construct dynamic atomic models that move and thus reflect the AFM movies. The static atomic structures of many proteins have been revealed by X-ray crystallography. However, the basic framework of structural biology has not been changed significantly since the first success by Professor Perutz in 1936. We hope that the high-speed AFM will enable a breakthrough in structural biology in the future.

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