High-speed atomic force microscopy of protein dynamics: myosin on actin and rotary enzyme F_1-ATPase

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INTRODUCTION

One frequent way to study protein function is to examine protein structure, as there is tight relationship between structure and function. The detailed structures of over 60,000 proteins have been revealed to date, however, these structures are merely static snapshots of the proteins, whereas proteins are highly dynamic. The dynamic behaviour of proteins in action has been studied by single-molecule fluorescence microscopy, but the protein molecules themselves are invisible in the observations. Hence, the simultaneous assessment of structure and dynamics has not been feasible, meaning that we have to infer how proteins function from gleaned data with significant resolution gaps. Directly visualizing functioning protein molecules at high spatiotemporal resolution has long been desired.

To materialize this long-quested dream, two decades ago Hansma's and my groups had independently embarked on the development of high-speed atomic force microscopy (HS-AFM). AFM enables the high resolution visualization of biological samples in solutions. However, it usually takes minutes to capture an image, and therefore moving objects cannot be imaged or can only be blurrily imaged. Through the creation of new techniques and the improvement of the first version of our instrument [1], HS-AFM has now been established [2] and an instrument is now commercially available from RIBM. The current HS-AFM can generally capture an image of protein molecules within 100 ms or less. Importantly, the function of fragile molecules is not disturbed by the interaction with a cantilever tip. As has been demonstrated in recent imaging studies [3-6], the visualized dynamic images of proteins can provide information inaccessible with other approaches, giving a great insight into how the proteins function.

Here we describe our experimental set-up for HS-AFM and report on its application to the study of two biological mechanotransducer systems: the movement of myosin V on actin filaments and the conformational changes in rotorless F_1-ATPase.

MATERIALS AND METHODS

HIGH-SPEED ATOMIC FORCE MICROSCOPE SYSTEM

A schematic of our tapping mode HS-AFM system is shown in Figure 1. A small sample stage (1.5 mm in diameter) is placed upside down and glued to the Z-scanner. The scanner can be displaced by up to 1 μm, 4 μm, and 800 nm in the X-, Y- and Z-directions, respectively. The resonant frequency of the Z-scanner is 450 kHz. The Z-piezoactuator (2x2x2 mm^3) is glued to the inner surface of a solid hollow cylinder at its four rims parallel to the displacement direction (inset in Figure 2) so that the Z-piezoactuator's centre of mass is maintained stationary [7].

To reduce the quality factor and hence increase the response speed of the Z-scanner, we developed a new Q-control method [8]. In a conventional Q-control method, a damping signal proportional to $-dz/dt$ is added to the driving signal. However, to measure the displacement or speed of the Z-scanner in real time is difficult. Instead, we added the output signal from a 'mock Z-scanner' constructed of an LRC circuit, as shown in Figure 2. The mock Z-scanner is characterized with a transfer function similar to that of the real Z-scanner. The amplitude detector can output amplitude signals every cycle of cantilever oscillation [2]. HS-AFM cantilevers made of silicon nitride are 10–20-times smaller than conventional ones. They are 6 μm long, 2 μm wide and 90 nm thick, which give the following mechanical properties: resonant frequency in air, 3.5 MHz; resonant frequency in water, 1.2 MHz; spring constant, 0.2 N/m; quality factor in water, 2. Small cantilevers are available from Olympus and NanoWorld.

The feedback bandwidth f_B representing the speed performance of the HS-AFM system reaches 110 kHz. The maximum possible imaging rate R_{max} is a function of f_B and imaging conditions (the scan size in the X-direction W; the number of scan lines N; the spatial frequency of sample height corrugation to be imaged $1/\lambda$) as well as of the maximum possible phase delay θ_{max} in the feedback operation that depends largely on the sample fragility:

$$R_{max} = 2\theta_{max}\lambda f_B/(\pi WN) \quad [9].$$

For example, under a realistic condition for imaging protein molecules by our HS-AFM (f_B = 110 kHz, θ_{max} = 20°, λ = 10 nm, W = 150 nm, and N = 100), R_{max} becomes 16.3 frames per second (fps).

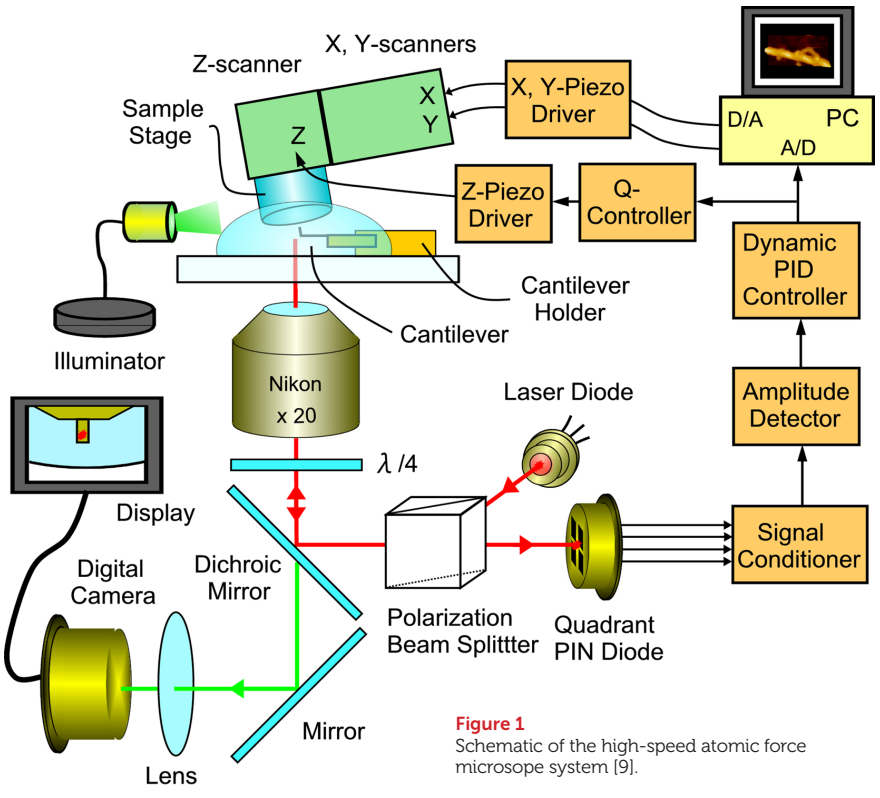


Figure 1
Schematic of the high-speed atomic force microsope system [9].

SAMPLE PREPARATION

Substrate surfaces onto which samples are placed are a key to successful HS-AFM imaging. For the observation of a motor protein myosin V (M5) walking on an actin filament, we used planar phospholipid bilayers containing biotin-lipid, formed on a mica surface [4]. We added a small amount of positively charged lipid in the bilayer to facilitate observing the characteristic sideways figure of M5. Partially biotinylated actin filaments were immobilized onto the lipid bilayer surface via streptavidin molecules with a low surface density.

For the observation of rotorless F_1-ATPase, we used a mica surface which was first treated with aminosilane and then with glutaraldehyde [5]. $(Lys)_7$ tags were introduced to the N-termini of the β subunits of F_1-ATPase. The tags were covalently reacted to the modified mica surface, resulting in the immobilization of F_1-ATPase with the C-terminal side at the top.

RESULTS AND DISCUSSION

WALKING MYOSIN V

Double-headed M5 functions as a cargo transporter in cells and moves processively along an actin filament toward the plus end of the filament in a hand-over-hand manner with a 36-nm advance for every ATP hydrolysis cycle (Figure 3a shows tail-truncated M5 (M5HMM)). 'Hand-over-hand' means that the two heads exchanges leading and trailing roles at each step, like 'bipedal walking'. We observed M5HMM interacting with actin using HS-AFM [4]. Figure 3b shows HS-AFM images of unidirectionally moving M5HMM (see Movie 1 [10]). The translocation velocity was 70% of that measured by fluorescence microscopy. When no positively charged lipid was included in the substrate, the velocity increased to 100 %, indicating no effect of tip-sample interaction on the motor activity. The velocity did not decrease with time throughout the imaging for 23 seconds, at 7 fps, despite the fact that each motor domain was tapped with the oscillating tip more than 60,000 times during this imaging.

In this experiment, the stepping behaviour could not be resolved because a step completed within a frame time. To slow down the stepping, we added more streptavidin on the substrate surface as a moderate obstacle to the advance. As shown in Figure 3c (see Movie 2 [10]), after trailing head detachment, the leading head spontaneously rotated from the reverse arrowhead orientation towards the arrowhead orientation but briefly halted by colliding with a streptavidin molecule placed in the way of its natural path, while the detached trailing head was distant from the actin filament and slightly rotated around the neck-neck junction. Then, the leading head overcame the streptavidin blockade to completely rotate to the arrowhead orientation. Accompanied by this further rotation, the trailing head was bound to a forward site of the filament to become a new leading head. The rotation of the leading head is the swinging lever-arm motion proposed by Hugh Huxley for muscle myosin.

When two-headed bound M5HMM was observed in the nucleotide-free condition, the leading head was often sharply bent, as shown in Figure 4a, whereas it was straight in the presence

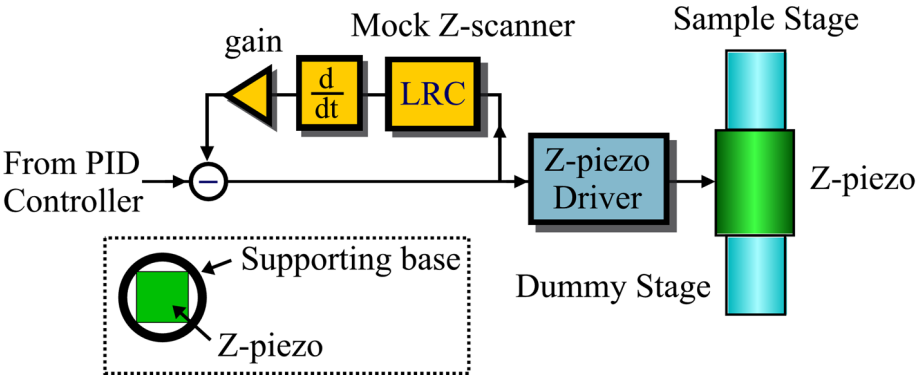


Figure 2
Active Q-control for damping Z-scanner vibrations by the use of a mock Z-scanner.

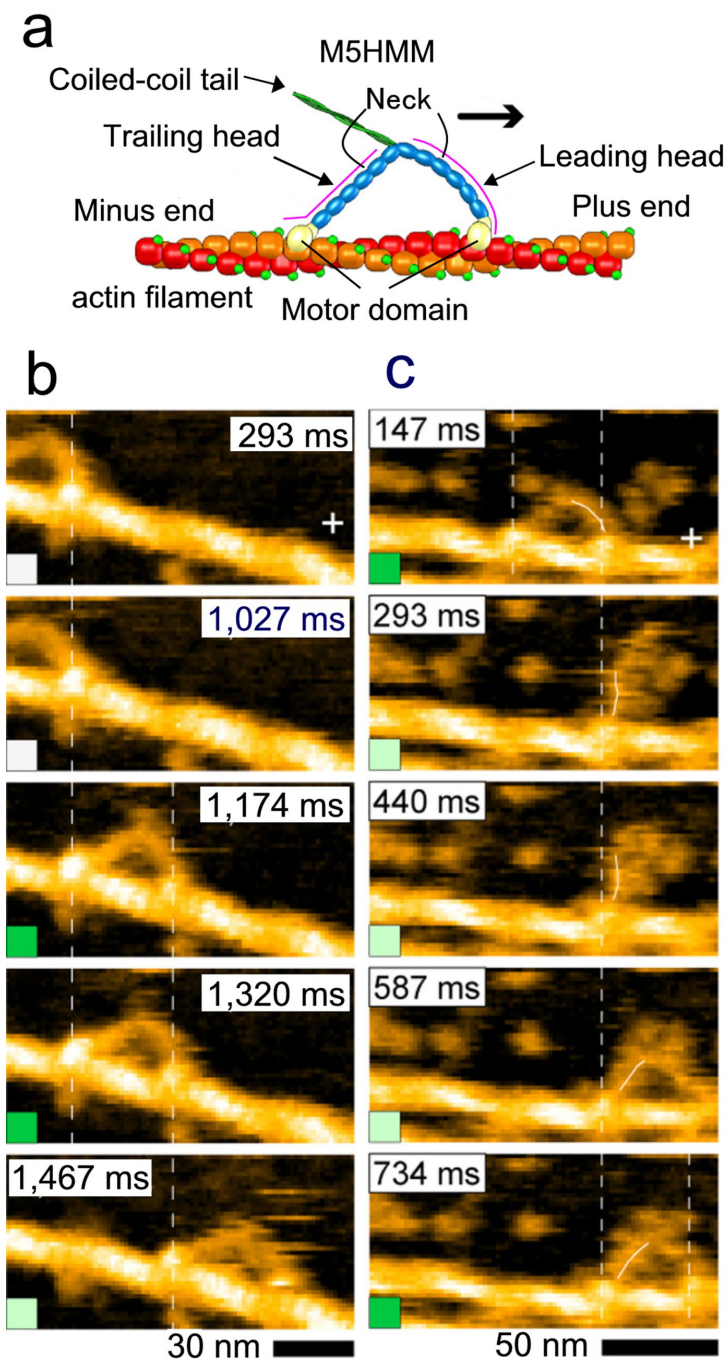


Figure 3
(a) Schematic of actin-bound M5HMM. (b, c) HS-AFM images showing (b) unidirectional movement and (c) hand-over-hand movement of M5HMM on an actin filament [4]. Frame rate, 7 fps.

of nucleotide, as shown in Figure 4b. Therefore, by observing the leading-head conformation, we can judge whether or not the leading head contains nucleotides. To estimate the rate constant of ADP dissociation from the leading head, we observed two-headed bound M5HMM in various ADP concentrations. It came out to be 0.1/s, meaning that ADP dissociates from the leading head every 10 seconds, on average. However, in the presence of ATP, M5HMM walks many steps in 10 seconds. Therefore, when M5HMM is walking in the presence of ATP, ADP never dissociates from the leading head. Thus, we conclude that ADP dissociation, subsequent ATP binding, and the resulting dissociation from actin only occur at the trailing head. This is the basis underlying the processive hand-over-hand movement of M5.

When two-headed bound M5HMM was observed in ADP, the coiled-coil tail was sometimes unfolded, as shown in Figure 5. Upon unfolding, the leading head rotated from the reverse arrowhead orientation to the arrowhead orientation, similar to the swinging lever-arm motion, indicating that intramolecular tension for the lever-arm swing exists in the two-headed bound M5HMM in ADP. This means that external energy is not required for tension generation. We observed a M5 behaviour (named 'foot stomp') in the presence of ATP, which reinforced this inference; the leading head briefly detached from actin and then rebound to actin, whereas the molecule remained at approximately the same position on the filament. After foot stomp, M5HMM stepped forward. Because phosphate (P_i) release occurs very quickly when the ADP-P_i-bound head is bound to actin, the leading head does not carry P_i before and after the foot stomp. These observations suggest that the chemical energy liberated by ATP hydrolysis is not used for force generation as well as forward movement of M5. External energy for forward movement is only required for the trailing head detachment from actin. The energy of ATP binding is used to detach the trailing head. The ATP hydrolysis reaction (not energy) is used to make it sure that the mechanical process proceeds unidirectionally. This idea, which should be verified henceforth, will overturn the common view that the energy of ATP hydrolysis is used for motor proteins to move.

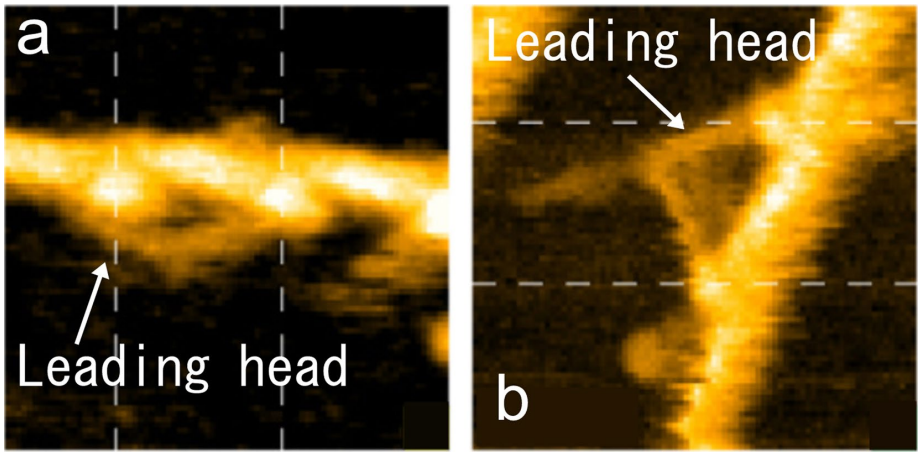


Figure 4
HS-AFM images showing (a) sharply bent leading head in nucleotide-free condition and (b) straight leading head in ADP [4].

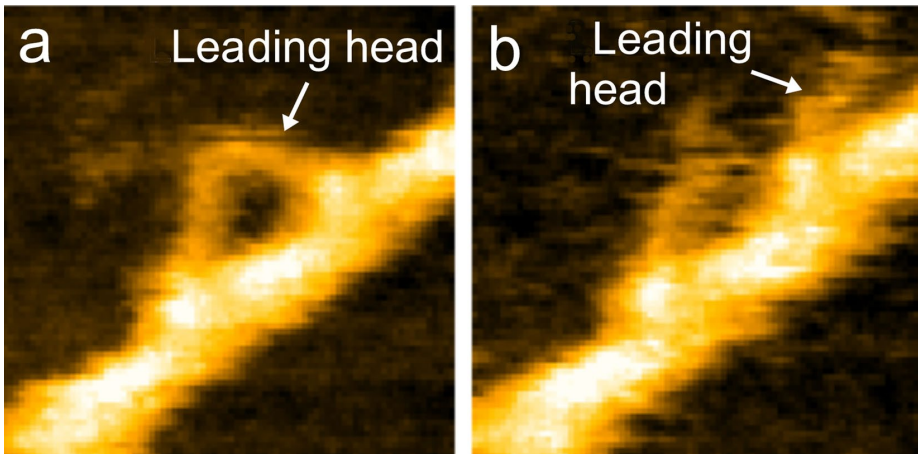


Figure 5
HS-AFM images showing (a) before and (b) after unfolding of coiled-coil tail of two-headed bound M5HMM in the presence of ADP [4].

ROTARY CATALYSIS OF ROTORLESS F_1-ATPASE

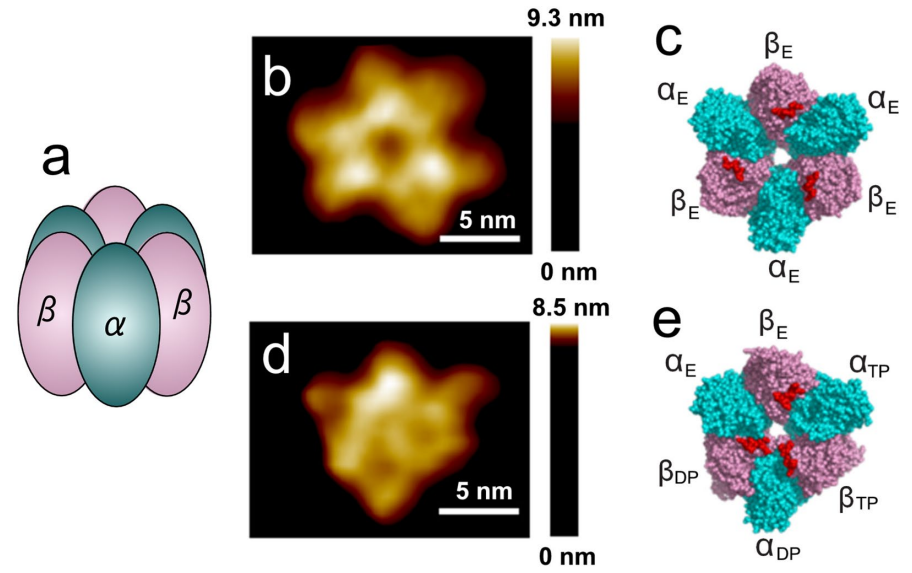
The minimum unit of F_1-ATPase, the $\alpha_3\beta_3\gamma$ subcomplex, is a rotary motor. The rotary shaft γ is inserted in the central cavity formed by the stator ring $\alpha_3\beta_3$, where the three α and three β subunits are alternately arranged. γ rotates in the counterclockwise direction as viewed from the C-terminal side of $\alpha_3\beta_3$. Three ATP binding sites are located at the α-β interfaces, mainly in the β subunits. In the ATPase cycle, three βs take different chemical states: one ATP-bound, one ADP-bound, and one nucleotide-free (empty). Thus, there is strong co-operativity between the three βs even though they are not in direct contact. It has been proposed that interactions with γ determine the conformational and catalytic states of the individual βs because γ has no symmetry and hence the three β-γ interactions are different.

We investigated this issue by observing $\alpha_3\beta_3$ (Figure 6a) using HS-AFM [5]. When nucleotides were absent, alternately arranged three subunits had higher protrusions than the other three (Figure 6b). From the crystal structure of $\alpha_3\beta_3$ (Figure 6c), the three subunits with higher protrusions were identified as βs. In the presence of AMP-PNP, $\alpha_3\beta_3$ looked triangular, because two of the alternately arranged three subunits were inwardly retracted. Moreover, only the subunit that was outwardly extended had a higher protrusion (Figure 6d). This subunit was identified to be nucleotide-free β from the crystal structure of nucleotide-bound $\alpha_3\beta_3$ (Figure 6e).

When imaged in ATP at 12.5 fps, each β exhibited a conformational transition between the outwardly extended high state (O state) and the retracted low state (C state), as shown in Movie 3 [10]. Only one β assumed the O state. When an O-to-C transition occurred at one subunit, a C-to-O transition occurred simultaneously at the subunit's counterclockwise neighbour. Thus, the O-conformation propagated counterclockwise, as shown in Figure 6f. These dynamic transitions indicate that the O-to-C transition occurs when ATP is bound to an empty β subunit, while the C-to-O transition occurs when an ADP-bound β subunit releases ADP. Therefore, the three subunits take different states in a counterclockwise arrangement, explaining the rotary catalysis by the $\alpha_3\beta_3$ subcomplex. Thus, we can conclude that the intrinsic co-operativity responsible for torque generation to rotate γ is elicited through β-β interplay alone, and that γ passively undergoes torque to rotate.

CONCLUSIONS

High-speed atomic force microscopy allows us to visualize directly protein molecules in action, at subsecond to sub-100-millisecond temporal and submolecular spatial resolution, without disturbing their function. Visualized dynamic images of molecules can provide information inaccessible with other approaches, giving a great insight into how the proteins function. The dynamics of molecules that appear in AFM



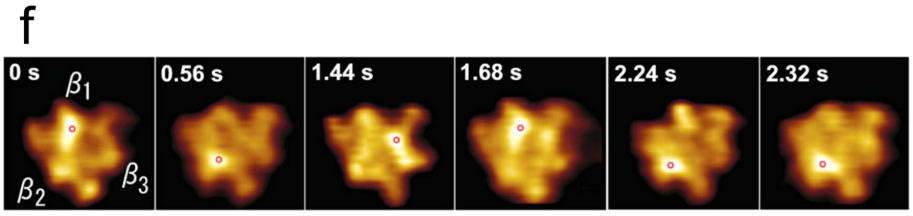


Figure 6
HS-AFM images of rotorless F_1-ATPase $\alpha_3\beta_3$ subcomplex [5]. (a) Schematic of $\alpha_3\beta_3$ subcomplex. (b) AFM image of $\alpha_3\beta_3$ in nucleotide-free condition. (c) Crystal structure of nucleotide-free $\alpha_3\beta_3$. (d) AFM image of $\alpha_3\beta_3$ in AMP-PNP. (e) Crystal structure of nucleotide-bound $\alpha_3\beta_3$. (f) HS-AFM images showing the conformational change of β subunits in ATP. The highest pixel in each image is indicated by the red circle. Frame rate, 12.5 fps.

movies can be interpreted straightforwardly without intricate analyses and interpretations, making it possible to attain firm conclusions.

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BIOGRAPHY

Toshio Ando is a Professor of Physics and Biophysics, and Director of the Bio-AFM Frontier Research Center at Kanazawa University in Japan. Toshio received a BEng in applied physics and a DSc in physics from Waseda University. After working for nearly seven years as a postdoctoral fellow and then an assistant research biophysicist at University of California San Francisco, he joined the faculty at Kanazawa University. He has been working on high-speed AFM for two decades.

ABSTRACT

AFM allows the visualization of biological samples under physiological solution conditions, at high spatial resolution. However, captured images are limited to snapshots because it takes at least 30 seconds to capture an image, whereas biological phenomena are highly dynamic. To overcome this limitation, my group has developed high-speed AFM. The dynamic processes and structural dynamics of protein molecules in action can now be visualized using HS-AFM. Here we describe our experimental set-up for HS-AFM and report on its application to the study of two biological mechanotransducer systems: the movement of myosin V on actin filaments and the conformational changes in rotorless F_1-ATPase.

ACKNOWLEDGEMENTS

This work was supported by JST/CREST and MEXT Japan.

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Microscopy and Analysis 27(3):10-13 (AP), 2013