Cutting Edge 1

Single-Molecule Nanobiotechnology

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Abstract

Epoch-making techniques for manipulating a single biological macromolecule have been developed recently and used to measure directly the chemo-mechanical reactions of a single molecule of actomyosin, the molecular motor of muscle. The dynamic properties and the unique operation of actomyosin molecules, which are different from those of man-made machines, suggest that the mechanisms of molecular machines are flexible and effective.

1. Introduction

Actomyosin, a complex of actin filaments and myosin motor proteins, is responsible for muscle contraction. The sliding movement of actin filaments relative to myosin molecules is driven by the chemical energy of ATP hydrolysis. A long-held model for this process hypothe-

ses that one ATP molecule is hydrolyzed by a myosin head, causing the myosin head to change its structure and pull the actin filament by one step.[1] This model is analogous to the principle of man-made machines that operate deterministically at energies much higher than the thermal noise. The myosin molecule, however, is nanometers in size and has a flexible structure, and it operates at an energy as small as the average energy of thermal noise. It is therefore very prone to thermal agitation. Actomyosin motors can thus operate under the strong influence of thermal noise, with high chemo-mechanical energy conversion (40% maximum). The mechanism underlying the actomyosin motor must therefore be essentially different from the mechanism one would predict by analogy to man-made machines. The working principle of the actomyosin motor can-

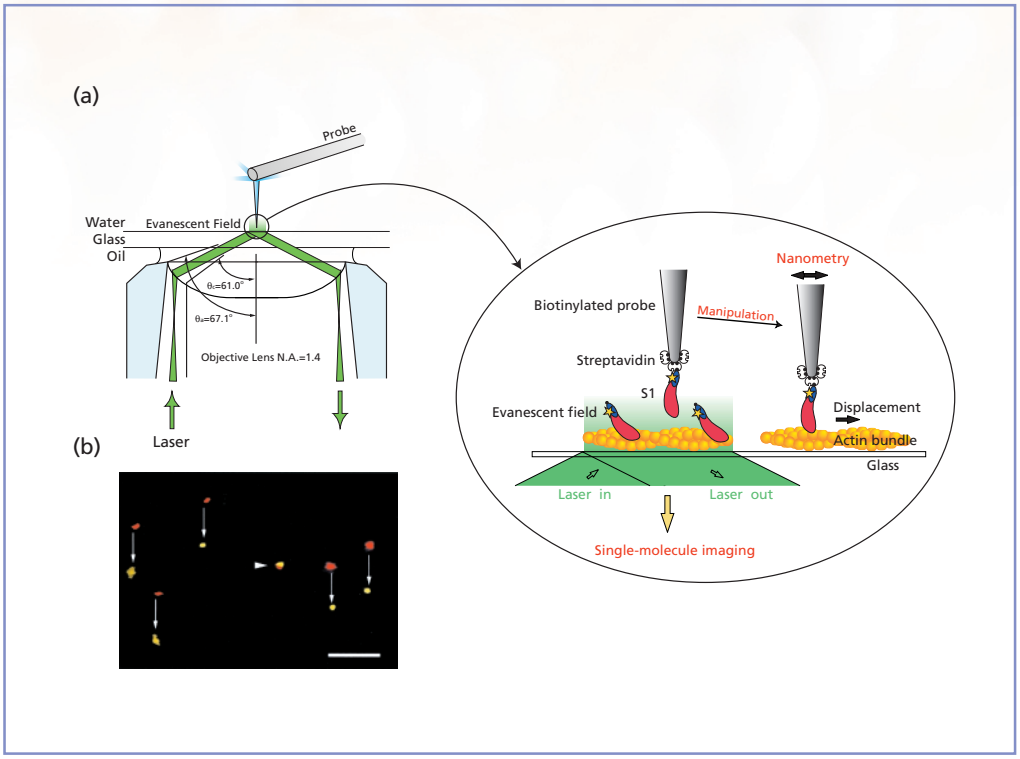


Fig. 1 Direct capture and manipulation of a single S1 molecule by a scanning probe.
(a) Schematic drawing of the experiment. A single S1 molecule, biotinylated and fluorescently-labeled with Cy3, was attached (at its tail end, through the biotin-streptavidin system) to a scanning probe and observed by objective-type TIRFM. The displacement produced when the S1 molecule was brought into contact with an actin bundle bound to a glass surface was determined by measuring the position of the needle with nanometer accuracy.
(b) Fluorescence images of single S1 molecules. The micrograph shows superimposed images of single S1 molecules either captured by the probe (arrowhead) or bound to actin bundles on the surface of the coverslip. The red and yellow spots respectively represent those seen in images before and after the stage was moved by a piezoelectric actuator. The captured S1 molecule (arrowhead) did not move with the stage but could be moved independently by piezoelectric scanners holding the needle. Bar: 5 μm.

not be elucidated without resolving the intrinsic characteristics of these molecular machines.[2)]

To explore the mechanism of the actomyosin motor, we developed a subtle technique using a scanning probe to manipulate a single myosin molecule.[3)] This technique has been able to resolve the process generating the displacements produced by a single myosin head. We have also demonstrated recently that single fluorophores in aqueous solution can be observed in real time by total-internal-reflection fluorescence microscopy (TIRFM) refined so that the background noise is very low[4,5)] and have used this technique to detect individual ATP turnovers of a myosin molecule by observing the single association/dissociation events of a fluorescent ATP analog, Cy3-ATP. Furthermore, by combining techniques for single-molecule imaging and nanomanipulation using an optical trap, we have developed an assay for simultaneously measuring individual ATPase and mechanical reactions of single myosin molecules while they are generating force.[6)] This assay shows how the ATPase reaction corresponds to the mechanical events in the actomyosin motor.

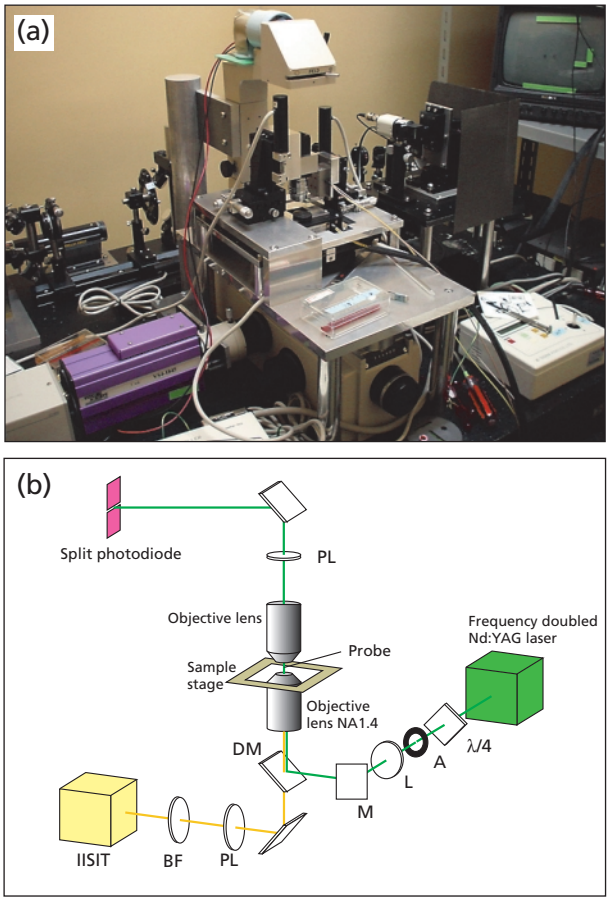


Fig. 2 (a) Experimental setup for manipulating a single myosin head by using a scanning probe. (b) Schematic of the system. λ/4, quarter-wave plate; A, diaphragm; L, focusing lens; M, mirror; DM, dichroic mirror; PL, projection lens; BF, bandpass filter; IISIT, image-intensified SIT camera.

2. Nanomanipulation of single myosin heads by a scanning probe

We first investigated the process generating the movement of single actomyosin molecules by using new technologies for the imaging and nanomanipulation of single biomolecules.[3)] By using scanning-probe-based technology (Fig. 1), we measured the tiny force that an individual myosin head exerts on actin - just piconewtons - and the tiny displacement it causes - just nanometers. The scanning probe, consisting of a fine glass needle (50-100 μm long and 0.3 μm in diameter) and a 5- to 7-μm-long ZnO crystal whisker with a very sharp tip (one whose radius of curvature was about 15 nm), was mounted on a three-dimensional piezoelectric scanner. The stiffness of the needles was low (0.01 - 0.03 pN/nm), and the forces and displacements produced by a single myosin head could thus be clearly detected.

Myosin head molecules were fluorescently labeled and biotinylated at the tail end, away from the ATP- and actin-binding sites on the myosin head, thereby avoiding damage due to labeling and interactions with the attached surface. The tip of the scanning probe was coated with streptavidin so that single myosin heads could be captured specifically at the biotinylation site. As seen in Fig. 1(b), single myosin head molecules captured on the tip of the scanning probe were visualized clearly, at a high fluorescence-to-background ratio, by an evanescent field produced in a simple way by objective-type TIRFM.[5)] In this system, the illumination mode is easily switching from epi-fluorescence microscopy to TIRFM by moving a mirror. Objective-type TIRFM has the advantage of providing a free space above the coverslip, so it is easy to combine with scanning probe microscopy.

The fluorescence of the myosin head captured on the tip of the scanning probe was characterized by a single, approximately Gaussian, intensity distribution and single-step photobleaching (data not shown), strongly indicating that only a single myosin head was captured. One was captured and manipulated by the fine scanning probe under a TIRFM and, in the presence of ATP, brought into contact with an actin filament bound to a glass surface. The displacements due to interactions between the myosin head and actin molecule were detected by using a differential pair of photodiodes to measure deflections of the needle with sub-nanometer accuracy.

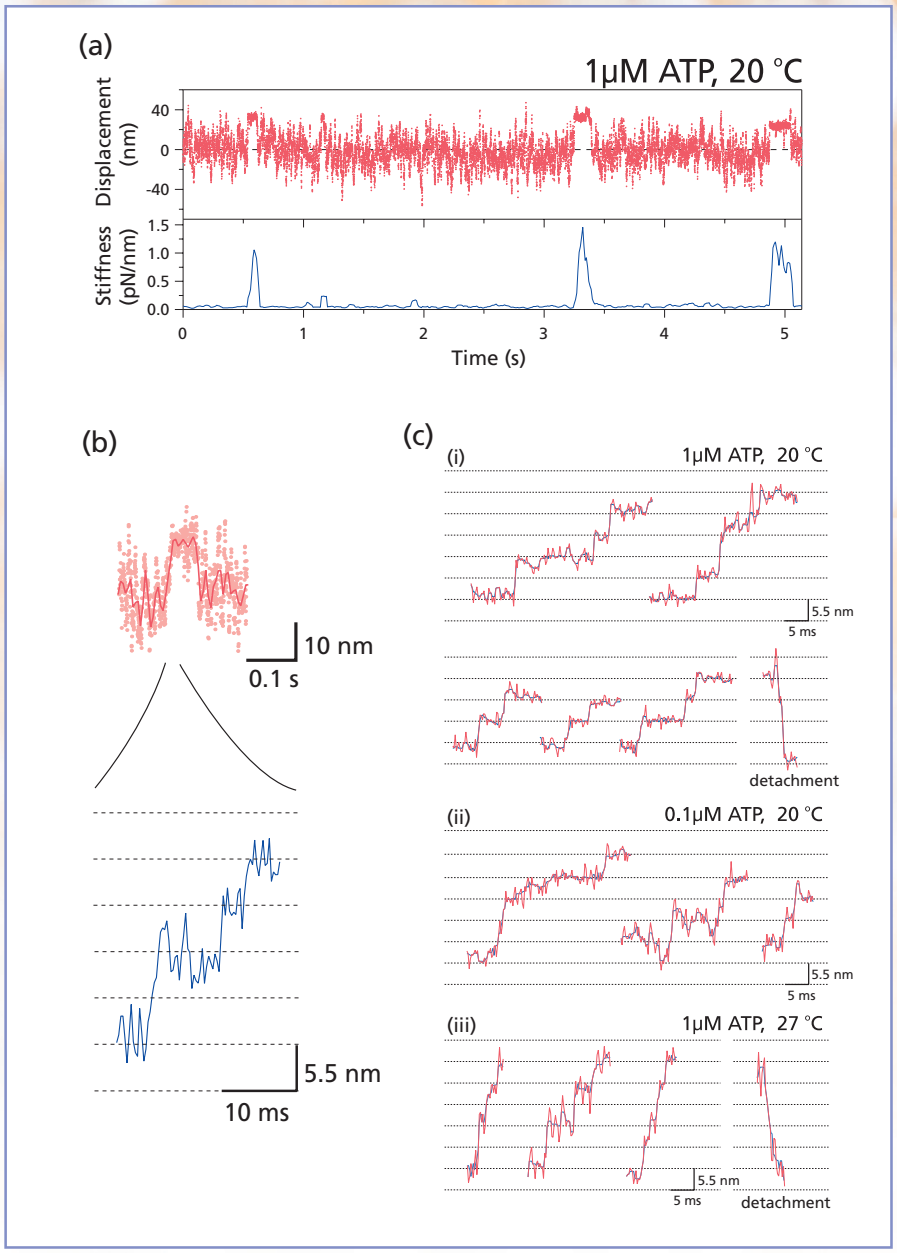


Fig. 3 Displacement caused by single S1 molecules.
(a) Upper: typical recording of the displacements made by an S1 molecule. Lower: stiffness calculated from the variance of the probe position.
(b) An example of a single displacement event and its rising phase on an expanded time scale.
(c) Records of the rising phase of displacements under various conditions: (i) 1 μM ATP, 20°C; (ii) 0.1 μM ATP, 20°C; (iii) 1 μM ATP, 27°C. Horizontal gridlines have been drawn at a spacing of 5.5 nm. In the falling phase of the displacements, stepwise movement was not observed (last traces in (i) and (iii)).

3. Stepping motion

When the myosin head was not associated with an actin filament, large thermal fluctuations of the probe were apparent. Their r.m.s. amplitude was about 13 nm. When the myosin head bonded to actin the r.m.s. amplitude of the fluctuations fell to less than 4.5 nm (Fig. 3(a), upper trace). Probe motion caused by actomyosin interactions could be clearly distinguished from thermal noise by monitoring the increase in stiffness, calculated as the reciprocal of the variance of the fluctuating probe position (Fig. 3(a), lower trace). The concentration of ATP was low in these experiments (0.1 or 1 μM), leading to prolonged actomyosin interactions and thus enabling individual mechanical events to be identified easily. When the myosin head was attached to an actin molecule, the stiffness of the probe-myosin-actin linkage was (at 20°C) 0.2-1.5 pN/nm, more than ten times the stiffness of the probe. The high degree of stiffness during the generation of displacements greatly improved the temporal resolution and signal-to-noise ratio, allowing us to resolve the elementary processes generating the displacements.

Displacements observed with a low temporal resolution took place abruptly (Fig. 3(a)), which appeared to be consistent with the conventional model. On an expanded timescale, however, we found that the displacements were not actually abrupt but instead developed in multiple steps (Fig. 3(b)). The time course of the rising and falling phases of displacements in the presence of ATP at different concentrations and temperatures is shown on an expanded timescale in Fig. 3(c). The steps at 20°C are clearer than those at 27°C because the dwell-time between them was longer. In the falling phase of the displacements (detachment of the myosin head from actin following ATP binding), the probe returned to the zero position with a 1/e time of 2-5 ms (Fig. 3(c), parts (i) and (iii), last traces), which was similar to the settling time of the free needle. No regular steps were evident during the falling phase. The size of steps seen during the rising phase was regular and consistent with the periodicity of adjacent actin molecules in an actin filament, 5.5 nm (Fig. 4(a)). The number of steps per displacement event varied stochastically from one to five (Fig. 4(b)), yielding overall displacements with sizes between 5 and 30 nm. Steps were not always forward but were sometimes backward (10% of the total number of steps). The size of the backward steps was also 5.5 nm. These patterns of steps indicate that the myosin head steps back and forth along the actin molecules in a filament by Brownian motion rather than by changing shape (Fig. 4(c)). Note that the individual displacements of single myosin molecules could not be determined directly by optical trapping measurements because the variance in start positions was large (~30 nm) and the signal-to-noise ratio was low because of the series compliance. As a result, only a mean displacement could be determined.[2)]

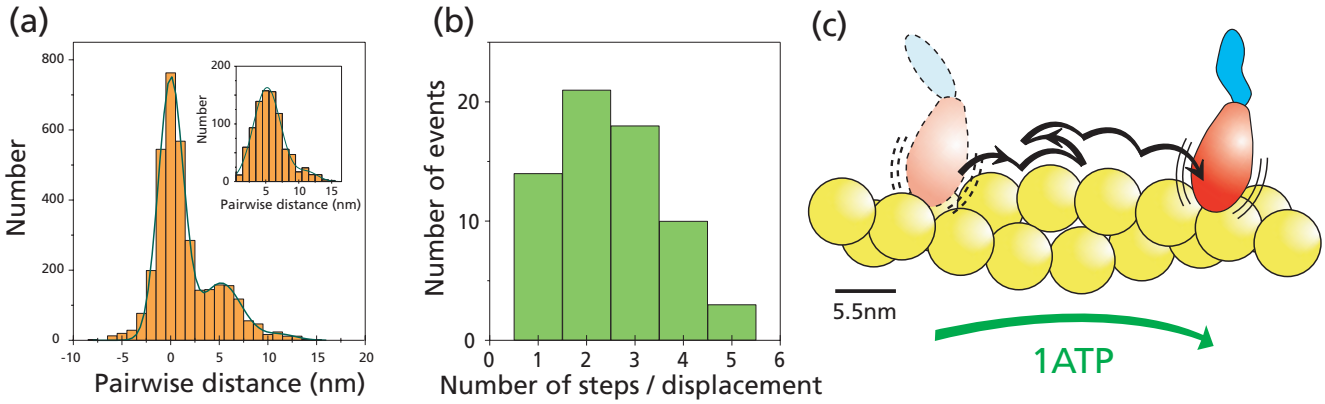


Fig. 4 (a) Histogram of the step sizes in the rising phase of displacements. Inset, histogram of the step sizes subtracting Gaussian distribution at 0 nm.
(b) Histogram of the number of steps per displacement.
(c) Model of an actomyosin motor.

We could determine the size of individual displacements directly in the present experiments, though, because the myosin head was directly attached to the working point and the series stiffness during actomyosin interaction could be several times that in the previous experiments. The signal-to-noise ratio in the present experiments was two to three times that in the previous experiments because the noise due to thermal fluctuations of the probe is inversely proportional to the square root of the stiffness. Thus, the high stiffness during actomyosin interaction allowed us to resolve the elementary process generating displacements.

4. Chemo-mechanical coupling

How are the 5.5-nm steps coupled to the biochemical cycle of ATP hydrolysis? To answer this question we measured the ATP hydrolysis reaction and the generation of displacement simultaneously[6] (Fig. 5). Individual ATP hydrolysis reactions of a single myosin head were measured by using TIRFM to monitor single fluorescently labeled ATP molecules associating and dissociating with the myosin head, and the individual mechanical events were measured by optical trapping nanometry. Measurement results are shown in Fig. 7, where part (a) shows the fluorescence image of individual Cy3-ATP turnovers produced by a single myosin head during displacement generation. The binding of a Cy3-ATP molecule to a myosin head was clearly observed as a fluorescent spot. All of the fluorescence intensities were similar (~1000 photons per second), indicating that the fluorescent spots were indeed due to single Cy3-ATP molecules. As shown in Fig. 7(b), changes in displacements were accompanied by changes in fluorescence intensity. Thus, each displacement corresponds to one biochemical cycle of ATP hydrolysis. That is, each 5.5-nm step in the rising phase of displacements was not coupled directly to a single ATP hydrolysis reaction. This implies that the mechanical reaction is loosely coupled to the biochemical cycle of ATP hydrolysis.

A myosin head that had been detached from an actin filament by the binding of Cy3-ATP reattached to the actin filament and generated a displacement. The generation of displacement was associated with the release of the bound Cy3-nucleotide, probably hydrolyzed Cy3-ADP (Fig.7 (c)). The timing, however, was complex. In about half of the events, the nucleotide release coincided (to within the temporal resolution of the system) with the generation of displacement (Fig. 8(a)). Surprisingly, in the

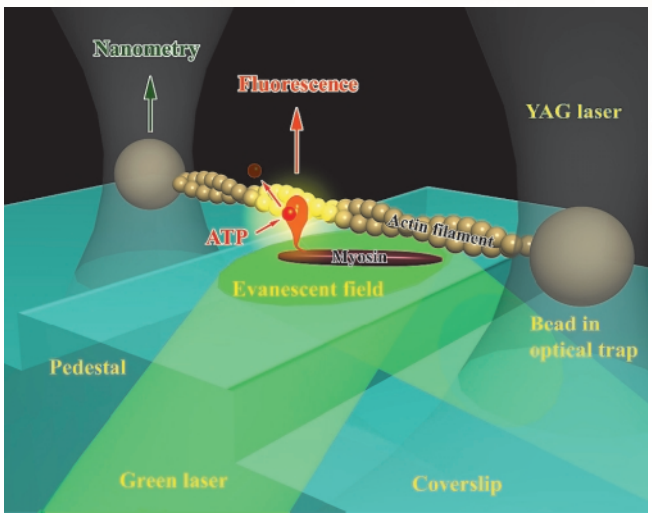
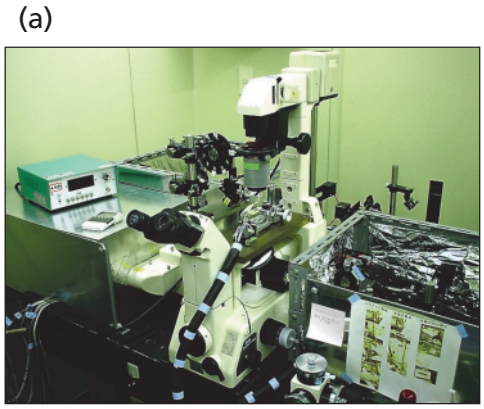


Fig. 5 Simultaneous measurement of individual ATP hydrolysis and the mechanical reaction of single myosin heads. A single actin filament with beads attached to both ends was suspended in solution by optical tweezers. The suspended actin filament was brought into contact with a single myosin head molecule bound to the surface of a coverslip. Displacements due to actomyosin interactions were determined by measuring bead displacements with nanometer accuracy. TIRFM was used to monitor individual ATP hydrolysis reactions as changes in fluorescence intensity due to association-(hydrolysis) -dissociation events of a fluorescently labeled ATP molecule with the myosin head.



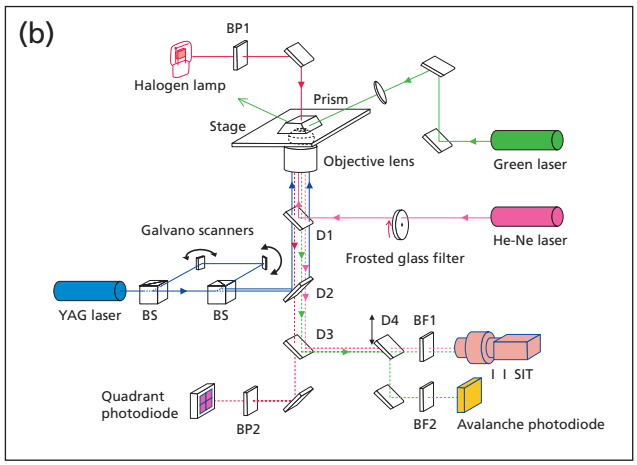


Fig. 6 (a) Setup for simultaneous measurement of individual ATPase and mechanical reactions.
(b) Schematic of the system. D, dichroic mirror; BF and BP, bandpass filter; BS, beam splitter cube.

other events the nucleotide dissociated from free myosin heads, which then reattached to the actin filament and generated displacements within 1 s (Figs. 8(b) and 8(c)). This indicates that the myosin heads could produce force and active displacements after the release of the bound nucleotide.

5. Model of the movement of the myosin head

The size of the small steps making up the larger displacements is the same as the distance between adjacent actin monomers in one strand of an actin filament (5.5 nm). Each step takes place stochastically and some of the steps are backwards. Multiple steps are produced during a single biochemical cycle of ATP hydrolysis and the number of steps in each displacement is variable (loose-coupling).

All these results indicate that the movement of myosin relative to the actin monomers

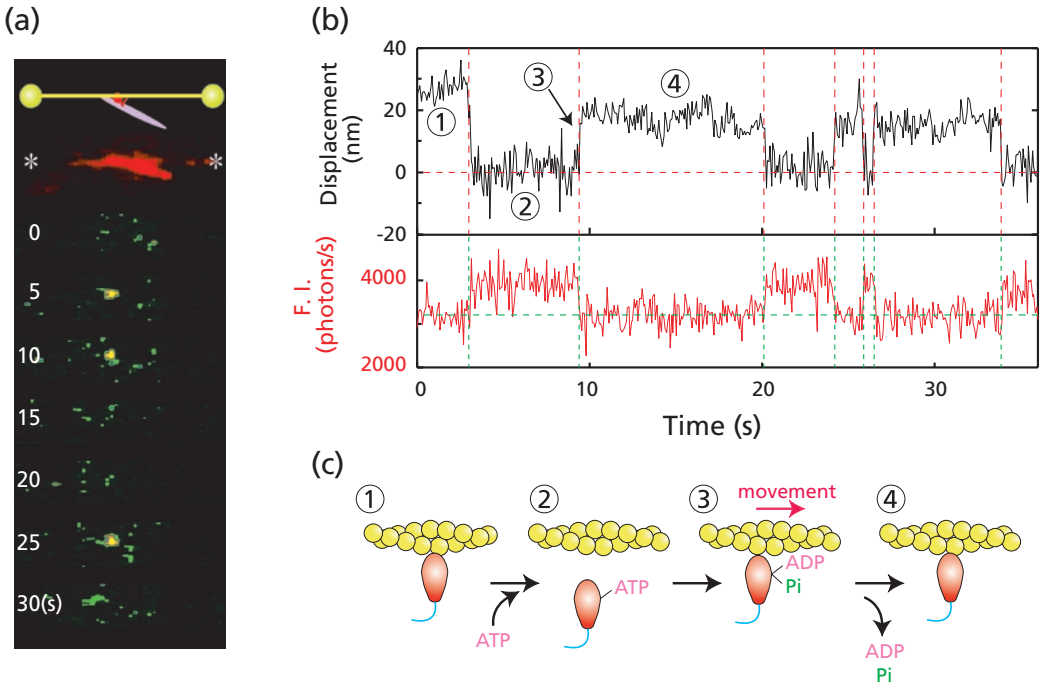


Fig. 7 (a) Fluorescence image of an association-(hydrolysis)-dissociation event of fluorescent ATP molecules with a myosin head during generation of displacements.
(b) Time course of the generation of displacements (upper trace) and of changes in fluorescence intensity (lower trace). Each displacement event corresponds to one biochemical cycle of ATP hydrolysis (c).

in an actin filament is a result of biased Brownian motion (Fig. 4(c)). Myosin heads are known to bind two adjacent actin subunits in rigor, and a myosin head may use these two binding sites to walk along an actin filament without detaching from the filament. Each step may be produced by a mechanism such as the thermal ratchet.[7] It is also possible that conformational changes within the actin filament play a major role in the generation of force.

Multiple steps would be produced for each molecule of ATP split if the chemical energy from ATP hydrolysis were stored in the myosin head or the actin filament and released gradually during successive actomyosin interactions. This idea challenges the widely accepted view that force generation is directly coupled to the release of bound ligands. The simultaneous measurements of ATPase and mechanical reactions show that the myosin head can attach to actin and generate force for a considerable time (>100 ms) after the bound nucleotide is released. The results presented here suggest that the myosin head or actin filament, or both, can store energy from ATP hydrolysis and release it productively in several packets of work.

If the movement of a myosin head is due to Brownian motion, there must be some mechanism by which random Brownian motion is biased into directional movement. The chemical energy from the ATP hydrolysis may not be used to generate the movement of the myosin directly but may instead be used to select unidirectional motions from Brownian motions. A mechanism by which myosin does not overcome but rather uses Brownian motion to effectively gain a good distance with minute energy could explain how myosin can walk under the strong influence of thermal agitation, with high efficiency of energy conversion.

6. Conclusion

Single-molecule imaging and nanomanipulation techniques have allowed us to evaluate the dynamic nature of the actomyosin motor directly, and further investigations using these techniques will certainly reveal the effective and sophisticated mechanism of other molecular machines. As shown in this paper, single-molecule detection techniques unveil dynamic properties hidden from averaged ensemble measurements. These techniques are being used to study an expanding range of life science, such as enzyme reactions, protein dynamics, DNA transcription, and cell signaling.[8]

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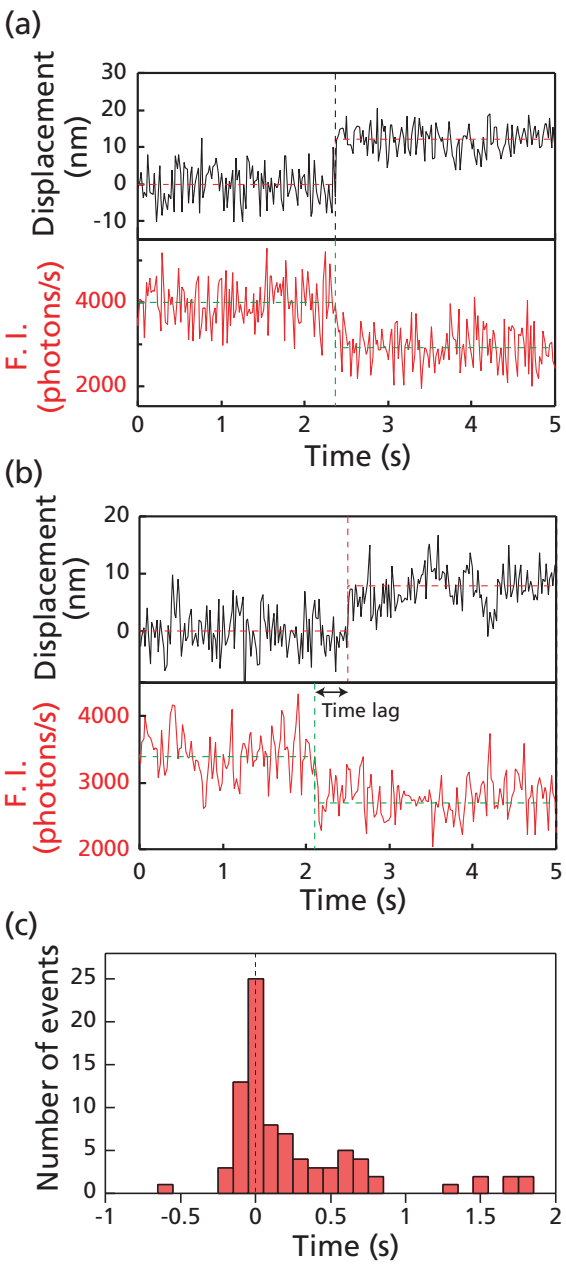

Fig. 8 Timing of nucleotide dissociation and the generation of displacement.
(a) The generation of a displacement coincided with the release of Cy3-nucleotide.
(b) The displacement was generated after the release of Cy3-nucleotide.
(c) Histogram of the delay times from the release of Cy3-nucleatide to the generation of displacements.

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