Single Molecule Detection in Life Science

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Abstract

In recent years, the development of single molecule detection (SMD) techniques has opened up a new era of life science. The dynamic properties of biomolecules and the unique operations of molecular machines, which were hidden in averaged ensemble measurements, have been unveiled. The SMD techniques have rapidly been expanding to include a wide range of life science. The experiments on molecular motors, DNA transcription, enzyme reactions, protein dynamics, and cell signaling are summarized in this short review.

Introduction

Biomolecules assemble to form molecular machines such as motors, DNA transcription processors, cell signaling processors, protein synthesizers, and folding chaperones. These molecular machines collaborate and function in living cells (Fig. 1). The structures of the biomolecules and the roles they play have been determined using recent advanced technology of structural and molecular biology. However, the structure of and the interaction between biomolecules change dynamically. It must therefore be important to study the dynamic properties of biomolecules further. In ensemble measurements involving a large number of molecules, however, the dynamic properties are averaged and cannot be observed. Recently, single molecule detection (SMD) techniques have made rapid advances with the major advances in technology. These techniques have allowed us to record the behavior of individual molecules in real time.

SMD was developed so as single molecules could be visualized and manipulated without damage. After success in imaging of single fluorophores on an air-dried surface [1], a single fluorophore attached to a protein molecule in aqueous solution was first observed in 1995 by using total internal reflection fluorescence microscopy (TIRFM) and conventional epi-fluorescence microscopy [2]. Single biomolecules are visualized as fluorescent spots when they are immobilized and their motion can be traced when they move.

Biomolecules, and even single molecules, can be caught by glass-microneedle [3,4] or by beads trapped by optical tweezers [5,6]. With this technique it is possible to manipulate them precisely.

Microneedle or a bead trapped by laser has the nature of a spring: When force is applied on the spring from the outside, the spring expands in proportion to the applied force [7]. Utilizing this nature, the force and displacement exerted by single molecular motors can be measured. The displacement of a bead and microneedle have been determined with an accuracy of sub-nanometer, much less than diffraction limit of optical measurements. This accuracy of the displacement corresponds to the sub-piconewton accuracy in the force measurement.
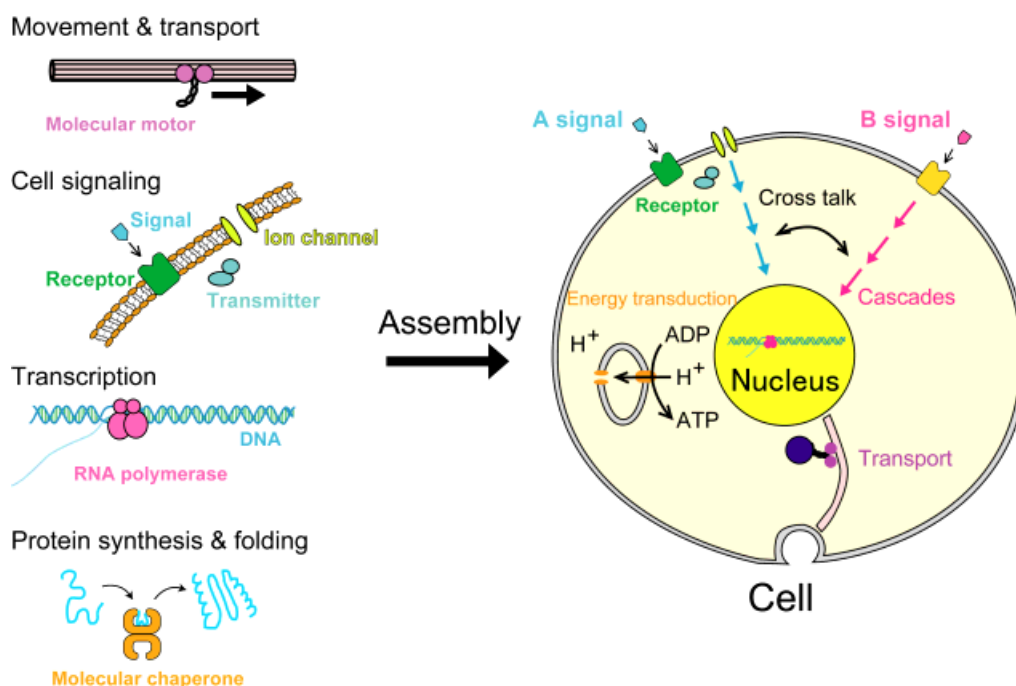
Fig. 1. *Molecular machines and cell. Cells are composed of many molecular machines and cell signaling networks run all over the cell from the outer membranes to the nuclear.*

Molecular Motors

Molecular motors are typical molecular machines, in which the characteristic features of proteins such as enzymatic activity, energy conversion, molecular recognition, and self-assembly are integrated [8]. SMD techniques have been extensively and very successfully used on molecular motors and this research has substantially contributed to the rapid progress recently made on these mechanochemical enzymes.

Myosin

Myosin is a molecular motor responsible for muscle contraction and other cellular motility [8]. Myosin molecules slide along actin filaments, fuelled by the chemical energy driven from ATP hydrolysis. The development of techniques for manipulation of a single actin filament and nanometry with a microneedle [3,4] and optical tweezers [6,9-13] has allowed individual mechanical events such as displacement and force to be measured from single molecules of myosin or its subfragment *in vitro*. A single myosin molecule has been found to generate mean displacements of 5 to 25 nm at nearly zero load and mean forces of 3 to 5 pN at high loads during a single ATP hydrolysis cycle. More recently, a measurement of the process of displacement and force generation with a high spatial and time resolution has been performed with a new assay: a single fluorescently-labeled myosin subfragment-1 (S1) molecule is manipulated with a very fine scanning probe and its mechanical events measured (Fig. 2A) [14]. S1 is the head portion of myosin containing the binding site for ATP and actin and has been proved to move as fast as intact myosin. The displacement records showed that the displacements do not take place in a single step but instead in several distinctive steps: A single step we used to measure, actually contains several steps (Fig. 2B). The steps take place stochastically and some of them (<~10 % of total steps) were backward. The step-size was 5.5 nm, coinciding with the interval between adjacent monomers in one strand of an actin filament. A series of steps could be produced by the hydrolysis of a single ATP molecule. Taking all these results into account, a new model has been proposed that suggests a myosin head can walk on actin monomers in its filament by Brownian motion (Fig. 2C, right panel). This model challenges a currently accepted model, the so called "lever arm swinging model" [15], in which the neck region of a myosin head swings relative to the main body of the head to generate force and displacement, and the swing motion is coupled tightly to the ATP hydrolysis cycle in a one to one fashion (Fig. 2C, left panel). Recently different type of myosins have been identified and characterized [12,16]. The diversity of the life bestow SMD a variety.
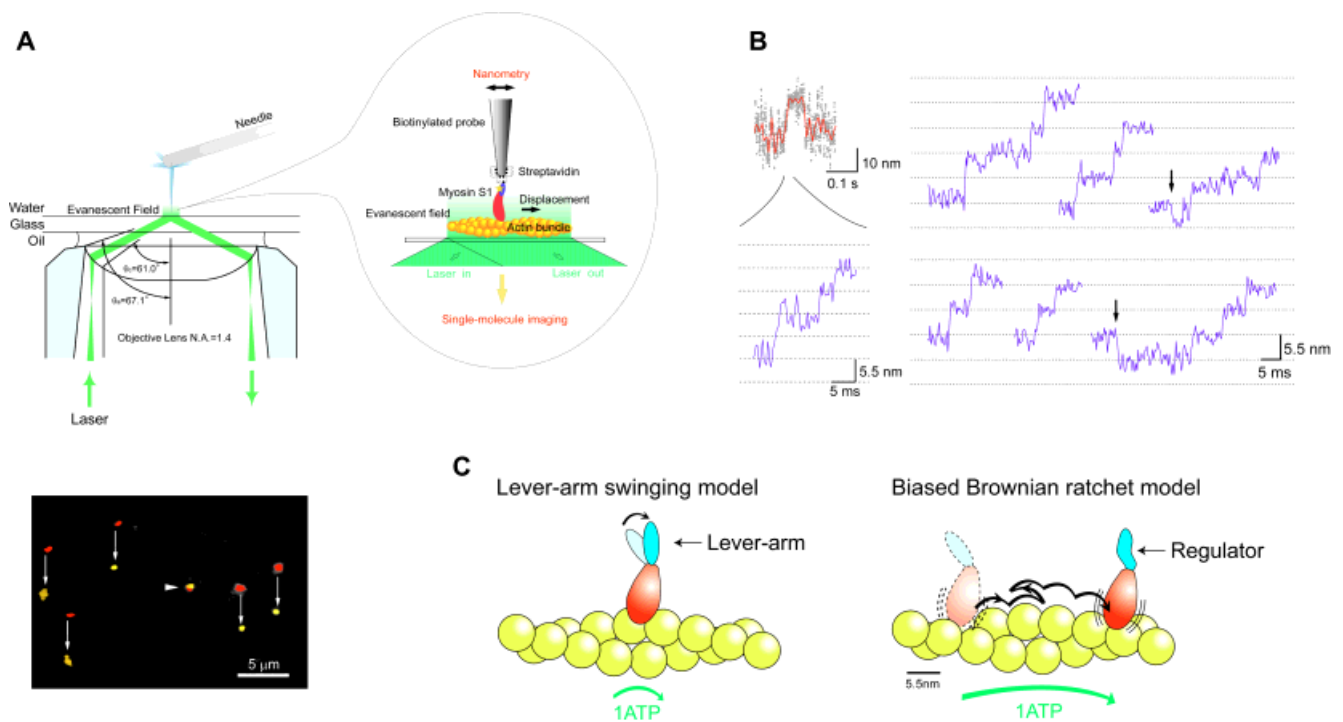
Fig. 2. *Sliding movement of a single molecular motor, myosin subfragment 1 (S1). [14] (A) Fluorescently labeled S1 molecules on the surface of the glass were illuminated by an evanescent field generated by the total internal reflection of laser. A single S1 molecule was captured by a scanning probe through the biotin-avidin system. The bottom panel shows an image of single S1 molecules when the stage was moved. The S1 molecule captured by the probe remained in the one position, while others moved with the stage, indicating that the single S1 molecule has been captured by the probe. (B) The S1 molecule captured by the scanning probe was allowed to interact with actin bundles in the presence of ATP and the displacement of S1 was measured by monitoring the displacement of the probe. The rising phase of the displacement records in a long time range (left top) has been expanded in a short time range. A 5.5 nm step could be clearly observed. The arrows indicate backward steps, which implies that the sliding movement is derived from thermal motion. (C) Two models have been proposed to explain the mechanism of movement of myosin. In the lever-arm swinging model, the myosin molecule moves in a single step. This step occurs by the rotational motion of the lever-arm relative to the head portion of myosin, coupled with the ATPase reaction. In the biased Brownian ratchet model, movement of myosin is driven by the Brownian motion and the ATP hydrolysis biases the direction of the movement.*

Kinesin

Kinesin is another molecular motor that transports cellular organelles along a microtubule. Kinesin works as only a few molecules in cells, in contrast to myosin, in which large number of molecules are usually organized into filaments and highly assembled structures. A single kinesin molecule moves continuously along a microtubule for long distances (up to several micrometers) without dissociating, and hence a series of single-molecule events can be relatively easily observed. Fig. 3A shows the imaging of a single fluorescently-labeled kinesin molecule moving along a microtubule by TIRFM [17]. Fig. 3B shows a high resolution measurement of the motion of a single kinesin using optical trapping nanometry [5,18-24]. A single kinesin molecule exhibits a stepwise displacement with amplitude of 8-nm by hydrolysis of one ATP molecule. An 8-nm step reflects the periodicity of the tubulin heterodimers in a microtubule. Based on this finding, a hand-over-hand model has been proposed, in which a kinesin walks on tubulin-heterodimers via its two heads in an alternating fashion (Fig.3C, top). Single molecules of the kinesin super-family and mutant kinesin molecules which are one-headed, have also shown continuous movement along a microtubule although the travel distance was smaller [25,26]. The motion was not smooth and the direction fluctuated in forward and backward directions. Thus, as for myosin, Brownian motion appears to play an essential role in this movement (Fig. 3C,

bottom) [27]. The motions of myosin and kinesin may be driven from Brownian motion[28], and the chemical energy from ATP hydrolysis may be able to bias the Brownian

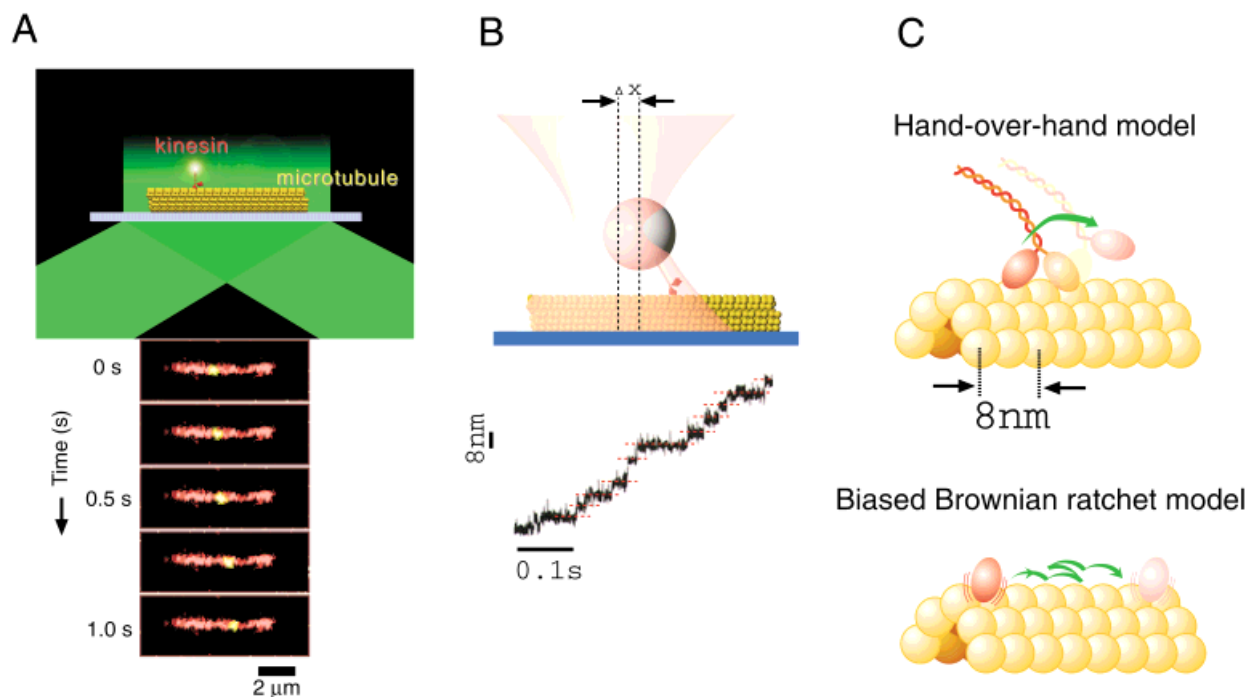motion to a certain direction. This problem is attracting much attention in the field of SMD.

Fig. 3. *Sliding movement of a single molecular motor, kinesin, along a microtubule. (A) Fluorescently labeled kinesin was visualized in TIRFM, while it was moving along a microtubule placed on the glass surface. Time records of the fluorescence image show that the kinesin (yellow) slides on the microtubule (red). [17] (B) A single kinesin molecule was attached to a bead trapped by a laser. Displacement of the two-headed kinesin was measured by monitoring the displacement of the bead. Displacement records that the step size is 8 nm. [5] (C) A single two-headed kinesin molecule moving in one direction in a hand-over-hand fashion (top panel). Thermal ratchet model has been supported by recent measurements of the kinesin super-family motors and deletion mutants of kinesin (bottom panel).*

F_1 Rotary Motor

ATP synthase (F_1F_0-ATPase) produces ATP from ADP and Pi in the F_1 portion by using proton- or sodium-motive force in the F_0 portion (Fig. 4A) [29]. The process is reversible. When hydrolyzing ATP in F_1, the enzyme pumps protons in F_0 in the opposite direction. ATPase and proton flow are coupled by the rotation of a shaft which links F_1 and F_0. The rotation of the shaft could be visualized by tracing the position of fluorescently labeled actin filaments attached to the top of the shaft (Fig. 4B) [30]. When the ATP concentration is lowered, a 120° step of the rotation could be clearly observed, which is consistent with the structure of this rotary motor (Fig. 4B) [31]. In this motor the rotational motion was tightly coupled with the hydrolysis of ATP, and this molecular machine was found to be very efficient: the work done at each step was almost the same as the energy released by hydrolysis of a single ATP molecule.

DNA Transcription

The initial steps of gene expression including the binding of RNA polymerase (RNAP) to DNA, the search for a promoter in the DNA sequence, where transcription starts, and the synthesis of RNA based on the information coded in the DNA. These steps are central regulatory mechanisms of gene expression and have been extensively investigated in kinetic and structural studies. Recently, SMD techniques have been applied to directly observe the process of gene expression [32]. Fig. 5A shows a single molecule of fluorescently-labeled RNAP undergoing linear sliding along DNA suspended in solution by optical traps. This observation provides direct evidence that the sliding motion is the mechanism used to searching for the promoter. When RNAP binds to the promotor of the DNA, transcription starts. The transcription process by a single RNAP has been directly followed by optical trapping nanometry (Fig. 5B)

[33,34]. The force generated by RNAP (>14pN) was much larger than the molecular motors, myosin and kinesin. Thus, we can directly visualize the early stage of the DNA transcription that we have only imagined. The experiments will further proceed to visualize the whole processes of the DNA transcription including the termination processes, in which many unanswered questions still remain (Fig. 5C).
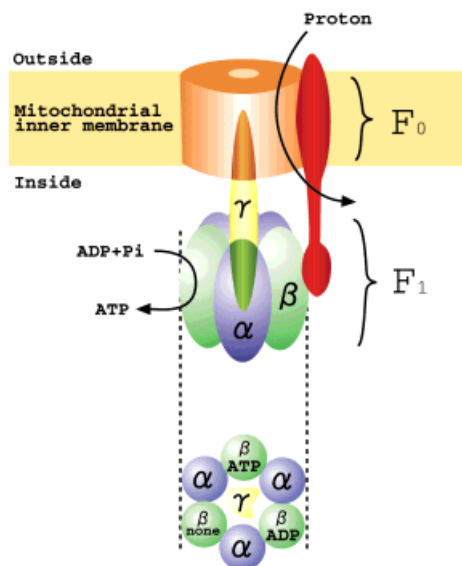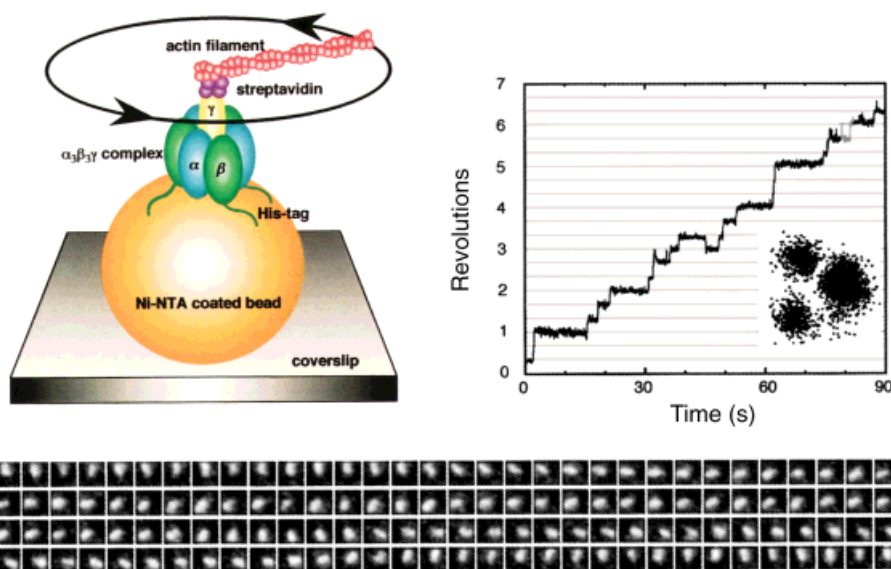

Fig. 4. *Rotary motion of an F_1 motor (A) Architecture of the F_0F_1 motor. A side view of the motor is shown in the top panel, while the bottom panel shows the top view of the F_1 portion. The hydrolysis of ATP at $(\alpha\beta)_3$ causes the rotary motion of the γ subunit relative to $(\alpha\beta)_3$. (B) Rotary motion of the F_1 motor was detected by visualizing the motion of a fluorescently labeled actin filament attached to the γ subunit, while $(\alpha\beta)_3$ is immobilized. Bottom panel shows the time record of the image of the actin filament. The trace of the revolutions shows a step rotary motion of 120. [29]*
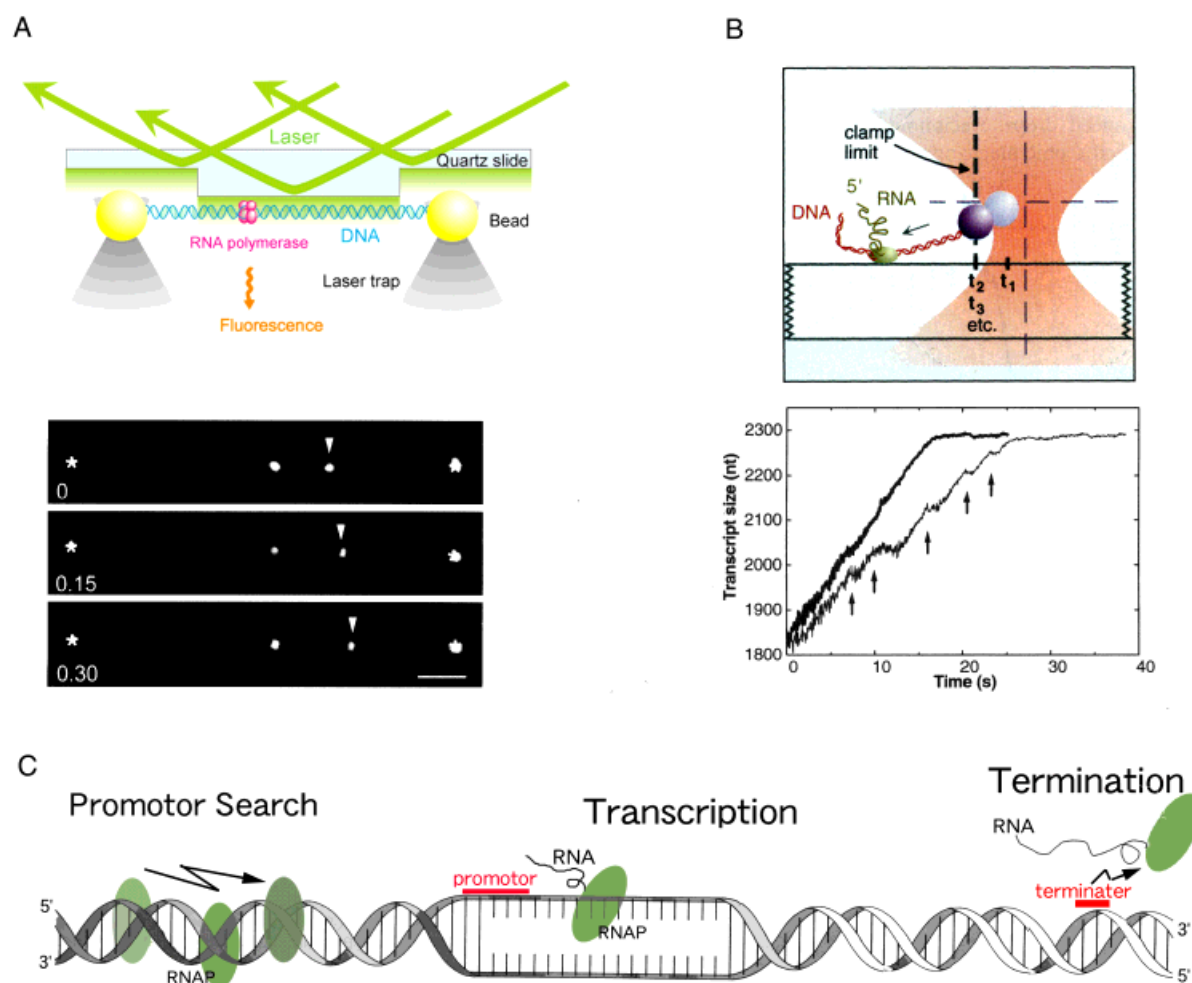
Fig. 5. *DNA transcription processes by RNA polymerase (A) Fluorescently labeled RNA polymerase (RNAP) was visualized on a single DNA molecule trapped by a laser through beads at both ends (indicated by asterisks). In the time records (lower panel) RNAP (indicated by an arrow) is moving along a DNA molecule which is suspended between two beads. [32] (B) When RNAP reaches a promotor on the DNA, RNAP moves along the DNA molecules while transcribing. This process was monitored by laser trap nanometry: The displacement of a bead at one end of the DNA molecule was measured, when the RNAP on the glass surface moved along DNA during transcription. (Reprinted with permission from Science [34], copyright 1998 American Association for the Advancement of Sciences) (C) Model for DNA transcription processes.*

Enzymatic Reaction

Motions of molecular motors and operations of most of other molecular machines are fueled by the chemical energy released from ATP hydrolysis. ATP hydrolysis is catalyzed by the enzyme, ATPase. Myosin, kinesin and F_1 are both motors and ATPase. In order to uncover the operations of these molecular machines, it is important to observe the individual cycles of ATP hydrolysis by single ATPase molecule. This has been achieved by using the single molecule imaging technique with TIRFM and the fluorescent ATP analog, Cy3-ATP (left panel Fig. 6A) [2]. Cy3-ATP is hydrolyzed in the same way as ATP [35]. The turnovers of

ATP hydrolysis were monitored by recording the fluorescence of Cy3-ATP at the position where a marked myosin molecule was located. The images in Fig. 6A middle shows the individual cycles of ATP hydrolysis by a single myosin molecule. In this observation, individual mechanical events are simultaneously measured using optical trapping nanometry (left panel Fig. 6A) [36]. The optical trapping nanometry has been developed and extensively used for the mechanical measurements of myosin [6,10]. The right panel in Fig. 6A shows the time traces of ATPase and displacement which has been measured simultaneously. The detachment of the myosin head from actin, monitored by the decrease in the displacement, occurred immediately

after the binding of ATP. However, the timing of the force generation was not uniquely determined after the dissociation of ADP hydrolysed from ATP: In half of the cases, the force generation occurred immediately after ADP release, but in the other half, it was delayed as much as 1 sec after dissociation of ADP. Thus, the mechanical events were not always coupled to the chemical reactions. The energy released from the hydrolysis of ATP is then stored and used for the generation of the force afterwards.

Using SMD, similar molecular memory effect have also been observed for flavoenzyme which catalyzes the oxidation of cholesterol by oxygen (Fig. 6B) [37]. Enzymatic turnovers were observed by monitoring the fluorescence from the active sites, flavin adenine dinucleotide (FAD), which is naturally fluorescent in its oxidized form but not in its reduced form. The reaction is stochastic. The enzymatic turnover was not independent of previous turnovers (right panel Fig 6B). This could be explained by the slow conformational fluctuations of the enzyme molecules between the two states, as detected by spectral fluctuation (see below).
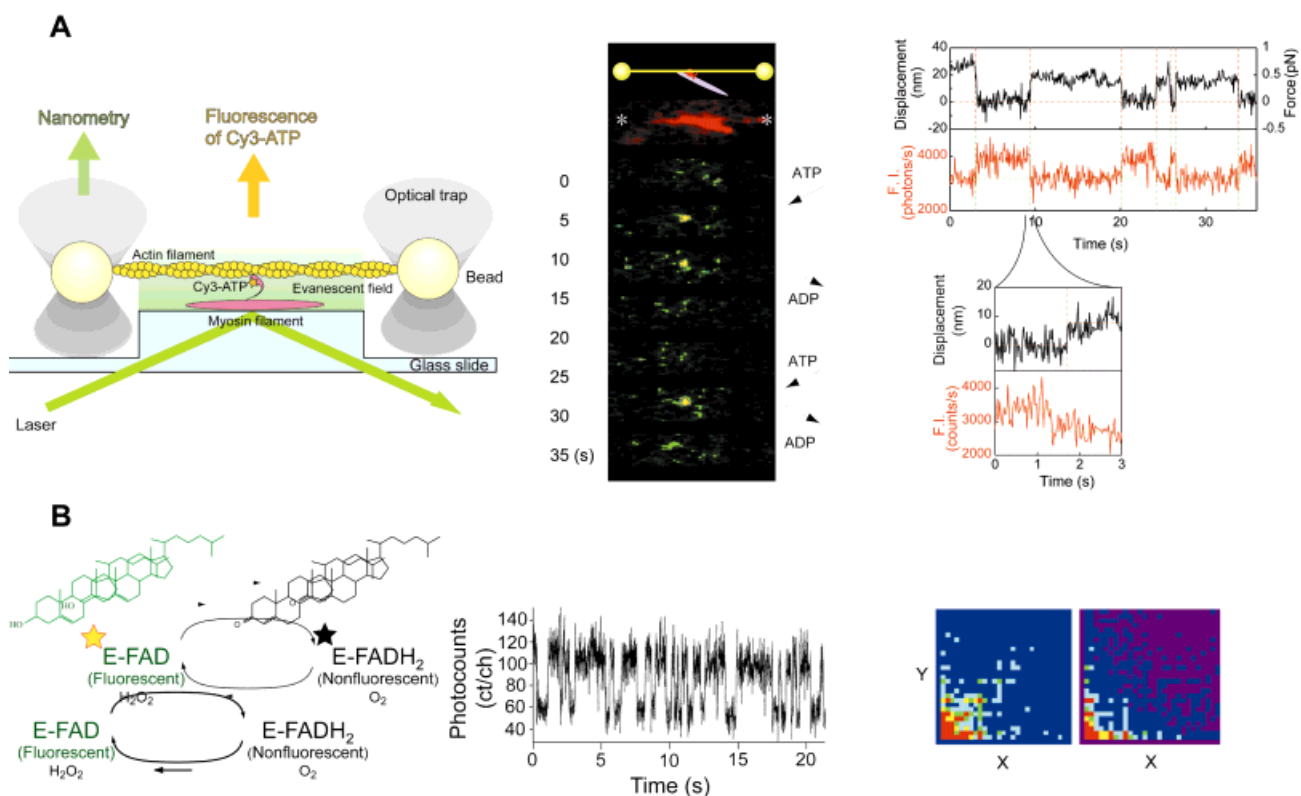

Fig. 6. *Turnover of an enzymatic reaction (A)(left) A schematic drawing how the ATPase by a single myosin molecule has been measured. A single myosin head was fixed on the glass surface and the turnover of the ATPase monitored by the fluorescence of labeled-ATP. The fluorescence could be visualized when fluorescent ATP was bound to myosin and no fluorescent detected when the binding site of myosin was unoccupied. To avoid any possible artifact of the direct binding of proteins to the glass surface, a single one-headed-myosin was incorporated into a head-less myosin filament. Time records of the Cy3-ATP images in the middle panel show binding at 5s and dissociation of Cy3-ATP at 15s. In this experiment, the force exerted by the myosin was measured simultaneously. When an actin filament was allowed to interact with a single myosin head, the displacement of an actin filament was measured at one end by optical trap nanometry, while the other end was fixed by a laser trap. (right) Simultaneous measurement of ATPase and force. When ATP bound to the myosin, myosin dissociated from the actin filament and bound to actin, again. Force was generated when ADP dissociated from actin after the hydrolysis of ATP to ADP. The rising phase of the displacement has been expanded showing that the force generation was delayed after the dissociation of ADP from actin (bottom panel). [36] (B) The turnover of the oxidization by flavoenzyme. (left) The turnover was visualized by the fluorescence of FAD, which is on when it is in the oxidized form (E-FAD). (middle) The on/off cycle of fluorescence was repeated in a time scale of seconds. (right) On-time of the turnover was not independent of previous turnover, but independent between two turnovers separated 10 turnovers.(Reprinted with permission from Science [37], copyright 1998 American Association for Advancement of Sciences)*
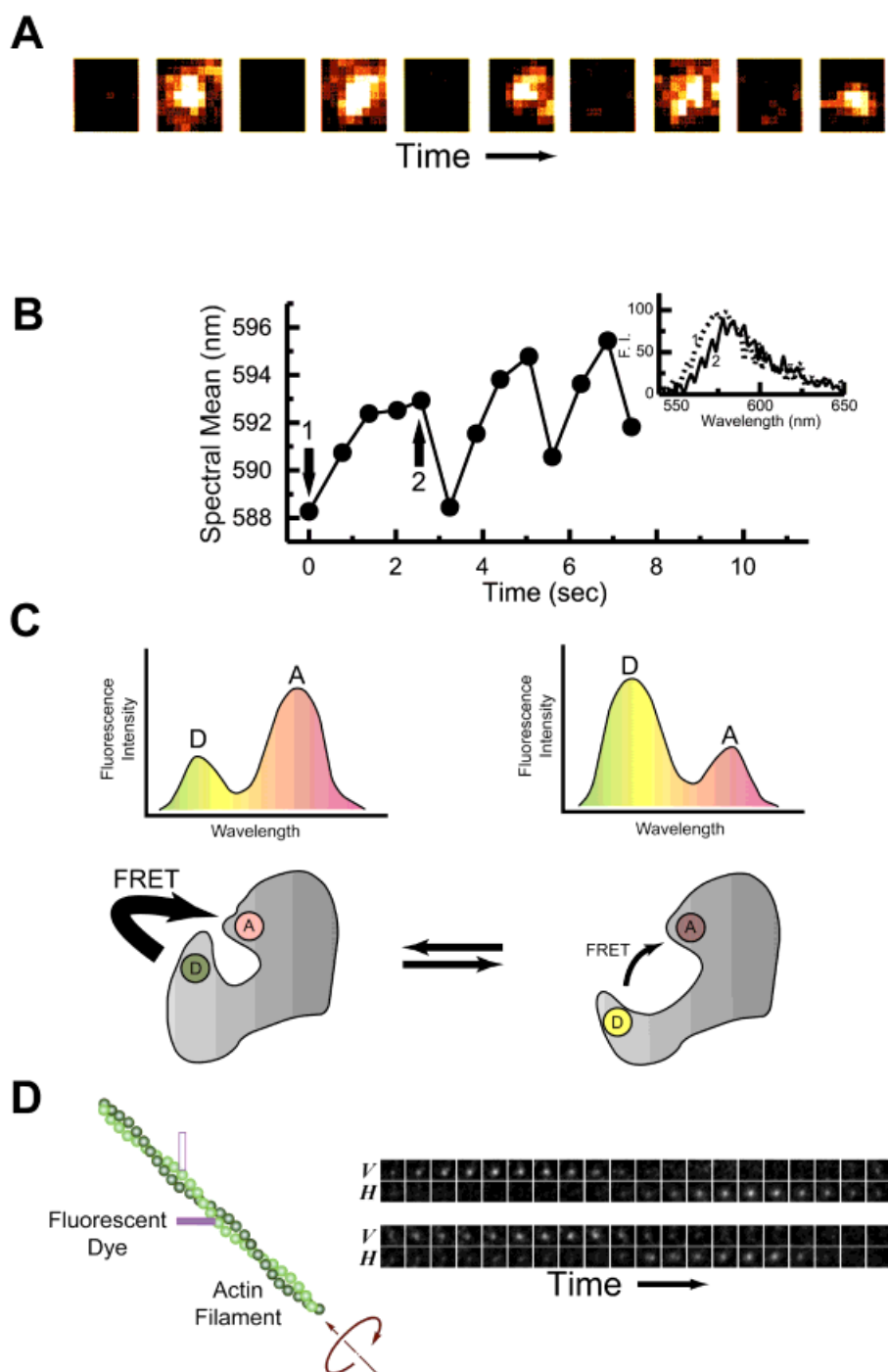
Fig. 7. *Protein dynamics revealed by single molecule fluorescence spectroscopy. (A) The fluorescence of green fluorescent protein (GFP) from the jellyfish Aequorea victoria shows repeated on/off cycles of emission (blinking). [43] (B) The fluorescence spectrum of tetramethylrhodamine attached to myosin S1 fluctuates on a time scale of seconds. The spectral mean was plotted as a function of time. The inset shows the two fluorescence spectra which give different spectral means. [44] (C) Fluorescence resonance energy transfer (FRET) is a technique that determines the distance between two probes on the proteins. When two probes called donor, D, and acceptor, A, are close, the excitation energy of the donor is transferred to the acceptor and the acceptor fluoreces, but the donor fluoreces when the donor and acceptor are far apart. The FRET efficiency is sensitive to protein conformation and to protein interactions. (D) Fluorescence polarization is used to determine the rotational motion of proteins. A fluorescent probe placed on an actin monomer within the filament rotates slowly while sliding along myosin molecules. [52]*

Protein Dynamics

Proteins, the main constituents of molecular machines, behave dynamically. Structural changes inside may occur. Fluorescence spectroscopic method is useful to monitor the conformational changes of proteins [38,39].

Green fluorescence protein (GFP) from jellyfish *Aequorea victoria*, a naturally fluorescent protein, has been used to mark proteins by fusion [40-42]. The time record of the fluorescence intensity of single GFP protein molecules show a repeated cycle of fluorescent emission on a time scale of seconds (Fig. 7A) [43]. This may be due to either slow conformational fluctuations or slow photochemical reactions.

The fluorescence spectrum is sensitive to the microenvironment of the fluorescent probe. In addition to spectral fluctuation in FAD described above, tetramethyrohdamine attached to the most reactive cysteine on the myosin S1 shows spontaneous fluctuations on a time scale of seconds (Fig. 7B) [44]. This may be due to slow conformational changes of this protein.

The fluorescence resonance energy transfer (FRET) method has been used as a ruler in the nanometer scale [45]. Combining this with SMD, it has been demonstrated that FRET can be used as a ruler at the single molecule level [46-50] and has been used for studying the dynamic changes in protein conformation (Fig.7C) [51].

Fluorescence emission is polarized unless it is depolarized due to free rotation of the dye within its fluorescence lifetime. The polarized emmision can be used to monitor the orientation of proteins [52-56]. Rotational motion of an actin filament has been measured as it moved along myosin molecules absorbed on a glass surface (Fig.7D) [52].

It has been believed that proteins have unique conformations [57]. Recently, this idea has been challenged: proteins rather populate in many conformational states, which give similar conformational energy [58]. SMD will direcrly give an answer to this question.

Protein Folding

The problem of protein folding is intriguing. Does every molecule fold using the same sequence of events or are many different routes visualized? This topic has attracted the attention of many investigators. In vivo, proteins fold with the aid of a molecular machine called a chaperone [59]. SMD will help understanding the mechanism of this machine [60].

Cell Signaling

Cell signaling is one of the major target areas of the SMD. Cells are very complex but have very well controlled systems consisting of many kinds of molecular machines [61]. Cells work very flexibly and autonomously, responding to external stimuli. The problem of how signals are transmitted and processed in cells is a central theme of the life sciences. Although there have been many approaches, it is still difficult to understand the mechanism of these processes. To monitor the behavior of single molecules is a unique and powerful approach. Cell signaling is triggered by signals from the outside and the first event of this process occurs on the cell membrane. SMD has been applied to monitor the Brownian movement of fluorescently labeled single lipid molecules in artificial membranes (Fig. 8A) [62,63]. It is possible to incorporate proteins such as signaling and channel proteins into artificial membranes to study the properties of single protein molecules (Fig. 8B)[64]. It is already possible to study the function of ion channels at a single molecule level using the patch clamp method [65,66].

Recently, it has been demonstrated that binding of single molecules of fluorescently-labeled epidermal growth factor (EGF) to its receptor (EGFR) and the following rearrangement of EGF-EGFR complexes can be seen directly on the apical surface of living cells by TIRFM (Fig. 8C) [67]. The autofluorescence of these cells is small enough to observe single fluorophores and the evanescent field produced in the interface between the cell surface and the medium, allows direct observation of single fluorophores on the apical surface.

Future Perspective

SMD allows various types of measurements to be made at the single molecule level. Various aspects of a protein are measured independently. To understand the mechanism underlying the function of proteins, it is important to directly relate several aspects that have been studied independently. In the case of molecular motors, the coupling between the chemical reaction of ATP and the mechanical reaction has been directly measured by combining two measurements as described above. What structural changes occur and how these are coupled to the reactions is the next important step. It is SMD that can visualize these events dynamically.

Biomolecules are as small as nanometers. They use the energy as low as thermal energy. However, they do work very efficiently and accurately. The SMD measurements have provided a clue to understand the mechanism. Biomolecules responds differently, depending on the environment. They even have a memory function. Developing new technology triggers new finding in life science. SMD will be such a technology.

Atomic force, scanning probe near-field, and confocal microscopies are also powerful tools to detect the behavior of single molecules, which are not described here because of limited space.
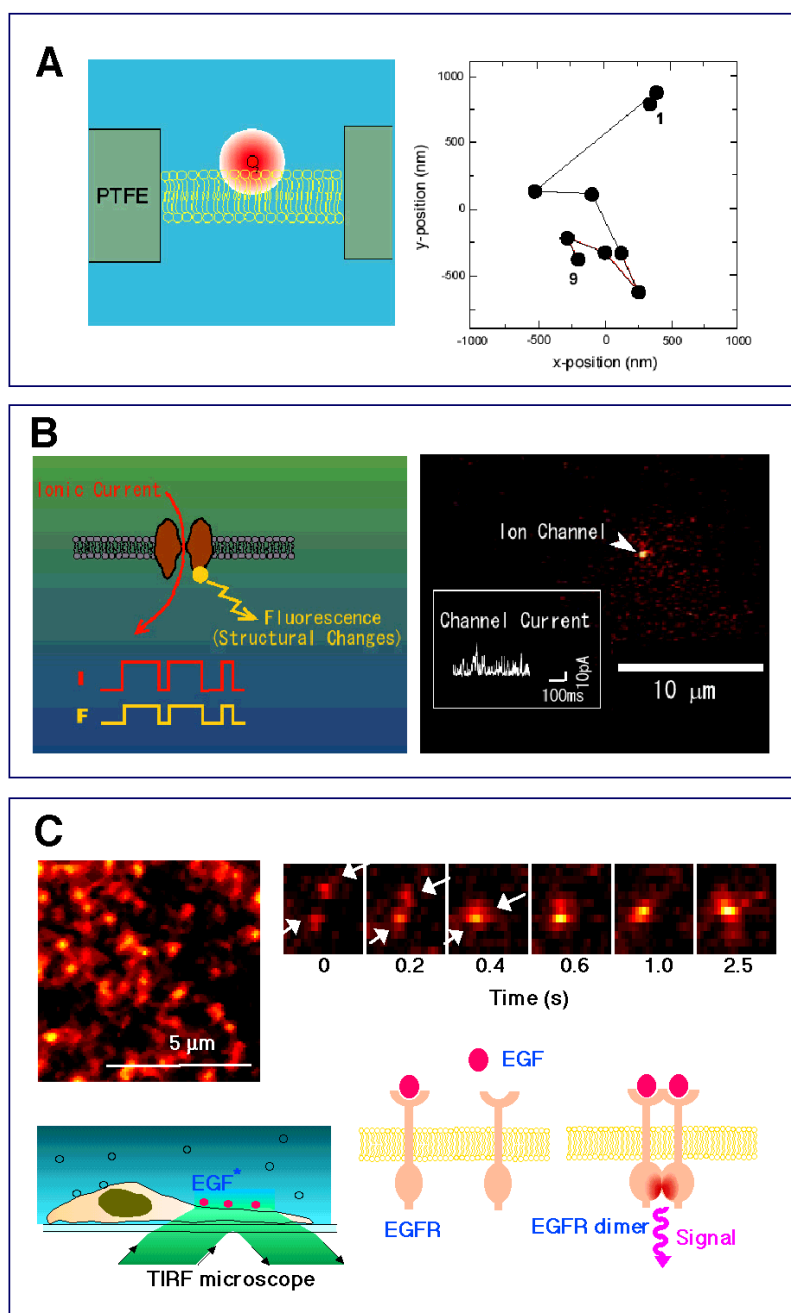
Fig. 8. *Cell signaling. (A) Lipid molecules move by thermal agitation. Small fraction of lipid molecules were fluorescently labeled and the Brownian motion monitored. [63] (B) Ion channels and their structural changes can be monitored by labeling with fluorescent probes. In addition, ion current from a single channel can be measured. It is possible to monitor the behavior of single channels simultaneously with iion current. (C) Fluorescently labeled epidermal growth factor (EGF) was visualized on the cell surface. A single EGF molecule can be seen as a spot and a time sequence of images shows dimerization of the EGF/EGF receptor, triggering transmission of signal. [67]*

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