Single molecule nanobioscience

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In recent years, the rapid development and progress of single-molecule detection techniques have opened up a new era of biological research. The advantage of single-molecule studies is that data are not obscured by the ensemble-averaged measurements inherent in classical biochemical experiments. These techniques are shedding light on the dynamic and mechanistic properties of molecular machines, both *in vivo* and *in vitro*. This review summarizes the single-molecule experiments that have been designed to investigate molecular motors, enzyme reactions, protein dynamics, DNA transcription and cell signaling.

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Single-molecule detection (SMD) techniques have recently attracted a great deal of attention in the field of life science[1–3]. How advantageous is SMD for investigations? Observing and manipulating biomolecules allows their dynamic behaviors to be directly revealed, as has been demonstrated for motor proteins. Reactions of biological molecules are generally stochastic. Therefore, even if the reactions of molecules are initiated at the same time, they cannot be synchronized, so the dynamic behaviors of individual molecules are averaged and hidden in ensemble-averaged measurements. *In vivo*, biomolecules work in dynamic and complicated heterogeneous systems, involving different kinds of molecules such as cell-signaling proteins. It is difficult to quantitatively detect dynamic behaviors of target molecules in such systems using ensemble-averaged measurements. The SMD techniques are expected to overcome these difficulties and have already been successfully applied to study the dynamic properties of biological molecules such as motor proteins, enzymes, RNA polymerase and cell-signaling proteins.

The SMD techniques are based on the two key technologies of single-molecule imaging and single-molecule nanomanipulation. First, the imaging technique will be explained. The size of biomolecules and even their assemblies are in the order of nanometers, so they are too small to observe by optical microscopy. To overcome this problem, biomolecules can be fluorescently labeled and visualized using fluorescence microscopy. Single fluorophores in aqueous solution were first observed in 1995 using total internal reflection fluorescence microscopy (TIRFM) and conventional inverted fluorescence microscopy[4]. The major problem to overcome when visualizing single fluorophores in aqueous solution is the huge background noise, which can be caused by Raman scattering from water molecules, incident light breaking through filters, luminescence arising from the objective lens, immersion oil and dust. In this system, the evanescent field was formed when the laser beam was totally reflected by the interface between the solution and the glass. The evanescent field was not restricted to the diffraction limit of light, thus it could be localized close to the glass surface, which resulted in the penetration depth (~150 nm) being several-fold shorter than the wavelength of light (Fig. 1a). Therefore, the illumination was restricted to fluorophores either bound to the glass surface or located close by, thereby reducing the background light. Furthermore, by careful selection of optical elements, the background noise could be reduced by 2000-fold compared with that of conventional fluorescence microscopy. This made it possible to clearly observe single fluorophores in aqueous solution. Fluorescence measurements from fluorophores attached to biomolecules and ligands allow the detection of, for example, the movements, conformational changes, enzymatic reactions and cellular signaling processes of biomolecules at the single molecule level.

The second key technology is single-molecule nanomanipulation. Biomolecules and even single molecules can be captured by a glass needle[5–7] or by beads trapped by optical tweezers[8,9]. Optical tweezers are used to trap and manipulate particles of 25 nm to 25 μm in diameter by the force of laser radiation pressure. The particle is trapped near the focus of laser light when focused by a microscope objective with a high numerical aperture. The optical tweezers produce forces in the piconewton range on the particles. Biomolecules are too small to be directly trapped by the optical tweezers, so they are generally attached to an optically trapped bead. Microneedles or a bead trapped by a laser act as a spring that expands in proportion to the applied force. Thus, the force and the displacement caused by the biomolecules can be measured. The displacement of a microneedle and a bead has been determined with subnanometer accuracy, much less than the diffraction limit of an optical measurement[6–9]. This accuracy of displacement corresponds to the sub-piconewton accuracy in the force measurements. Thus, the mechanical property of biomolecules can be determined directly at the single-molecule level. Furthermore, when the single-molecule nanomanipulation and imaging techniques are combined, simultaneous measurements of mechanical and chemical reactions of single biomolecules are possible.

Movement of a microtubule-based kinesin motor

The SMD techniques were first used to study molecular motors. The example discussed here is that of a microtubule-based kinesin motor, which transports organelles along a microtubule. Kinesin is composed of two heavy chains, each comprising a force-generating

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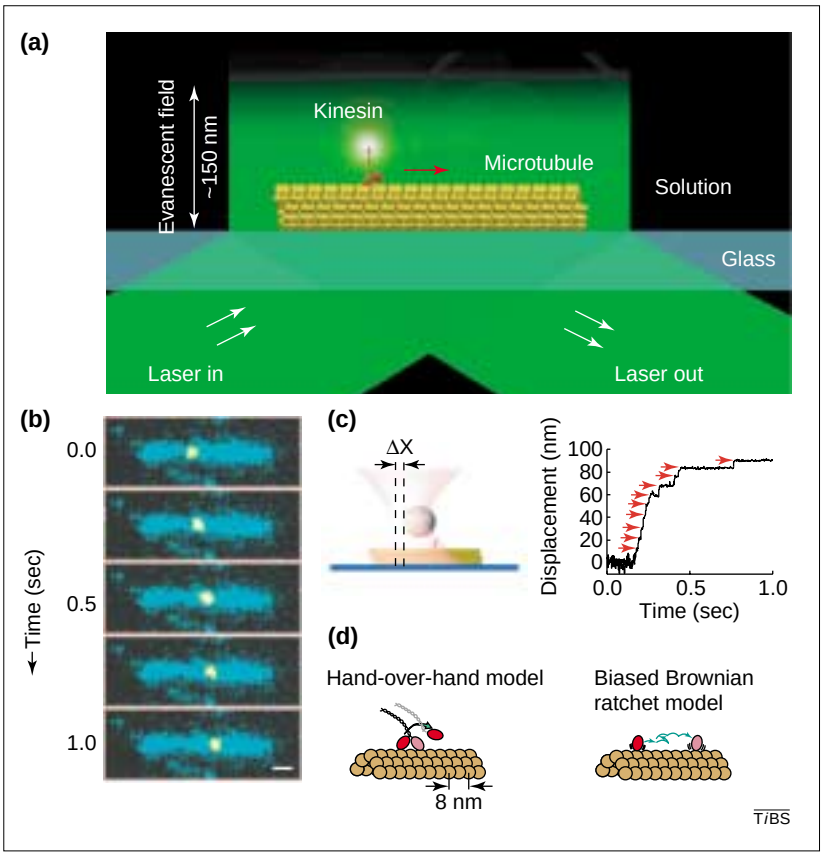


Fig. 1. Single molecule imaging of the movement of a fluorescently labeled kinesin motor by total internal reflection fluorescence microscopy[10,13]. (a) The experimental setup[4]. The laser light was incident on the interface between a glass surface (high refractive index) and the solution (low refractive index). Above the critical angle, the light was totally reflected and the evanescent field was produced just beyond the interface in the solution with a 1/e penetration depth of ~150 nm. The background noise was >2000-fold lower than that of conventional fluorescence microscopy. A fluorescently labeled kinesin was visualized while it moved along a microtubule placed on the glass surface. (b) Time records of the fluorescence image showed that the kinesin (yellow) moves along the microtubule (blue)[13]. Scale bar = 5 μm. (c) A single kinesin molecule was attached to a bead trapped by a laser (pink shading). Displacement (ΔX) of the kinesin along the microtubule was measured by monitoring the displacement of the bead with nanometer accuracy. Displacement records (right) indicated that the step size (i.e. distance between two sequential red arrows) is 8 nm (Ref. 8). (d) A hand-over-hand model has been proposed to explain the processive movement of the two-headed kinesin[11]. In this model, the two heads work in a coordinated manner; one head remains bound to the microtubule while the other steps from the αβ-tubulin dimer behind the attached head to the dimer in front. The overall movement is 8 nm. A biased Brownian ratchet model has been proposed to explain the processive movement of the one-headed kinesin superfamily, KIF1 (Ref. 12) and deletion mutant of kinesin[13]. In this model, the one-headed motor walks or slides along the microtubule by Brownian (thermal) motion biased, for example, by a potential gradient along a microtubule.

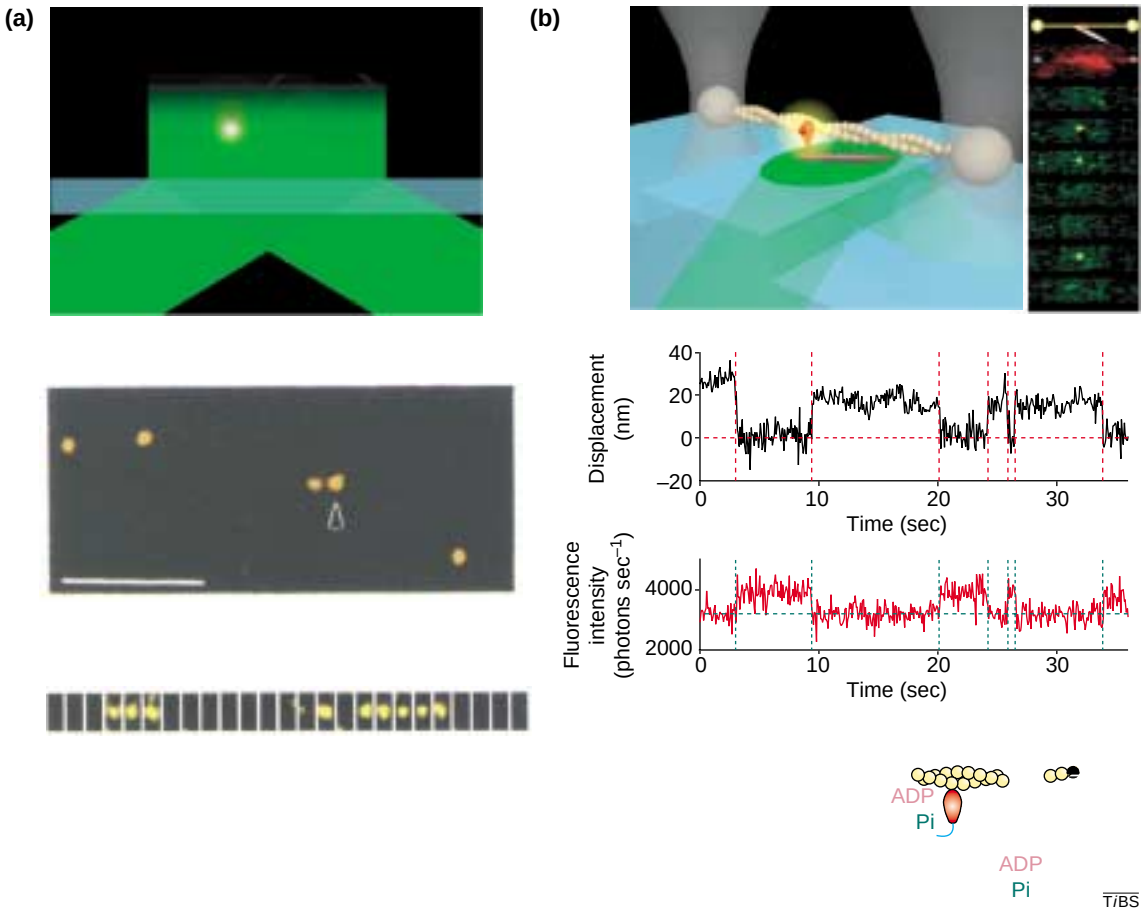
globular domain (head), a long α-helical coiled-coil and a small globular C-terminal domain (tail). Microtubules are cylinders comprising parallel protofilaments, which usually number 13 or 14 when reassembled *in vitro*. In turn, protofilaments are linear polymers of α and β tubulin dimers. Movement of single kinesin molecules along a microtubule has been directly observed by TIRFM (Ref. 10). Kinesin molecules, fluorescently labeled at the tail-end without damage, were added to microtubules adsorbed onto a glass surface in the presence of ATP (Fig. 1a). Figure 1b shows a series of fluorescence images of a single kinesin molecule moving along a microtubule, demonstrating directly that a single molecule of kinesin can processively move for long distances along a microtubule without dissociating.

Movement of single kinesin molecules was measured with nanometer accuracy by optical trapping nanometry[8] (Fig. 1c). Kinesin has been shown to move along a microtubule with regular 8 nm steps, coinciding with the α–β tubulin dimer repeat. Based on this finding, the processive movement of kinesin has been explained by a hand-over-hand mechanism, in which one head remains bound to the microtubule while the other detaches and moves forwards[11] (Fig. 1d). However, it has recently been shown that a member of a kinesin family (KIF1), which has only one force-generating head[12], and a one-headed deletion mutant of kinesin[13], can move processively. To explain these findings, an alternative biased Brownian ratchet model has been proposed[12] (Fig. 1d). In this model, the one-headed motor walks or slides along the microtubule by Brownian (thermal) motion. However, Brownian motion is random, so this movement must be biased in one direction, possibly by the production of a potential slope along the microtubule.

ATPase turnovers of an actin-based myosin motor

In general, the motions of molecular motors and the operations of other biomolecules are fueled by the chemical energy released from ATP hydrolysis. Kinesin and myosin are both motor proteins and ATPases. To uncover how the biomolecules work using the chemical energy from ATP, it is crucial to observe the individual cycles of ATP hydrolysis by single ATPase molecules. This has been achieved using the single-molecule imaging technique TIRFM in combination with the fluorescent ATP analog, Cy3-ATP (Fig. 2a)[4,14]. This method was first applied to an actin-based myosin motor that is involved in muscle contraction and other cellular motility. Although myosin is approximately twofold larger in size than kinesin, the ATP-binding domains of these two proteins have similar structures. Cy3-ATP is hydrolyzed by myosin in the same way as ATP (Ref. 15). The rate of the biochemical cycle of ATP hydrolysis averaged for many events or individual myosin molecules was consistent with that obtained by a conventional biochemical method using a suspension of myosin[4,14].

To date, the states of bound ATP and ADP cannot be distinguished because the signal to noise ratio of the measurement system is not high enough to detect the difference in fluorescence between the Cy3-ATP and -ADP forms. If fluorescent nucleotide analogs become available in which fluorescence in the ATP form is sufficiently different (>twofold) from that in the ADP form, and if the measurement system is further improved, then it will be possible to directly monitor the process of ATP hydrolysis. In addition to those of ATPases, the enzymatic turnovers of single cholesterol oxidase molecules have been observed by monitoring fluorescence from flavin adenine dinucleotide in the active site[16].



Simultaneous observation of the ATPase turnover and mechanical events of an actin-based myosin motor

To investigate how the mechanical event of myosin corresponds to the ATPase cycle, the single-molecule imaging technique was combined with optical-trapping nanometry to simultaneously measure individual ATPase cycles and mechanical events of a single myosin molecule[17] (Fig. 2b, top). Dissociation of the myosin head from actin corresponded with the binding of ATP, and association of the myosin head with actin and generation of displacement were followed by dissociation of a nucleotide (most likely

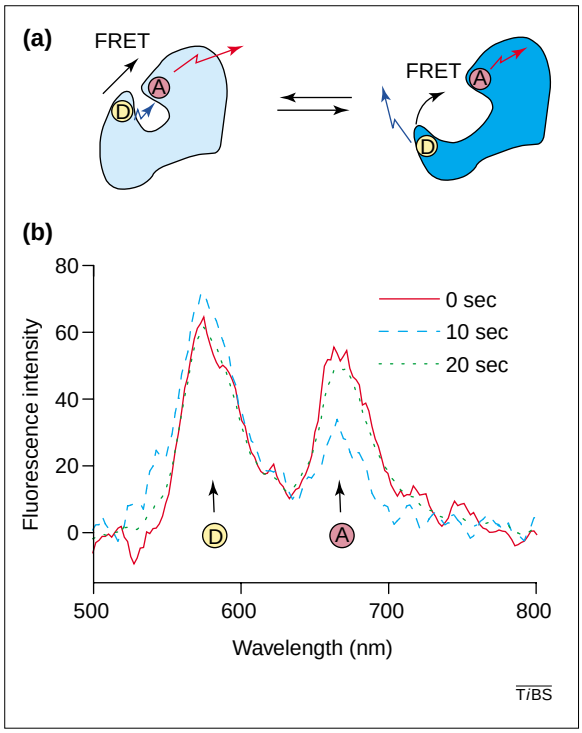


Fig. 3. Protein dynamics revealed by fluorescence resonance energy transfer (FRET)[20]. (a) FRET determines the distance between two probes on a protein. When two probes, called donor (D) and acceptor (A), are close, the excitation energy of the donor is transferred to the acceptor and the acceptor fluoresces (fluorescence is depicted by a kinked arrow extending beyond the surface of the protein). However, when the donor and acceptor are far apart, the donor fluoresces. The FRET efficiency is sensitive to protein conformation. (b) Traces show time records of spectra from a donor and an acceptor attached to a myosin molecule, each of which changes in a flip-flop fashion, indicating that the myosin undergoes conformational changes spontaneously in the time range of seconds.

ADP). Each displacement corresponded to a single ATP molecule (Fig. 2b, middle and bottom).

Understanding the process of a displacement became essential to investigating how myosin works using the chemical energy from ATP. Optical-trapping nanometry cannot resolve this process because the displacement is determined indirectly through a long actin filament and optically trapped beads, which have an unknown elasticity. Thus, the signal to noise ratio is not high enough to resolve the process. To overcome this problem, a more direct method combining a scanning probe and single-molecule techniques has been developed[7]. A single myosin head was attached to the tip of a scanning probe and the process of a displacement was resolved by measuring the displacement of this probe with nanometer and millisecond accuracies. The results showed that a myosin head moved along an actin filament with regular 5.5 nm steps and underwent five steps to produce a maximum displacement of 30 nm per displacement (i.e. representing the ATPase cycle). As the step size coincides with the actin monomer repeat (5.5 nm), and each 5.5 nm step is not directly coupled to the ATPase cycle (loose coupling), the results strongly indicate that the myosin head walks along the actin monomer repeat using biased Brownian motion (Fig. 2b bottom; Fig. 1d). This idea challenges the widely accepted view that the movement is caused by a large conformational change in the myosin head, tightly coupled to the ATPase cycle in a one-to-one fashion (tight coupling)[18]. Recently, many investigations have shown that the neck region of the myosin head undergoes conformational changes relative to the main body of the head, depending on the form of the bound nucleotide; this has been observed in both crystal and solution structures[19]. On the basis of these findings, the lever arm swinging model has been proposed, which postulates that the neck region of the head acts as a lever arm, and that tilting the lever arm produces the force and displacement. However, conformational changes in the neck region during isometric tension in muscle are much smaller than that expected from the model[19].

Protein dynamics

The dynamic behavior of proteins is crucial for their function. Fluorescence resonance energy transfer (FRET) is a direct method of monitoring the structural changes of proteins[2]. When the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor fluorophore overlap, and they are located close to each other (in the order of nanometers), the excited energy of the donor is transferred to the acceptor without radiation, resulting in acceptor fluorescence. Thus, we can determine the distance between the donor and acceptor fluorophores attached to two different sites on a protein by monitoring the color of the fluorescence (Fig. 3a). Figure 3b shows the fluorescence spectra of a donor and an acceptor fluorophore attached to a myosin molecule[20]. The fluorescence intensities of the donor and acceptor varied spontaneously in a flip-flop fashion, indicating that the distance between the donor and acceptor changes in the range of hundreds of angstroms; that is, the structure of myosin is not stable but instead thermally fluctuates. These results suggest that myosin can go through several metastable states, undergoing slow transitions between the different states[16]. This idea could explain the hysteretic or refractory behavior observed in some metabolic enzymes. These behaviors are thought to be important in the regulation of metabolic pathways in which several enzymes compete for the same intermediate[21]. Single-molecule FRET can be used to observe conformational changes of proteins upon binding ligands and during work in real time, and during association and dissociation with ligands or partner biomolecules both *in vitro* and *in vivo*[2].

Polarized fluorescence is also useful for monitoring protein dynamics[2]. Rotational motions of an actin filament[22,23] and myosin[24] were observed at the single-molecule level. Simultaneous measurements of the FRET or polarized fluorescence, and the mechanical or ATPase turnover events of a myosin molecule during force generation, will elucidate how the conformational changes in myosin are involved in force generation.

DNA transcription

The initial steps of gene expression include the binding of RNA polymerase (RNAP) to DNA, the search for a promoter in the DNA sequence and the synthesis of RNA based on the information encoded by the DNA. These steps are central regulatory mechanisms of gene expression and have been extensively investigated[25]. Harada *et al.*[26] have observed single, fluorescently labeled RNAP molecules interacting with a single

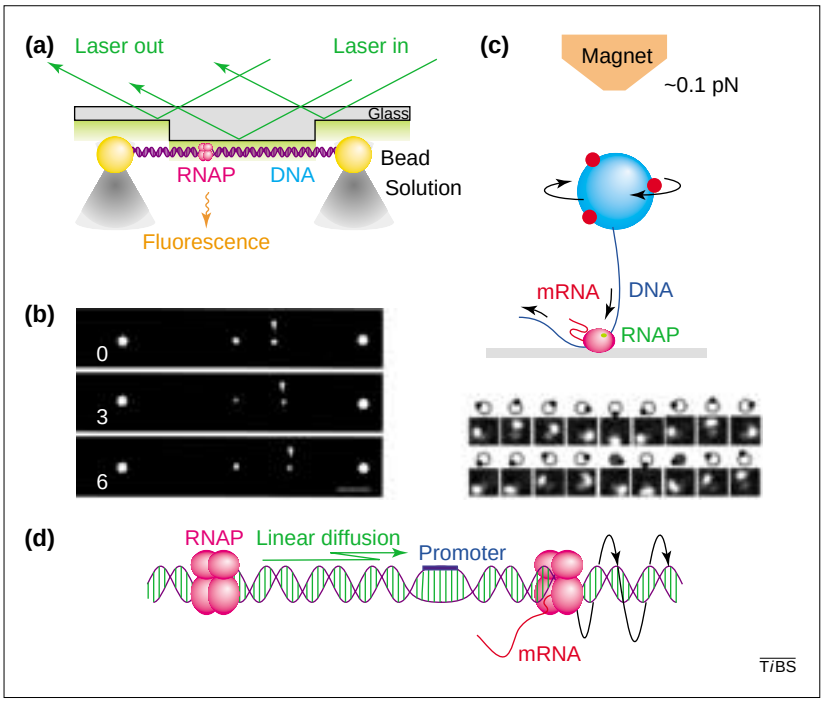


Fig. 4. Interaction of RNA polymerase (RNAP) with DNA. (a) Single molecules of fluorescently labeled RNAP were visualized on a single DNA molecule trapped by a laser (gray shading) at both ends through beads[26]. (b) Fluorescence images of a single RNAP. In the time records, RNAP (indicated by arrowheads) underwent thermal linear diffusion along a DNA molecule, which had been suspended between two beads (shown by asterisks). Images were taken every three seconds. Scale bar = 2 μm. (c) Observation of DNA rotation by RNAP. The top panel depicts the observation system. Rotation is determined by monitoring daughter fluorescent beads (20 nm; red) attached to a magnetic bead (850 nm; blue). The bottom panel shows snap shots of rotating beads at 532 msec intervals at NTP concentration of 50 μM. This figure is adapted, with permission, from Ref. 30. (d) A model of promoter search and transcription by RNAP. RNAP searches along the DNA for the promoter by thermal linear diffusion and rotates around DNA (black arrows) during transcription.

molecule of DNA suspended in solution using optical traps (Fig. 4a). Figure 4b shows a series of fluorescence images of a single molecule of RNAP undergoing linear sliding along DNA. The kinetic studies have proposed some mechanisms for promoter searching based on the results of the binding of RNAP molecules to specific and nonspecific sites on the DNA. These include sliding, intersegment transfer and simple dissociation and/or association reactions[25]. This observation (Fig. 4b) provides direct evidence that a sliding motion is one mechanism used for the search of promoters (Fig. 4d). The association and dissociation rate constants of RNAP could be also determined, depending on the sequence of DNA and on the mechanical strain exerted on the DNA. These values proved difficult to determine in solution because DNA molecules aggregate to form a network structure[25]. The transcription process was directly monitored by measuring the displacement or rotation of DNA during the interaction with RNAP by manipulating DNA with optical and magnetic tweezers[27–30]. Figure 4c depicts RNAP-mediated DNA rotation during transcription[30]. One end of DNA is attached to a magnetically trapped bead and the other interacts with a RNAP molecule adsorbed onto the glass surface. Rotation of DNA is determined by monitoring the rotation of the bead. The results showed that the rotation rate is consistent with high-fidelity tracking. The rotation per base pair is as much as 35° and should, in principle, be detectable. Therefore, this method could resolve individual steps of transcription in real time.

Cell signaling

Cell signaling is one of the major target areas for investigations using single-molecule imaging. Cells are complex but have well-controlled systems comprising many kinds of molecular machines. The problem of how signals are transmitted and processed in cells is a central theme in the life sciences.

Cell signaling is triggered by external signals that initiate at the cell membrane. Recently, we demonstrated that binding of single molecules of fluorescently labeled epidermal growth factor (EGF) to its receptor (EGFR) can be observed in living cells[31]. Fortunately, the autofluorescence of living cells is usually small enough to enable observation of single fluorophores. The evanescent field could be produced either in the interface between the glass surface and the medium, or between the cell surface and the medium, by carefully adjusting the incident light. Thus, single fluorophores could be observed on both basal and apical surfaces of a cell (Fig. 5a, left top). Following binding, dimerization of EGF–EGFR complexes, which is essential for signal transduction, could be directly visualized on the surface of living cells (Fig. 5a, left bottom and right). Dimerization of the EGFR (Fig. 5b, right) induces autophosphorylation on cytoplasmic Tyr residues. Such autophosphorylation was also observed at the single molecule level using perforated cells. After stimulation with Cy5-EGF, cells were incubated with Cy3-labeled antibody, Cy3-mAb74, that recognizes autophosphorylated EGFR (Fig. 5b, right). Using the same method, association and dissociation of individual Cy3-cAMP molecules with the cell surface of *Dictyostelium* amoebae during chemotaxis were investigated[32]. The dynamic behaviors of bound Cy3-cAMP on the cell surface could be observed and these correlated strongly with the chemotactic response of the amoebae during chemotaxis (Fig. 5c). These results provided important insights into how amoebae detect the small concentration gradient of Cy3-cAMP (attractant) in a very noisy environment, which then determines the orientation of their movement.

This method can also be used to visualize single fluorescent molecules in living cells if the number of fluorescent molecules to be controlled is small. GTP-binding proteins, including those belonging to the Ras and Rho families, are key molecules in many cellular signal-transduction pathways. For example, after stimulation of cells with EGF, activated Ras induces translocation of a mitogen-activated protein kinase, Raf 1, from the cytoplasm to the plasma membrane (Fig. 5d). We observed this pathway in living cells using green and/or yellow fluorescent protein (GFP and YFP) fused to Ras and Raf 1. Fluorescence from these proteins is strong enough to observe at the single molecule level[33–35], and so can be used for these experiments. After their activation and translocation,

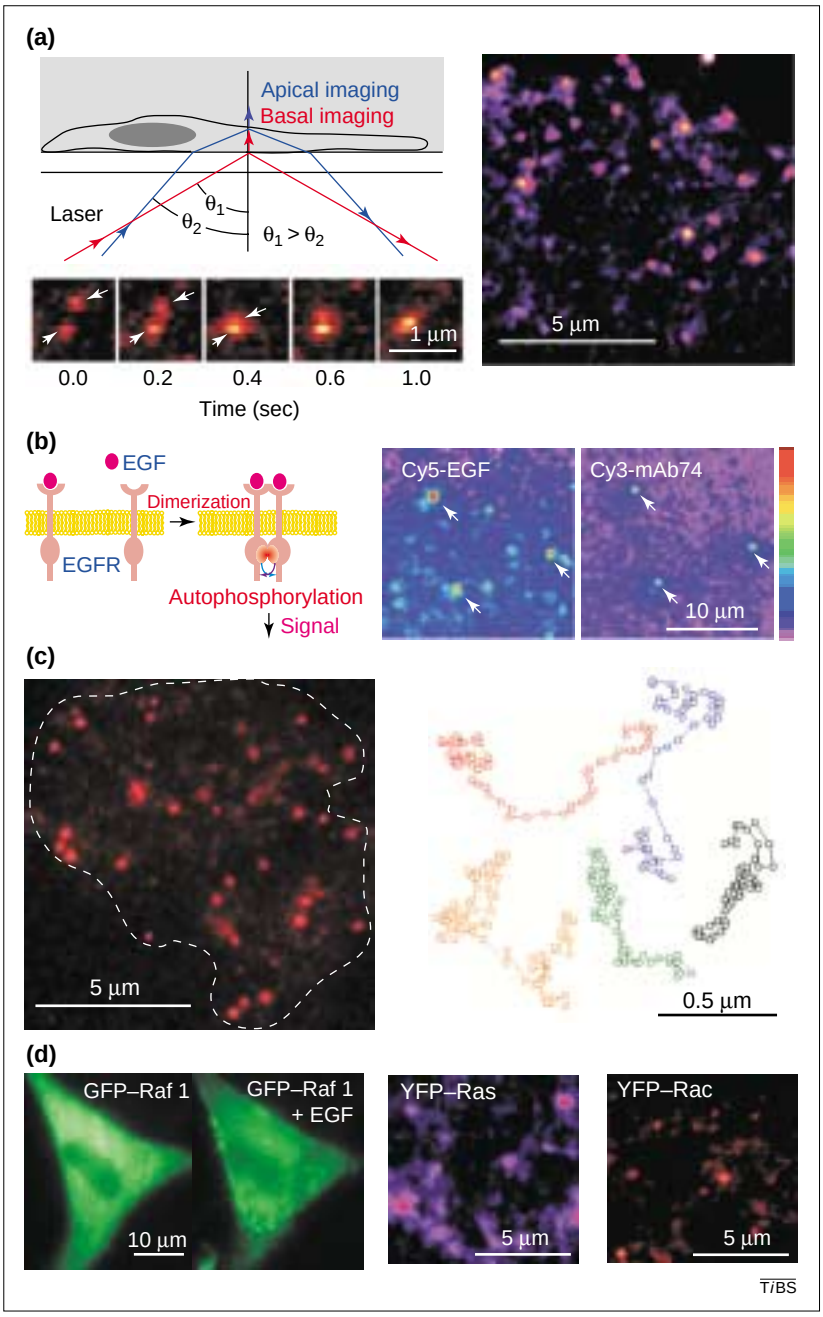


Fig. 5. Single molecule imaging of cell signaling[31,32]. (a) Visualization of EGF–EGFR dimerization on the plasma membrane of living A431 cells. (Left, top) Optics. θ_1 and θ_2 are the incident angles of a laser. Mouse EGF was conjugated with a Cy3 fluorophore at the amino terminus and added to the culture medium of living A431 cells under a TIRFM. (Right) Fluorescence images of Cy3-EGF molecules bound to the receptors on the apical surface of a living cell. (Left, bottom) Dimerization of EGF–EGFR complexes. Two spots of single Cy3-EGF–EGFR complexes collided at time 0.4 sec and then moved together. (b) Single-molecule detection of the autophosphorylation of EGF–EGFR complexes. A431 cells were perforated by streptolysin O and stimulated with 10 ng ml^{-1} Cy5-EGF for 1 min. Phosphorylation of EGFR was detected by Cy3-mAb74 (anti-active EGFR antibody). Images were acquired 15 min after the addition of Cy3-mAb74. Cy3-mAb74 bound at the position of clusters of Cy5-EGF (bright spots depicted by arrows). The fluorescence intensity was indicated by the colour spectrum at the side. (c) Cy3-cAMP on the surface of a chemotactic amoeba. (Left) Visualization of single molecules of Cy3-cAMP bound to its receptor on a *D. discoidium* amoeba moving along a chemotactic gradient of Cy3-cAMP. (Right) Typical trajectories of lateral movement of individual Cy3-cAMP–receptor complexes on the cell surface. Geometrical centers of individual Cy3-cAMP molecules at 33-msec intervals were linked to show the trajectory. The trajectories were plotted against the substrate. (d) Imaging of small G proteins and Raf1 kinase in living cells. Cells were transfected with cDNAs encoding YFP–Ras or –Rac, or GFP–Raf1. (Left) GFP–Raf1, which localized in the cytoplasm of a cell in the resting state, translocated to the plasma membrane after 10 min of stimulation with a 10 sec pulse of EGF at 100 ng ml^{-1}. (Center and right) Fluorescent spots of YFP–Ras and –Rac beneath the plasma membrane were visualized by TIR microscopy. Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; TIRFM, total internal reflection fluorescence microscopy.

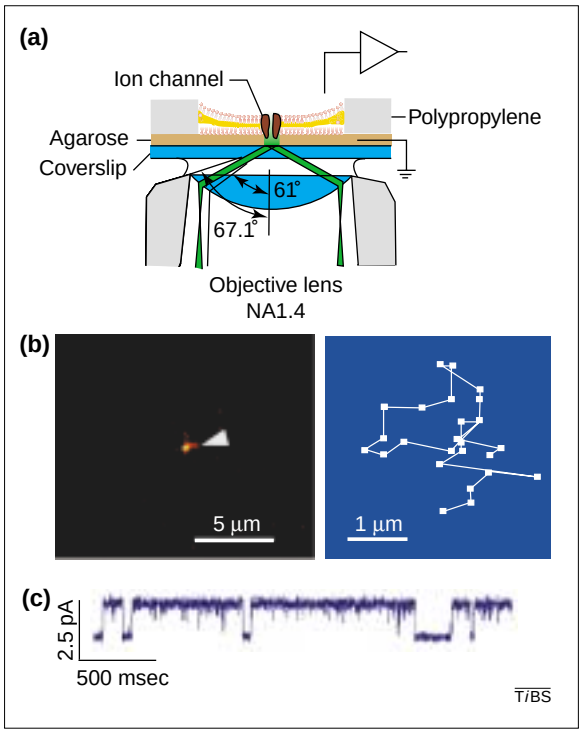


Fig. 6. Simultaneous electrical and optical measurement of single ion channels[37]. (a) The experimental setup used for these measurements. Fluorescently labeled ion channels were incorporated into a membrane formed on the agar-coated glass and observed by an objective type of total internal reflection fluorescence microscopy[14]. The incident light angle was set at between 61.0° and 67.1°. The numerical aperture (NA) of an objective lens was 1.4. Ion current from a single channel was measured simultaneously. (b) An image of a single ion channel (left) and trajectory of its Brownian motion in a membrane (right). (c) Current recording from a single ion channel indicated in (b). Ion current changes in a stepwise fashion, corresponding to opening and closing of a channel.

single GFP (or YFP)–Ras and –Raf 1 molecules were found to diffuse rapidly beneath the plasma membrane. Another small GTP-binding protein, Rho, and its relatives Rac and Cdc42, which are key molecules in controlling cytoskeleton organization, could also be observed in living cells at the single-molecule level by visualizing their fluorescent fusion proteins (Fig. 5d)[32].

Thus, the single-molecule imaging technique has allowed us to directly and quantitatively detect the location, movement, recognition and local turnover of various kinds of cell-signaling molecules in living cells. This technique can be a very powerful tool for studying the dynamic processes of cell signaling.

Ion channels

Ion channels precisely regulate the ionic flow across cell membranes and generate ionic gradients that are responsible for nerve and muscle excitability. Single ion channel current recording[36] has shed light on the kinetics and pharmacological properties of many kinds of ion channel; however, their detailed mechanisms are still being elucidated. The ability to monitor the conformation and chemical state of ion channels, combined with measurements of single channel ion currents, would aid research in this area. Combining the planar lipid bilayer and single-

molecule imaging technique, the ion current and fluorescence image of single ion channels have been simultaneously observed[37] (Fig. 6). Single potassium channels, which were conjugated with fluorescently labeled hongotoxin, which specifically binds to the channel, were visualized in living cells[38]. Thus, the mobility of single ion channels and their distribution (including colocalization) were directly observed both in the artificial membrane and on the surface of living cells. By combining this technology with FRET, we will be able to directly observe the interaction between a single ion channel and its regulator proteins or ligands (including drugs), and the following changes in conformation and ion current *in vitro* and *in vivo*.

Future perspectives

Recently, life science has made remarkable progress. This progress has been made possible by the identification of functional proteins and studies on the characteristic (structural and functional) properties of proteins as revealed by molecular cell biology, structural biology and molecular genetic approaches. The DNA sequences of not only invertebrates but also vertebrates (including human) are now available. Thus, research is charging into the post-genomic era. In this era, we tend to emphasize the concept that the structure explains the function and we endeavor to understand the mechanisms of the proteins and molecular machines based on this concept. However, knowing the function of proteins and molecular machines is not a simple task. Moreover, we could not understand the function of these proteins even if we knew their structures. Proteins and molecular machines are not simple and their function cannot be learned in analogy to artificial machines. Proteins and molecular machines have a size in the nanometer range, and a dynamic structure. In addition, the input energy to the molecular machines is comparable with thermal energy; molecular machines function at a very high efficiency when exposed to thermal agitation. This is contrasted by artificial machines, which use much higher energy than thermal energy to work rapidly, accurately and deterministically. For such reasons, it is necessary to understand the dynamic properties of proteins themselves and their interactions with each other. The SMD techniques have been developed as techniques to directly monitor the dynamics of proteins and molecular machines and have rapidly expanded to include a wide field of biological sciences. Combined with nanotechnology from the engineering field, the SMD techniques will prove more powerful in the future. Thus, SMD will govern and lead the future direction of research in proteins and molecular machines. (Atomic force, scanning probe near-field and confocal microscopies are also powerful tools, but are not described here because of space restrictions.)

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