
3 Biotechnology and Neurotechnology –Biological and Neurological Information in Telecommunications Technology–

3-1 The Single Molecule Nanometry for the Bio-supermolecule Research

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In order to understand functions of bio-supermolecules, it is important to characterize their behaviors by observing and manipulating them directly at a single molecule level. The newly developed technique; single molecule nanometry makes it possible to perform such characterization. Here, we introduce the principles, system configurations, and applications of the single molecule observations, the single molecule manipulation, and their derivative techniques mainly from the technical point of view.

Keywords

In vitro motility assay, Single molecule observation, Laser trap, Single molecule manipulation, Nanometry

1 Introduction

A group of bio-molecules known as protein motors are power source of almost all movement inside a living body, like cell movement, and muscle contraction. For example, muscle contraction is caused when two types of proteins, actin, and myosin, regularly arrayed in muscles, slide against each other. Flagellar movement, the driving force for the “swimming” motion of microorganisms, is caused when dynein molecules regularly aligned on one microtubule within the flagellum slide against an adjacent microtubule. One type of axonal transport within neurons occurs when kinesin molecules carrying vesicles move over microtubules. These protein motors interact with protein filaments using the chemical energy released when an

ATP molecule is hydrolyzed. Protein motors can thus be regarded as molecular machines that convert chemical energy into mechanical energy like force, and initiate sliding movements (Fig.1). These molecules differ from artificial motors in that they are extremely small, on the order of 10 nm, and they operate at normal temperatures. Although the size of the molecules is of a scale that means they are strongly influenced by thermal fluctuation, they are able to utilize the input energy, which is of almost the same order as the thermal energy to generate smooth motion. It is clear that there must be some sort of ingenious mechanism controlling this random thermal fluctuation to extract uni-directional motion. Resolving this mechanism could lead to the creation of energy and information converters based on completely new principles. We there-

fore view this topic as an extremely significant subject of research.

Numerous technical limitations have impeded investigation of the functions of these protein motors. Thus, many researchers have performed experiments focusing on muscle cells, the flagella themselves, or suspensions of purified protein motors; they then attempted to infer the characteristics of single molecules based on the results of these experiments. However, these experimental systems included innumerable protein molecules. (For example, several trillion protein motors are present in a muscle cell 50 micrometers in diameter and 1 mm long, and several tens of thousands of protein motors are found in a single flagellum.) As a result, only ensemble averages could be obtained, and a significant amount of information was averaged out and thus lost. In light of this, we decided to develop a method of direct observation and manipulation of a single protein motor. Although an artificial motor may easily be observed and manipulated, special measurement techniques must be developed to handle a delicate protein molecule as small as several nanometers in diameter which must also function in aqueous solutions. In this paper, we will describe various measurement techniques we have applied in our Protein Biophysics Group to track the behavior of a single bio-molecule with high precision.

2 Techniques for direct observation of bio-molecules

To understand the functions of bio-molecules at the molecular level, it is important to observe the behavior of a single functioning bio-molecule directly under a microscope. This section describes a method for visualizing protein motor behavior directly under a light microscope. This method is used to understand motion in a living body, such as muscle contraction and cell movement at the molecular level.

2.1 Visualization of protein filament

First, we will discuss a method to visualize

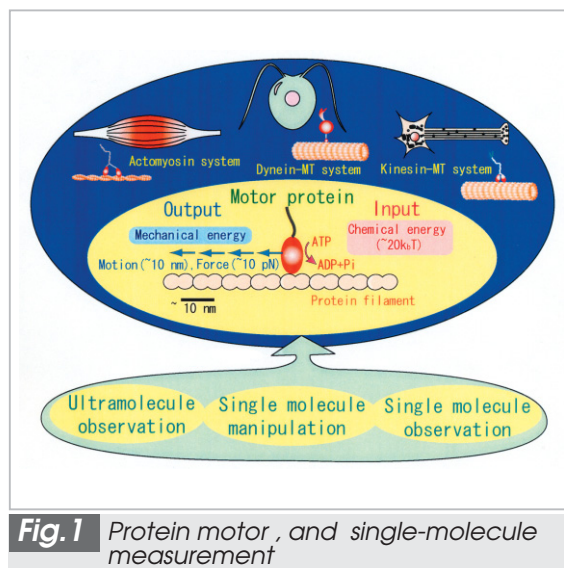


Fig. 1 Protein motor, and single-molecule measurement

protein filaments, such as actin filaments and microtubules. These protein filaments are much thinner than the wavelengths of visible light, and thus they cannot be observed using the bright-field observation technique which uses transmitted light. Thus, to observe these filaments without losing their activity (as opposed to viewing them when inert under an electron microscope), the filaments are marked with fluorochromes, and viewed under a fluorescence microscope. Based on the principle that enables viewing of a distant star as a light spot against a dark night sky, this method allows direct visualization of objects too small to observe as a fluorescent image floating against a dark background. To visualize a single filament in this manner, a number of devices are required: a fluorescence microscope, a camera, a video recorder, a monitor, and an image-processing unit (Fig.2). When setting up the fluorescence microscope, the selection of objective lens and dichroic cube is particularly important. Using an oil-immersion objective lens of 100 X and with a large numerical aperture (approximately 1.3), fluorescence from the fluorochrome can be collected with minimum loss. The dichroic cube separates the excitation light and the fluorescence, and should be selected to yield the optimum spectral characteristics based on the light source, and fluorochrome used. The optimum selection will suppress background light

caused by leaking or scattering of the excitation light, and will allow for clear extraction of target fluorescence only. A 100-W extra-high-pressure mercury lamp is normally used as the excitation source; intensity should be adjusted using an ND filter. To observe the fluorescence obtained with the microscope as a video image, a highly sensitive camera system is required (for example, an EB-CCD, em-CCD, or I.I. CCD camera) featuring sufficient sensitivity to detect the weak light generated from a single filament.

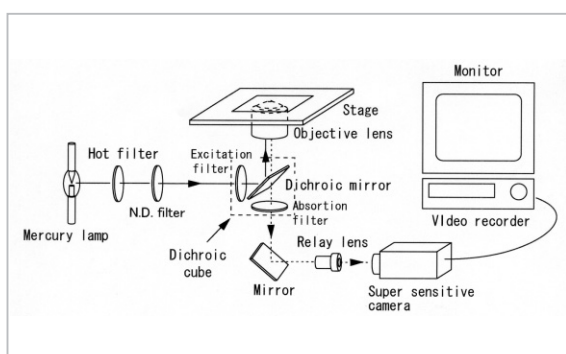


Fig.2 Experimental system for visualizing protein filaments

When labeling the filaments with a fluorochrome, serious consideration should be given to the selection of fluorochrome and to the method of introducing the fluorochrome into the filament. In a system with a mercury lamp as the excitation source, tetramethylrhodamine and fluorescein are typically selected as fluorochromes. These fluorochromes satisfy the required conditions: they are bright (with superior quantum yield and large molar absorption coefficients), and they take a long time to be bleached. As such, these fluorochromes prove suitable in experiments that require imaging of only a limited number of fluorochrome molecules—as in this case, in which a single filament is to be viewed. Various types of additional fluorochromes satisfying these conditions have also been developed, including cyanine dyes (Cy3, Cy5) and BOD-IPY dyes. With a laser as the light source, an increasing range of excitation wavelengths is available, allowing greater flexibility in the selection of fluorochrome in accordance with

the needs of the experiment.

Two methods are available to introduce the fluorochrome into the filament: one involves the use of a filament-stabilizing reagent (phallotoxins for actin, and taxol for microtubules) bonded to the fluorochrome; with the other, the fluorochrome is bonded directly to highly reactive amino acid residues (such as the SH, and NH groups) on the protein surface. A typical example of the former method involves labeling the actin filament with phalloidin-tetramethylrhodamine (PHDTMR) [1][2]. This method is frequently used because the procedures involved are simple, and the labeled actin filament shows sufficient biochemical activity and motility. The latter method is used not only for labeling filaments but also to label proteins in general [3]. Various fluorochromes with different reactive groups (including maleimide, succinimidyl esters, and isothiocyanate groups) are commercially available, and can be selected according to the amino acid residue to which the fluorochrome is to be introduced for protein labeling. The filaments prepared with these methods can be visualized as a video image as shown in Fig.3. Applying this system, sliding movements of various types of protein motors were reconstituted *in vitro*, yielding significant experimental results.

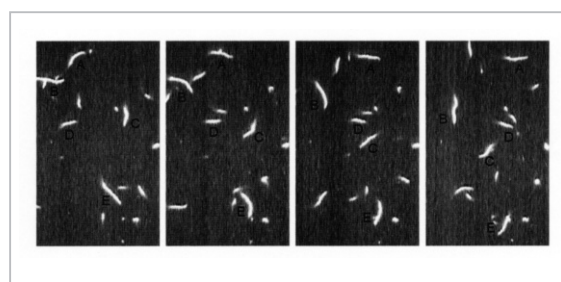


Fig.3 Video image of fluorescence-labeled actin filament

Images of actin filaments sliding on a cover slip coated with myosin molecules are displayed every 0.5 s.

2.2 *In vitro* motility assay

To analyze, and evaluate the functions of proteins, specific techniques are required to measure and observe these functions in real

time in solutions. For protein motors in particular, a technique to reconstitute the motor function *in vitro* is indispensable. Such a technique is provided by *in vitro* motility assay. This method entails fixing the protein motor to a glass substrate or to a small bead, allowing the visualized protein filament to interact with the protein motor, and observing its movement using a video camera. Fig.4 schematically shows two assay methods, which differ in the method of positioning: surface assay (see Fig.3) and bead assay. These methods can be used not only for functional analyses but also as basic techniques to extract motion and forces generated by protein motors. When fixing the protein motor to the surface, great care should be taken not to arrest motor activity. Trial and error is the only available approach in this case, as each type of molecule has its own distinct surface characteristics. It is sometimes effective to modify a part of the molecule using molecular biological techniques to enable bonding (such as antigen-antibody bonding and avidin-biotin binding) in which the modified site is specifically identified as a target surface for protein attachment.

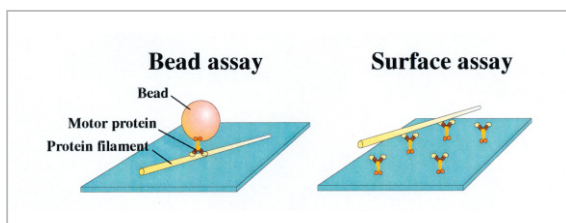


Fig.4 *In vitro* motility assay

2.3 Visualization of a single molecule

Next we will discuss a method of visualizing a single bio-molecule. A single bio-molecule is viewed through visualization of its single-molecule fluorochrome label [4][5]. This involves the detection of objects 100 times darker than the fluorescent actin filament discussed above. For this reason, the experimental system must suppress background light which impedes visualization of the single molecule. The detector and the fluorochrome

should also be selected with extreme care. Given these requirements, an experimental system must be designed to incorporate a refined method of visualizing a single filament.

The main causes of background light in fluorescence observation are (1) the light generated by undesired fluorescent molecules in the sample, and (2) leakage of excitation light scattered by the optical filters. Problem (1) is resolved through the use of an evanescent field as excitation light. An evanescent field is generated when light is totally reflected at the interface between the slide glass and the sample solution [6]. This light is localized in the region near the glass surface at a depth of approximately 100 nm, and thus selectively excites only the molecules near the glass surface. In this way, the background light is significantly reduced relative to the method of epifluorescent-illumination in which all fluorescent molecules are excited in the direction perpendicular to the surface.

Figure 5 shows an experimental system employing a second-harmonic YAG laser (532 nm) for visualization of a single molecule labeled with Cy3 (absorption/ fluorescent wavelengths: 550/ 570 nm). This system involves placement of a trapezoid prism via glycerol above a sample mounted on an inverted microscope. A laser beam is emitted to the sample through the prism at an angle greater than the critical angle (66 degrees on the interface between quartz and water). The laser beam is collimated with a condenser lens (in this example, one with a focal distance of approximately 50 mm) to illuminate an area 100 μm in diameter on the sample. Laser power of several milliwatts is sufficient in this case. Here, non-fluorescent glycerol must be used. The glass slide and the prism should feature low fluorescence, and must be thoroughly washed.

In addition to the method described, another technique (to be described later) is available for generation of an evanescent field, in this case using an objective lens with a large numerical aperture [7]. The leaking indicated

as problem (2) above is minimized through examination of the spectral characteristics of the absorption filters. Since a laser is used as the excitation source, it is also effective to use a notch filter that blocks the excitation wavelength. Detection of the fluorescence image of a single molecule is generally enabled by amplifying the detected photons with the addition of an extra image intensifier in front of the highly sensitive camera described above. The most frequently used fluorochrome for single molecule observation is Cy3 due to its brightness and durability against photo bleaching. Other candidate fluorescent molecules include Cy5, tetramethylrhodamine, Alexa532, GFP, and related molecules.

Figure 6 shows an image obtained using this system in which a single Cy3AT(D)P molecule is bonded to a single-headed myosin

molecule [8]. The experiment involves adsorption of a co-polymerized filament on the glass surface composed of single-headed myosin and a myosin rod labeled by Cy5 (molar ratio of 1:1,000; single-headed myosin molecules are distributed sparsely in the filament) on the glass surface (see the far-left panel in Fig.6), and an observation is enabled through the addition of 10 nM of Cy3ATP. Here, only the Cy3AT(D)P molecules bonded to the myosin molecule are observed as a clear fluorescent spot (see second and subsequent panels in Fig.6). Dissociated Cy3AT(D)P molecules move with rapid Brownian motion (diffusing approximately $1 \mu\text{m}$ per ms), and thus are not observed as spots; instead these molecules increase background light uniformly. Therefore, flashing bright spots indicate the association and dissociation of the mole-

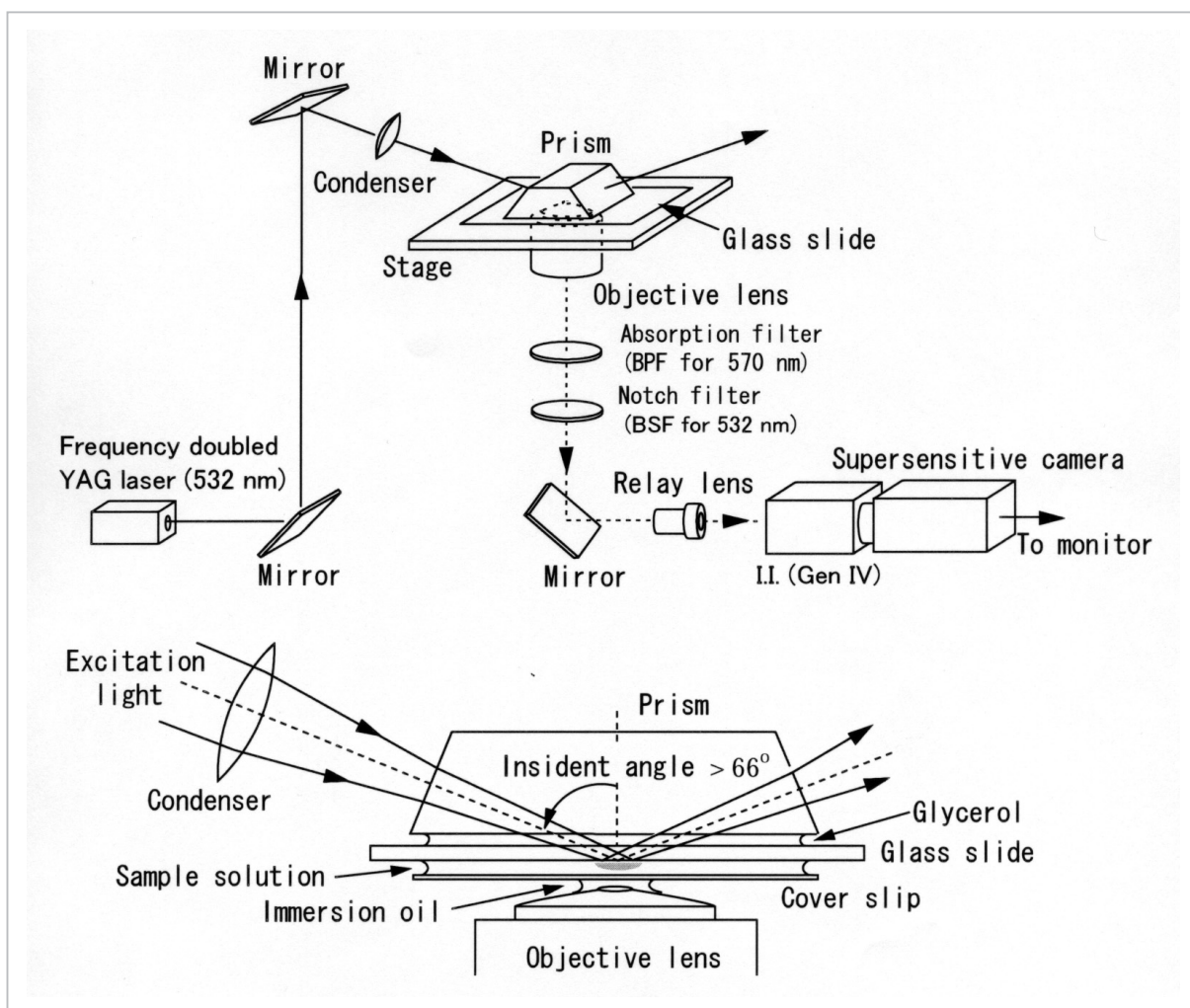


Fig.5 Experimental system for single-molecule visualization

cules. In the example shown in Fig.6, association and dissociation occur at four sites on the filament. To observe a single molecule clearly, it is necessary to decrease Cy3ATP to 50 nM or less in order to suppress background light. This single-molecule imaging system can be applied to purposes other than the visualization of ATP hydrolysis by motor protein molecules [4][5][7][8], including study of the movement of protein motors along a filament [9][11].

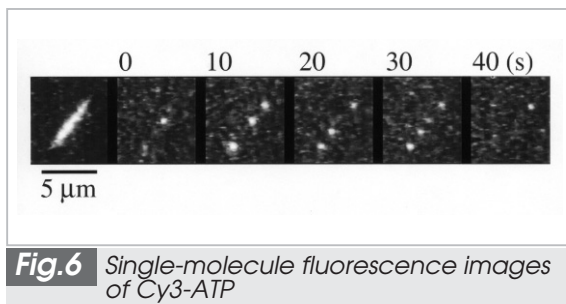


Fig.6 Single-molecule fluorescence images of Cy3-ATP

2.4 Detection of orientation of a single fluorescent molecule

The single-molecule visualization system discussed above can be further developed into an experimental system to detect the orientation of a single fluorescent molecule [12] (Fig.7). In this system the polarization of the evanescent field is rotated on the sample surface, and the orientation of the fluorescent molecule bonded to a protein motor fixed to the glass surface is determined with a precision of 5 degrees. In this experimental system, an objective-lens-type total internal reflectional illumination system is employed. The emergence angle of the excitation laser and its plane of polarization are rotated using a polarizing plate and a wedge-shaped prism which are placed in the illumination route, and rotated simultaneously. The laser beam is condensed on the back focal plane of the objective lens, and is allowed to pass through the edge of the lens to generate the evanescent field. As a result, the polarized evanescent field excites the fluorescent molecule bonded to the protein with fluorescence intensity subject to sinusoidal variation with the rotation of

the polarization angle. The peak position observed in this experiment provides the data required to calculate the angle of the fluorescent molecule bonded to the target molecule. This method has enabled identification of the three ATP binding sites on a rotary protein motor known as F1-ATPase at the single-molecule level. A related study has since directly linked 120-degree rotation of the molecule to the association and dissociation processes of ATP on the three bonding sites at the single-molecule level.

3 Single-molecule manipulation, and nanometry

For detailed investigation of mechanical characteristics and the processes in which force is generated in a bio-molecule, it is necessary to observe movement on a molecular scale (several nanometers) and on a time scale corresponding to the speed of the movement (several milliseconds). Here we will discuss a laser-trap-nanometry technique that enables observation through a combination of molecular manipulation using optical tweezers [13] with a nanometric method based on the use of optical sensors [4].

3.1 Laser manipulation of a molecular motor: the laser trap

A laser trap is used to catch a minute object using light. The principle behind such a device may be simply stated as follows: when a laser beam is condensed using an objective lens with a large numerical aperture, the intensity at the focus is extremely large where light is concentrated (Fig.8). The intensity rapidly decreases at a small distance from the focus. This means that the electric-field gradient of the light is extremely large near the focus. When a small spherical dielectric material (for example, a polystyrene bead with a diameter ranging from several micrometers to several tens of nanometers) approaches this region, it is trapped near the focus due to the large electric-field gradient. Applying this principle, the trapped particle can be manipulated with complete control by moving the

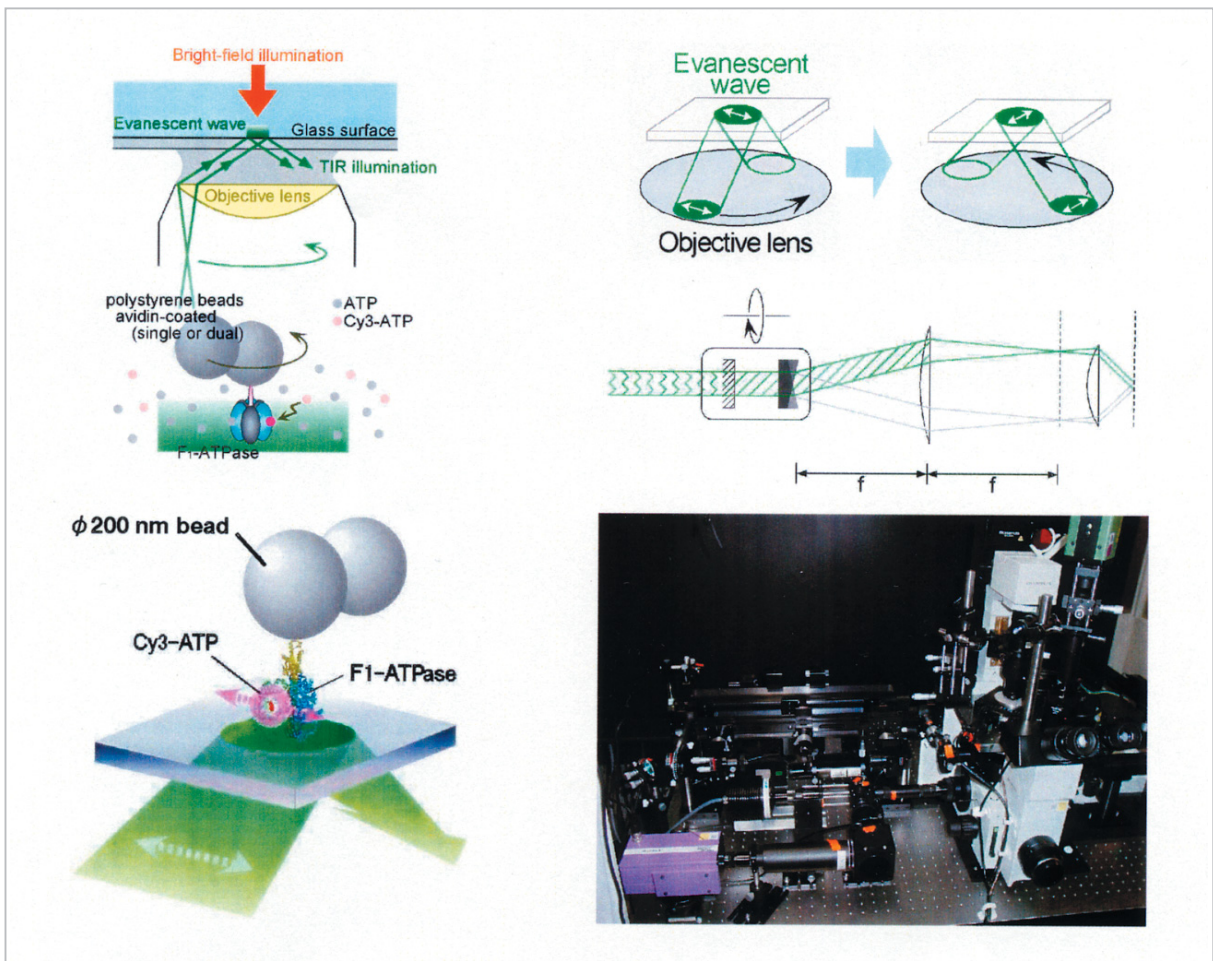


Fig.7 Orientation detection of a single fluorescent molecule
 Composition of the apparatus(upper), schematic of the measurement(lower left), and appearance of the apparatus(lower right).

position of the focus three-dimensionally. The trapped bead is also known to receive a restoring force approximately proportional to the displacement from the stable position. Thus, this system can be used as a microscopic tension gauge. As bio-molecules are generally too small to be trapped directly by the laser trap, a single bio-molecule is fixed to the surface of a bead in a manner that does not damage its activity, and the bead is manipulated with the laser. Adjustment in the content ratio of the mixed molecules and beads makes it possible to attach a single molecule alone to the bead surface. The time resolution achievable within this system depends on the ratio between the viscous drag on the bead and the spring constant of the optical tweezers. For example, the time resolution is approximately 1 ms when trapping a bead with a diameter of

0.2 micrometers and a spring constant of 0.004 pN/nm.

3.2 Observing nanometer-scale motion

In mechanical measurement of a protein motor placed on a protein filament fixed to a cover glass, the laser trap captures the bead on which the protein motor is fixed. The observation system then tracks—with high sensitivity—the generated displacements and forces as functions of time. Here, the resolution of the light microscope is several hundreds of nanometers at best, due to the restrictions imposed by the diffraction limit of light. Thus at first glance it may seem impossible to detect the displacement of a molecule as small as several nanometers. However, resolution in this case is defined as the ability to distinguish

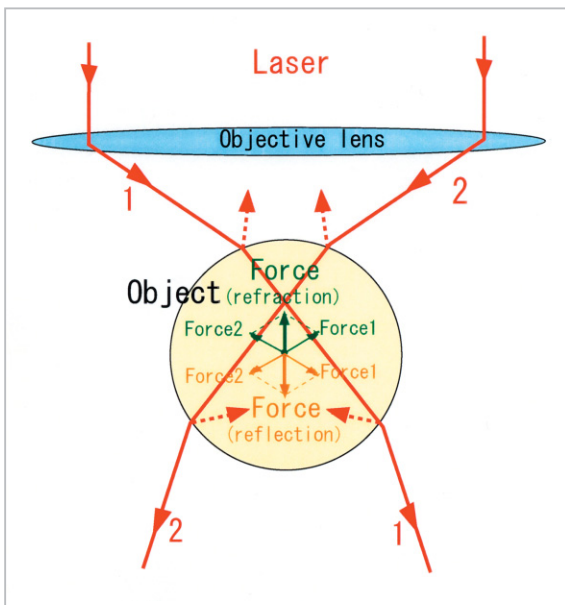


Fig.8 Principle of laser trap

between two adjacent points, while the detection of a change in position of a single point is free from this restriction. To determine the position of the center of mass of the bead, we

project its image—magnified 1,000 times—to the center of a quadrant photodiode sensor, and detect the difference in light intensities at horizontally or vertically aligned sensors (Fig.9). When the bead moves 1 nm under these conditions, the image on the detector moves 1 μm with a corresponding change in intensity difference. This method allows for detection of a displacement as small as the size of a hydrogen molecule, that is, displacement on a scale of 0.1 nm. As the spring constant of the laser trap is also known, the force generated by the bio-molecule can be estimated from Hooke's law with precision on the order of piconewtons. Applying this system of measurement to the dynein-microtubule system, a stepwise trace is obtained as shown in Fig.9, reflecting the movement of a single molecule. Elaborate analyses of the step size and the timing of step generation (together with the feedback of these results to biochemical data and to the results of molecular-struct-

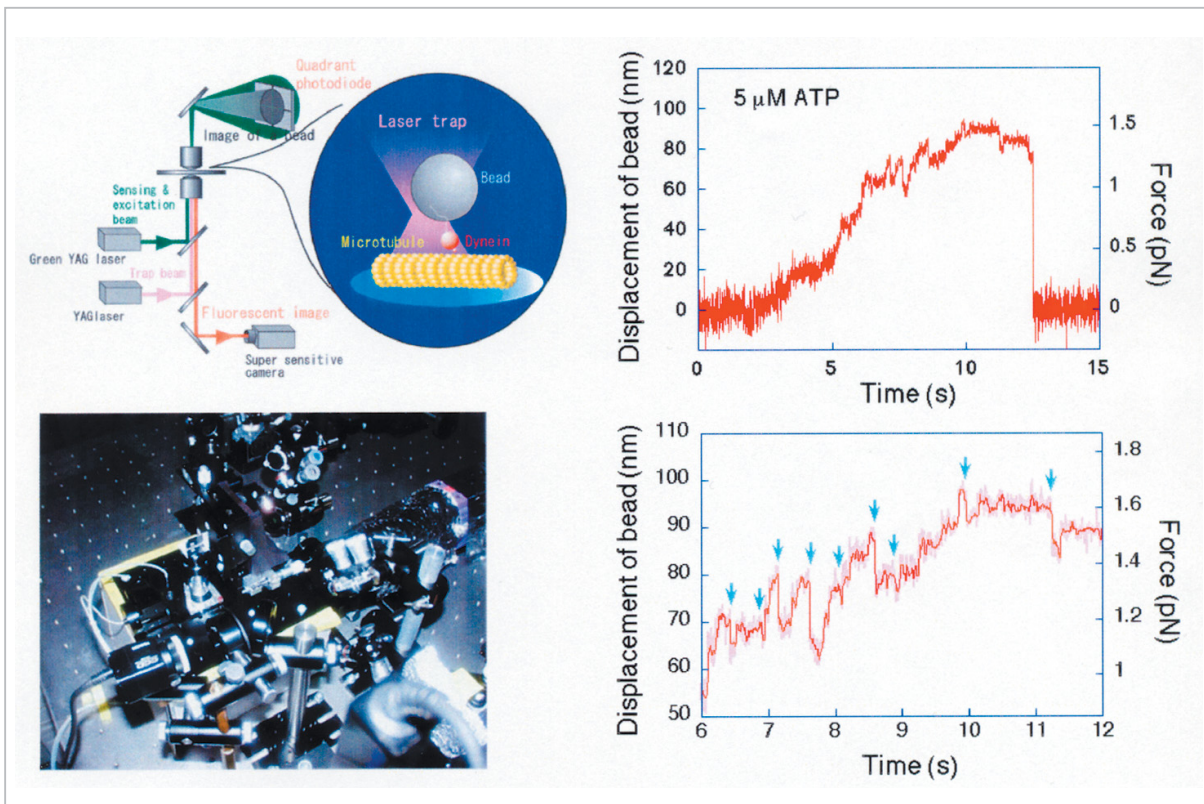


Fig.9 Laser-trap nanometry

Schematic and picture of the apparatus (left) and the displacement produced by a single dynein molecule detected by this apparatus (right). 8 nm steps corresponding to the structural repeat of microtubules are observed.

ture analyses) have rapidly clarified a series of previously unknown details relating to the mechanisms of generation of bio-molecular functions [15]-[17].

3.3 Observing the events within steps: feedback control of the probe position

To further investigate the details of the force-generating mechanism in protein motors, we are developing a technique to measure the minute forces generated between

molecules without losing positional information. This next-generation technique also entails the measurement of single molecules.

The laser-trap method described above cannot be used to measure instantaneous events. The steps observed in the displacement traces are completed in an instant, as the beads move freely pulled by the protein motor, and this renders it impossible to analyze the events that occur in the discrete steps. Nevertheless, the most important events related to the generation of force are considered to occur

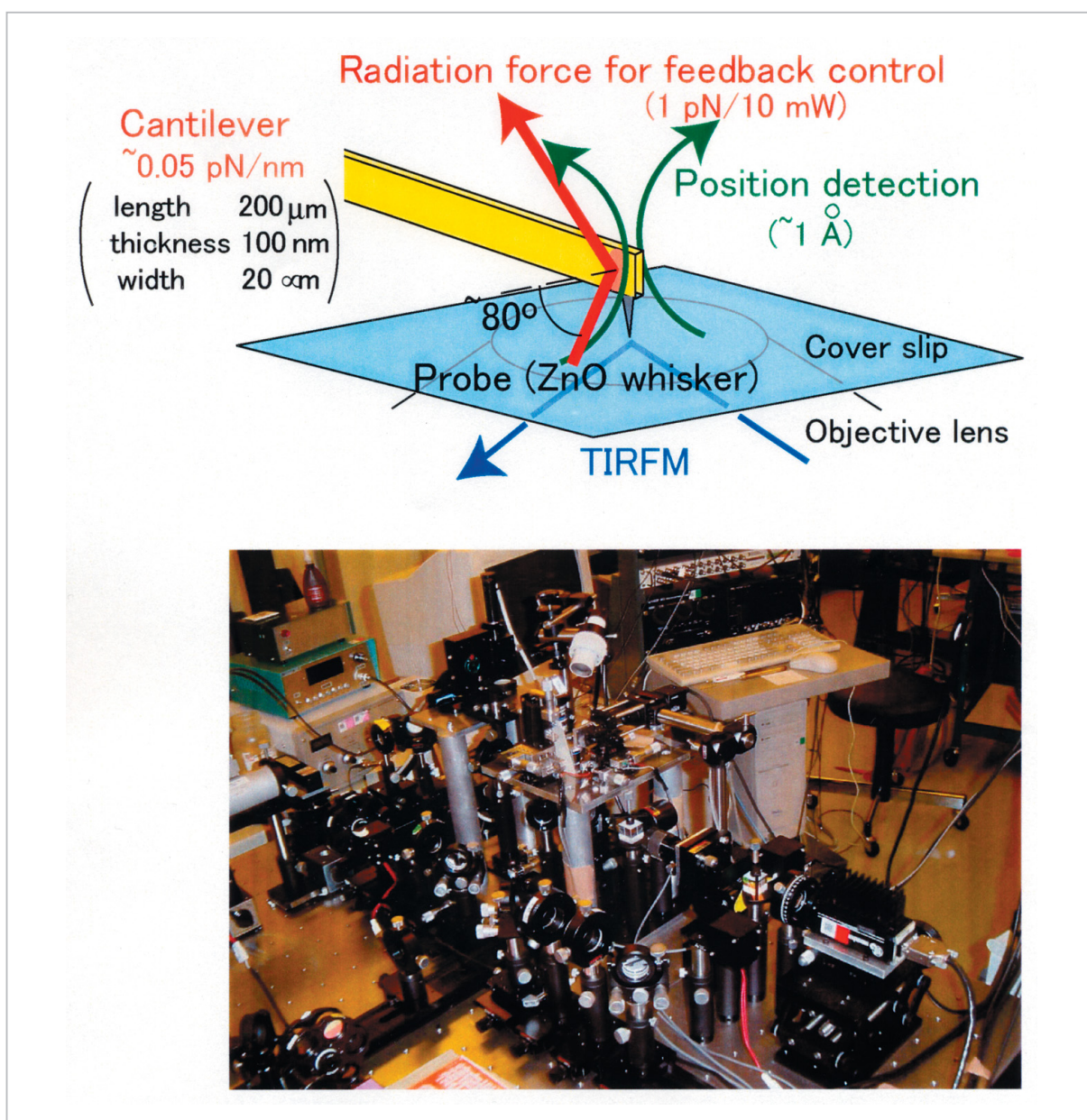


Fig. 10 Configuration of the position-control-type sliding-force measurement system
A schematic illustration of a probe part in the apparatus (upper) and a picture of the apparatus (lower).

at this instant, and it is therefore extremely important to detect the forces generated in each of the transient steps. To enable this measurement, we constructed a feedback system to control the position of the probe using the radiation pressure of light (Fig.10). This system now enables us to control the position of the tip of the probe for force measurement with a precision of 0.4 nm in terms of the root mean square. This means that it is now possible to measure force with the probe positioned at any desired site on the protein filament. Scanning the protein filament using a probe attached to a protein motor is strongly expected to enable measurement of the mechanical interaction potential formed between these molecules.

4 Conclusion

This paper describes a number of cutting-

edge techniques for visualizing, manipulating, and measuring a single molecule. In the past several years, the research results obtained using these techniques have added extensively to our knowledge of the functions of protein motors. The techniques developed here and the outcomes of studies employing these techniques will certainly be extremely useful beyond the narrow field of studies of protein motors, extending to our understanding of biological phenomena at the molecular level. We would be extremely pleased if the current study proves helpful in broadening understanding of the sublime functions shared by living creatures unconsciously for billions of years; we are also hopeful that the technical designs obtained here will provide a basis for application of these techniques to new technologies in the future.

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