Microscopies for the Biological Functions

Hiroaki KOJIMA

To understand the mechanism of cellular and bio-molecular functions, it is crucial to develop the method for characterizing their dynamics and structures by observing and manipulating them directly in the physiological conditions. Here, we introduce the principles, system configurations, and applications of the microscopy at single molecule level, the laser trap nanometry, and the reconstitution technique of bio-molecular functions mainly from the technical point of view.

1 Introduction

Because biological systems continue to survive by adapting to a complex and constantly changing environment, they have acquired outstanding characteristics through the course of evolution such as autonomy, self-organization, energy efficiency, and advanced robustness. In order to achieve this, they implement strategies from a variety of perspectives that are completely different from systems built by people. We are studying the outstanding information functions of biological systems, and targeting biomolecular systems that form their foundations, in order to acquire technology that we will be able to make use of. Of all the biomolecules, nanometer sized protein molecules and complexes formed by them play major roles in a variety of biological functions including the communication of information and movement. In a living organism, the movement of substances signifies communication of information. Intracellular communication of information and intercellular communication through hormones are typical examples of this. We have focused our research on molecular protein motors responsible for movement within living organisms, and other related molecules.

Organisms exhibit a huge variety of methods of biolocomotion, but breaking them down to a biomolecular level reveals that they rely only on a few types of universal protein motors (Fig. 1).

They are divided into molecular systems for rotational motion (bacterial flagellar motor, F1 ATPase) and linear motion (actin-myosin system, kinesin-microtubule system, dynein-microtubule system) depending on their style of movement. With the exception of bacterial flagellar motors, which draw energy from proton concentration gradients, protein motors draw energy from a chemical substance known as ATP (fuel molecule) to achieve unidirectional motion. The bacterial flagellar motor moves by spinning a rotational axis found within the protein assembly. By comparison other protein motors achieve unidirectional motion through the movement of molecular protein motors along polymerized, filament-shaped rails. Some of their characteristics to take note of are (1) the input energy is around 10 times the thermal fluctuation energy, and they function with high efficiency in an energetically extremely noisy environment, (2) the sizes of molecular devices are on a scale of nanometers making them difficult to mimic using current technology, and (3) they form high order molecular assemblies through self-organization to realize advanced functions. The input factor of ATP consumption and the output of molecular motion and other force generating processes are easy to physically quantify making them suitable for analyzing the expression of biomolecular functions. Moreover, a carefully designed reconstituted system of these motor functions will enable the building of an information communication system that makes use of these biofunctions.

So far, we have successfully developed our own technology for recreating biomolecular functions, micros-copy techniques, and manipulation technology, and
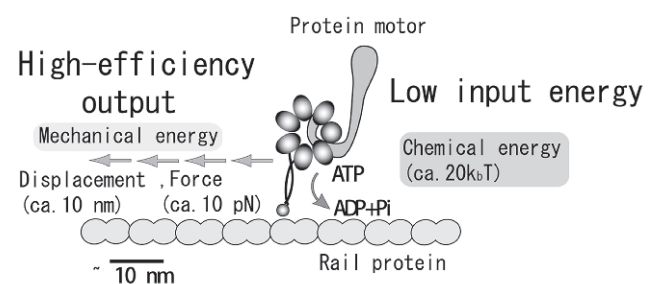

Fig. 1 A protein motor and its size

wielded these technologies in our efforts to analyze the functions of molecular protein motors, and build micromachines based on protein motor systems. In the following paragraphs we will report on our methods of research in these initiatives.

2 Functional analysis of protein molecule functions with the protein motor as an example

2.1 Conditions necessary for assessing biomolecular functions

Figure 2 is a schematic diagram of the flagella of a eukaryote and their internal structure; an example of a biological system made of protein motors. Flagella are cellular organelles used for propulsion found in microorganisms such as Euglena and Chlamydomonas. They are used to move underwater by flexing them in a way similar to the movement of the arms in breaststroke. Within the flagella are 9 pairs of longitudinally arranged rail proteins (microtubules) between which several tens of thousands of protein motors (dynein) generate the flexing motion of the flagella by creating a shear force between the closely packed microtubules.

Let us take this example to look at an outline of the necessary conditions for observing the movement of molecules. The smallest unit responsible for generating the movement of flagella is the dynein molecule, which is extremely small at around 20 nanometers, and the microtubule being around 25 nanometers in diameter. This means that it requires a method of observation with nanometer resolution, exceeding the diffraction limit of light. In general, the refractive indices of proteins are also close to that of water, making them virtually invisible underwater, so it is necessary to devise a way of enhancing the contrast of molecular imaging. Furthermore, the energy consumed per cycle is 20 kT (k: Boltzmann's constant,
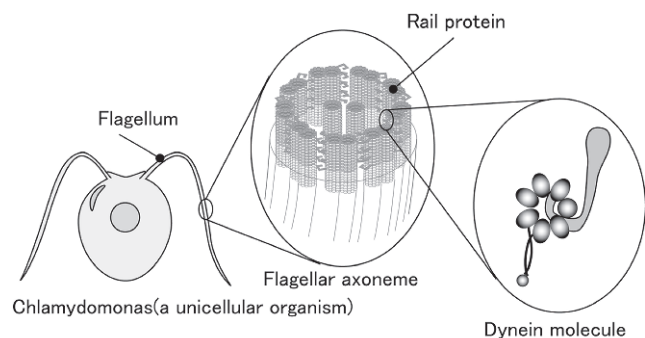
T: Absolute temperature, kT is an order of thermal fluctuation), so the force generated by one molecule is several piconewtons. This is extremely weak, and measuring it requires a highly sensitive method. Making this function also requires the preparation of conditions similar to those inside the living organism (solution environment, surface conditions, etc.), and microscopy that can handle this is limited to optical microscopy technology. It is only when these difficulties are overcome that we will be able to analyze functions through direct observation and handling of biomolecules, much in the same way as analyzing the structures and functions of calculators or engines.

2.2 Direct observation of the movement of biomolecules: *In vitro* motility assay

An *in vitro* motility assay is a way of extracting a molecular protein motor system from a living organism and binding it to an artificial glass substrate for example, to reconstitute motor functions (Fig. 3). It begins with the establishment of a method of extracting molecules, setting the conditions for the solution, and examining methods of binding the molecules to the surface of the substrate, after which an assay is carried out on the molecular protein motor extracted from the biological specimen. *In vitro* motility assay systems have several variations as the result of their geometry. Figure 3 shows methods known as the surface assay and the bead-based assay.

Sufficient contrast is achieved by neither method under optical microscopy for direct observation of the biomolecules, so tags such as fluorescent dyes with high quantum yield (Cy3, TMR, GFP, etc.) or beads (polystyrene, silica, etc.) are bound to enhance contrast
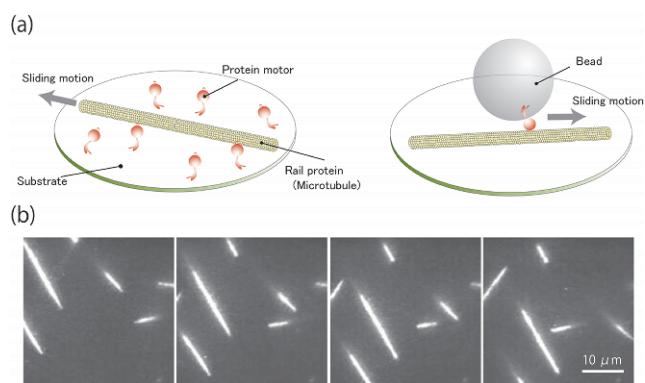

Fig. 2 The structure of a flagellum and a dynein molecule

Fig. 3 *In vitro* motility *assay*
(a) Schematic diagram of surface assay (left) and bead-based assay (right)
(b) Video sequence of surface assay in dynein‐microtubule system taken every second

with the background of gold colloid for example. This is how the movement of molecules is tracked and assessed. It will suffice to have a fluorescence microscope with an objective lens with a large numerical aperture (N.A. = 1.3 is ample), or a dark-field microscope with a highly sensitive CCD camera. Once an assay system is established, the number of molecules involved in motion can be changed by controlling the concentration of molecules bound to the surface of the substrate, or by controlling the mixing ratio of beads to molecules. This concentration or mixing ratio can be lowered to the limit to statistically lower the number of molecules involved in motion down to just one, to
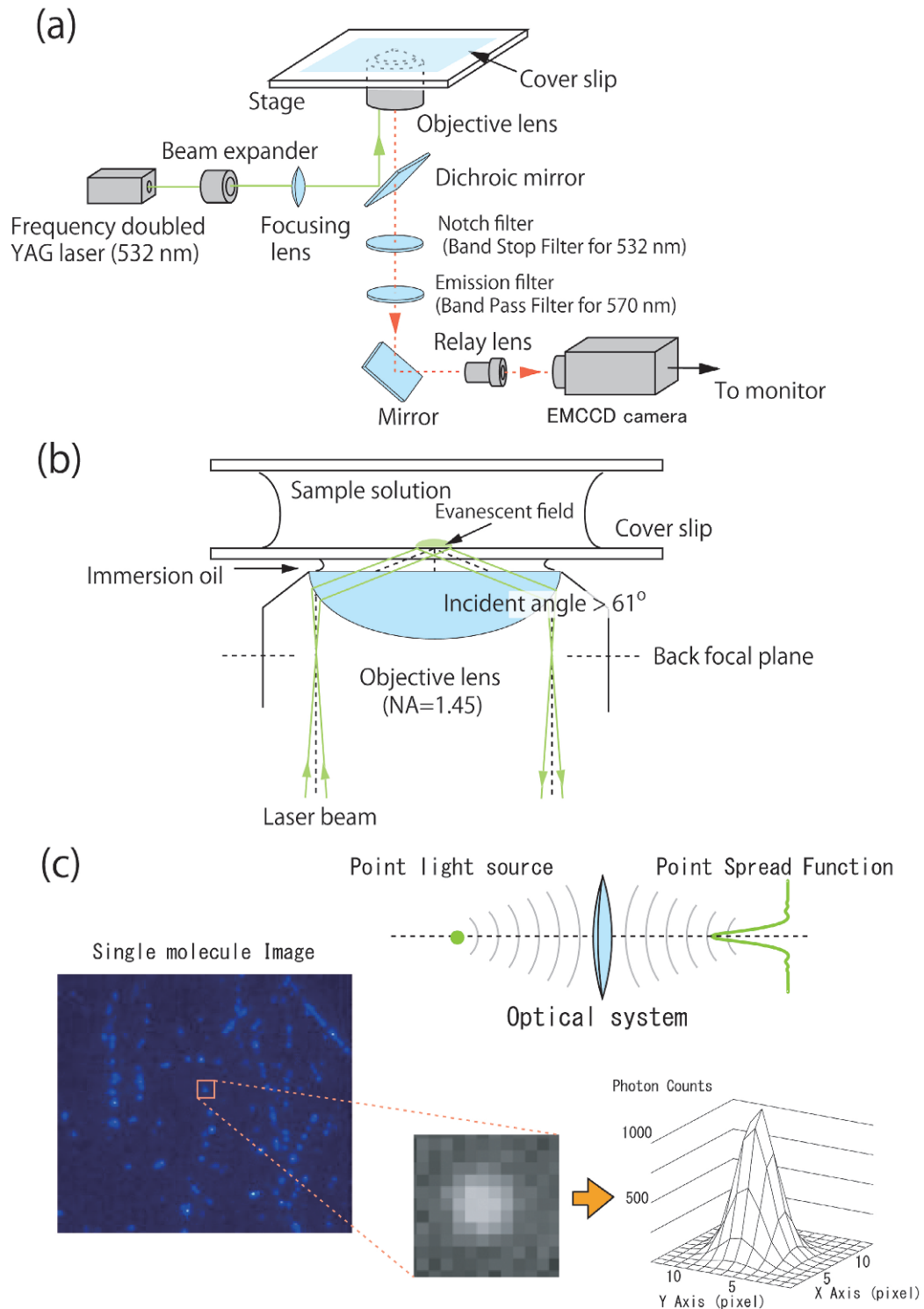
Fig. 4 Total internal reflection fluorescence microscopy (TIRFM method)
    (a) Example of optical path in objective lens-type total internal reflection microscopy
    (b) Magnified diagram of area around sample
    (c) A fluorescence image of a single molecule of Cy3 tagged kinesin and the shape of the point spread function obtained from it

observe its behavior. This method can be developed to make it possible to directly observe the behavior of a single molecule without relying on statistics. But this requires microscopes to be taken one step further.

2.3 Direct observation of a single biomolecule: Total internal reflection fluorescence microscopy

In order to directly observe a single molecule, one must begin by making it fluoresce. This is achieved by fluorescent tagging of the targeted protein molecule or biologically active substance. The tags used must be bright and they must not fade easily. Cy3, GFP, quantum dot, etc., are used depending on the purpose. Binding one of these tags to one molecule enables the ascertainment of how many molecules there are by observing the strength of fluorescence under a microscope. The fluorescence from these tags is extremely faint, and their fluorescence is indistinguishable from the background light under normal fluorescence microscopy. So a special method of lighting them up known as total internal reflection microscopy is used to create an evanescent field on the surface of the glass substrate. In this method, the excitation light is confined to an area within 100 nm near the surface of the glass to create conditions with low background light (total internal reflection fluorescence microscopy: TIRFM). Under these conditions, only molecules that come close to the surface and enter the evanescent field are observed as light spots. In total internal reflection microscopy there are two methods, namely the prism method and objective lens method. Figure 4 shows the objective lens method in total internal reflection microscopy, and an actual image of a single molecule observed. To enable total internal reflection at the interface between the glass and water, an objective lens with a large numerical aperture (N.A. = 1.45) is used and a highly sensitive EMCCD camera with little noise is used. It is sufficient for the laser excitation light to be 1 mW at the surface of the sample. For more details on theoretical studies of TIRFM, refer to[1][2].

The position of the fluorescent dye is detected with nanometer-level precision by TIRFM. In principle, the position of the center of fluorescence is detected with high precision exceeding the diffraction limit of light. The image of the fluorescent dye (wavelength: λ nm) or positional light source created by the objective lens of the microscope is an airy disc pattern (Fig. 4). The profile of this central region can be approximated by a two-dimensional Gaussian function in which the standard deviation δ is

0.5λ/N.A. $\approx$ 250 nm (Equation (1)).

$$P(x, y) = \frac{1}{\sigma_x \sigma_y \sqrt{2\pi}} \exp[\frac{-(x-\Delta x)^2}{2\sigma_x^2}] \cdot \exp[\frac{-(y-\Delta y)^2}{2\sigma_y^2}] \qquad (1)$$

This is known as the point spread function, and it can be detected by an EMCCD camera. The position of the dye can be found by identifying the center of the point image, or by least-squares fitting of a Gaussian function on the point image. The precision with which this position can be ascertained is dependent on the level of noise in the image detection system, and the standard error, $\delta n^{-0.5}$, regulated by the number of photons, n, that arrive at the camera. Using this method enables ascertainment of the position of the fluorescent dye to within a margin of error of several nanometers under general experimental conditions. Refer to[3]–[5] for details on the technological background.

2.4 Trapping a biomolecule: The laser trap method

The laser trap method is an experimental method of using light to trap a micrometer scale microobject to manipulate it. Concentrating laser light with a Gaussian profile using an objective lens with a large numerical aperture focuses the light so it becomes extremely intense. But the intensity of light falls away sharply the moment you move away from the focal point. In other words, the electric field gradient is extremely large in the vicinity of the focal point. And when a small, spherical dielectric (e.g., a bead of several microns to several dozen nanometers in diameter) approaches this point, it becomes trapped by the powerful electric field gradient (Fig. 5) [6][7].

This effect can also be explained through ray optics. The difference in the refractive indices of the microobject and water results in the laser being refracted at the interface. A force is then exerted on the bead as a reaction to the change in power of the light. The irradiation of the bead by a cone of light exerts a force on it, which attains equilibrium near the focal point, causing the bead to be trapped there. The force exerted by the total reflection of a single photon on the surface of a mirror is around 10^{-15} pN, and it is extremely weak, but using a light source of around 100 mW−1 W will generate a force of around 30 pN. This is very convenient because it is about the same level as the force exerted by a molecular protein motor, or the force required to dissociate DNA base pairs apart. Biological specimens absorb light well in the visible light range, while they absorb little light in the near infrared light range. To avoid damage to biological specimens with the use of high
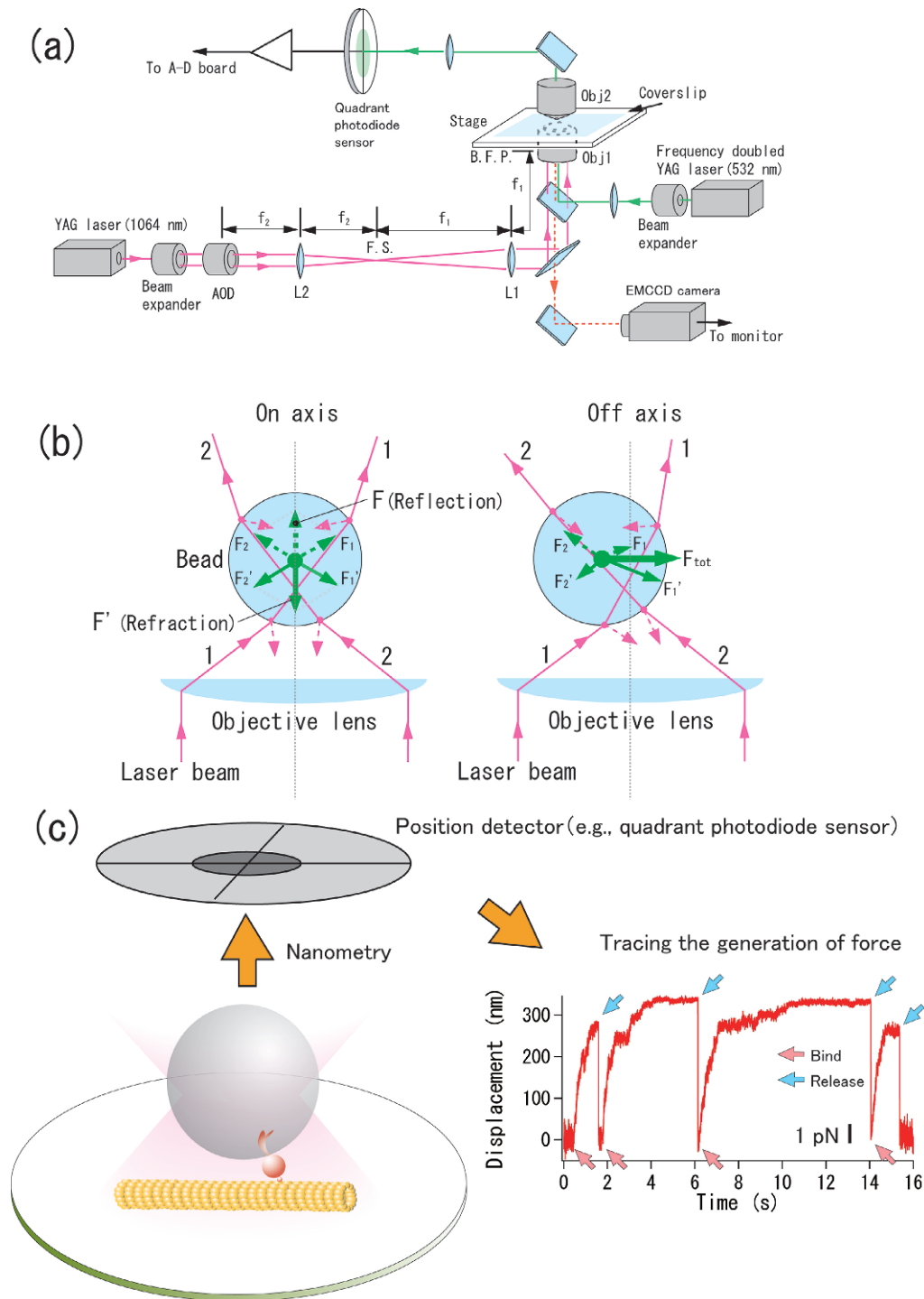
Fig. 5 The process of force generation in laser trap nanometry and a molecular protein motor
(a) An example of an optical system in laser trap nanometry (b) Explanation of the force exerted on bead in a
laser trap (c) Assessment of the force generated by a molecular protein motor

intensity laser irradiation, a near infrared laser (a YAG laser with a wavelength of 1,064 nm, or a YLF laser with a wavelength of, e.g., 1,047 nm) is normally used as the light source for laser traps[8]. The position of the laser trap within the specimen can be moved at high speed by scanning the galvanometer mirror or the diffraction grating (AOD and EOD) located by the aperture stop of the laser irradiation optical system. This enables free manipulation of the

trapped particle inside the specimen. Trapped beads like this are known to be subject to a restoring force approximately proportional to the displacement from the stabilization point, allowing it to be used like a microscopic spring balance.

Biomolecules are too small for the laser trap to trap directly. So the bead-based assay mentioned earlier is used to manipulate beads by binding molecules to their surface

without deactivating them. The time resolution that regulates the limit of speed of phenomena observable under this system is determined by the ratio of its viscous resistance to the beads, to the spring constant of the laser trap. When a bead 0.2 microns in diameter is trapped under a spring constant of 0.004 pN/nm, this is around 1 ms.

To examine the function of a molecular protein motor in detail, it is necessary to ensure adequate resolving power relative to the size of the molecule (around 10 nm) and unit step generation time (several ms). For this reason, the movement of the bead trapped by the laser trap is more often detected by an analog assessment method using a photodiode rather than by using a video camera. A method often used is to ascertain the position of the bead by projecting an image of it onto a quadrant photodiode sensor (QPD), then measuring the differential output. In order to acquire adequate output relative to the background noise, it is necessary to obtain a bead image with sufficient contrast, and choose the right bright or dark field illumination with a laser as its light source in accordance with the size of the bead, the quality of the material and the structure of the microscope system. By this method, known as nanometry, it is easy to attain a resolution of around a sub-millisecond to sub-nanometer [9][10].

We have so far applied the laser trap method and nanometry to dynein-microtubule systems mentioned earlier[11]–[13], myosin-actin systems responsible for substance transport within plants[10][14], and kinesin-microtubule systems vital to intracellular substance transport[15], and carried out assessments of microscopic dynamics (Fig. 5) to measure movement and force with precision on molecular scales (nanometers and piconewtons). As a result, we succeeded in investigating the size of unitary stroke as a molecular protein motor progresses along the rail molecule, the maximum force generated, the change in motor activity due to load, the cooperativity between molecules when they gather into a group, etc. Through this we identified the specifications of protein motors, and acquired new knowledge on the mechanisms involved, such as proposals for a model on how the functions of protein molecules are expressed.

2.5　Obtaining the blueprint for biomolecular Systems: Structural analysis of the protein motor system

To understand the grand design of biomolecular systems, it is important to investigate the design of biomolecules and complexes formed by them, or in other words investigate their structures in detail and the relationship with their functions. So far we have made full use of electron microscopy and other imaging methods with high spatial resolution to identify the internal structures of protein motor molecules and their changes, and the 3-dimensional positioning of molecules within molecular complexes.

Detailed analysis of the structure of a single molecule was carried out on a dynein molecule mentioned above. The dynein molecule moves in one direction along the microtubule through the bond dissociation of ATP molecules. To investigate how the dynein molecule itself changes structure, and how this leads to a motor function, it is necessary to assess the shape of the molecule with resolution exceeding the size of the dynein molecule. We took raw images of dynein molecules with shape distributions obtained through electron microscopy, and divided them into several groups of similar shapes. We then made full use of the single particle analysis method to average out the images in each group to obtain high quality images of molecules, and identify the changes in molecular shape due to differences in the state of ATP bonding under high resolution (Fig. 6)[16].

Based on these observations, we proposed a model for explaining the movement of the dynein molecule from the changes in their structure, and it has become recognized widely as an important working hypothesis in current dynein research.

Dynein molecules found in flagella are arranged in an orderly manner within the large-scale structure of the flagellum, generating a higher order vibratory movement instead of the simple one-way sliding movement seen in a
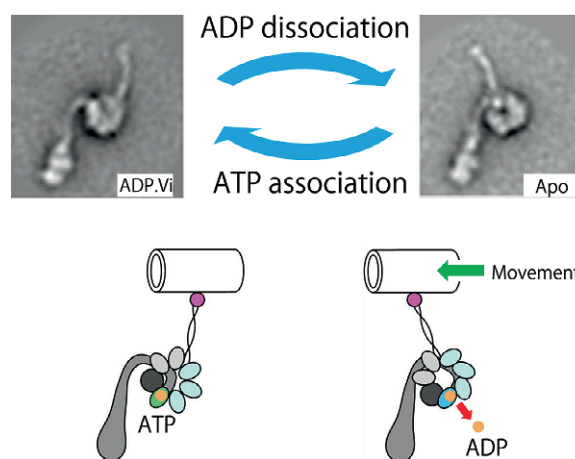

Fig. 6　Single particle analysis of dynein molecules, and a model of its movement
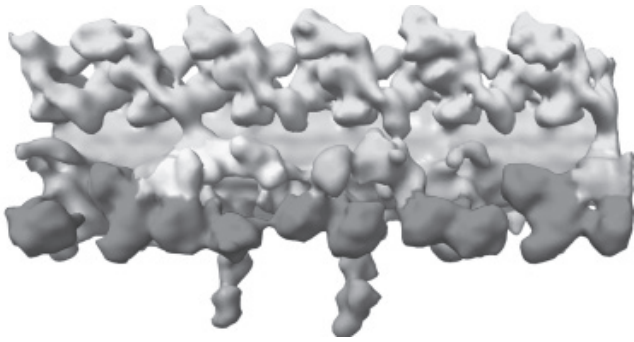
Fig. 7 The result of analyzing the 3-dimensional positioning of molecules within a flagellum through electron tomography

single molecule. In order to analyze the mechanism behind the expression of functions when multiple devices come together to function as a high order system, it is important to acquire knowledge of the 3-dimensional positioning or in other words the blueprint of the devices within the system. It is based on this perspective that we have used electron tomography technology to acquire knowledge on the 3-dimensional positioning of dynein molecules within the flagella of eukaryotic organisms (Fig. 7)[17][18]. The results obtained in this research give us information on the spatial and functional coordination between devices that make up the flagellum system, and they are proving to be very useful in designing research into bridging the gap between functional units and systems.

2.6 Controlling the flow of biomolecules: Building a micromachine that operates on protein motors

We have been searching for building principles based on our belief that it is possible to build micromachines using the functions of the biomolecules themselves by combining an *in vitro* motility reconstitution system with nanostructure building technology.

Starting with an *in vitro* motility reconstitution system in which molecular protein motors move in random directions, turning this into useful motion to build micromachines will mean developing research by (1) controlling the trajectory of movement, (2) controlling the direction of movement, (3) designing functions that make use of the movement, and (4) systemizing the function. So far we have achieved up to (2) or (3).

We have used the photolithography method as an effective strategy for controlling the trajectory of movement. We discovered a way of etching the surface of substrates in a way that allows the targeted protein motors to bond without deactivating them. Through this method,

we acquired technology for reconstituting the movement of filaments on etched "highways" with micron-scale widths. We also succeeded in changing the direction of movement of filaments known as microtubules, whose movement is driven by molecular protein motors, by making use of their property of changing direction when they come to a wall, to make them move along it. Based on this method, we discovered a highly effective way of controlling the direction of movement of microtubules by preparing a path for them consisting of arrowhead patterns, and we succeeded in achieving unidirectional movement necessary for building micromachines[19]–[21].

It has now become possible to etch an even more complex "wall" on the surface of substrates, making it possible to build micron-scale circuit blocks with functions such as the ability to trap and concentrate moving microtubules, induce unidirectional movement, and prompt scanning within patterns (Fig. 8).

Initiatives like this to build new devices by fusing nanostructure building technology with *in vitro* motility reconstitution systems began at KARC (Advanced ICT Research Institute), and researchers all over the world are now implementing initiatives using the same approach.

3　Preparing for the future

We have been carrying out research in an effort to sow the seeds of innovation for the next generation of information communication technology based on the search for the operating principles of biomolecular systems and the building principles of micromachines driven by biomolecular systems.

By taking the former approach, we succeeded in acquiring an understanding of the design of eukaryote flagella from the level of a single biomolecule right up to the biomolecular system level, by making use of high precision assessment methods to examine the structure of biomolecules. Furthermore, in regard to functional assessment, we made it possible to examine the behavior and input-output correlation of biomolecules at a single molecule level, in a way that feels like the molecules are being handled directly, through the development of biomolecular function reconstitution systems and advanced assessment technology. We also obtained many results in elucidating the mechanisms behind the movement of biomolecules including molecular protein motors.

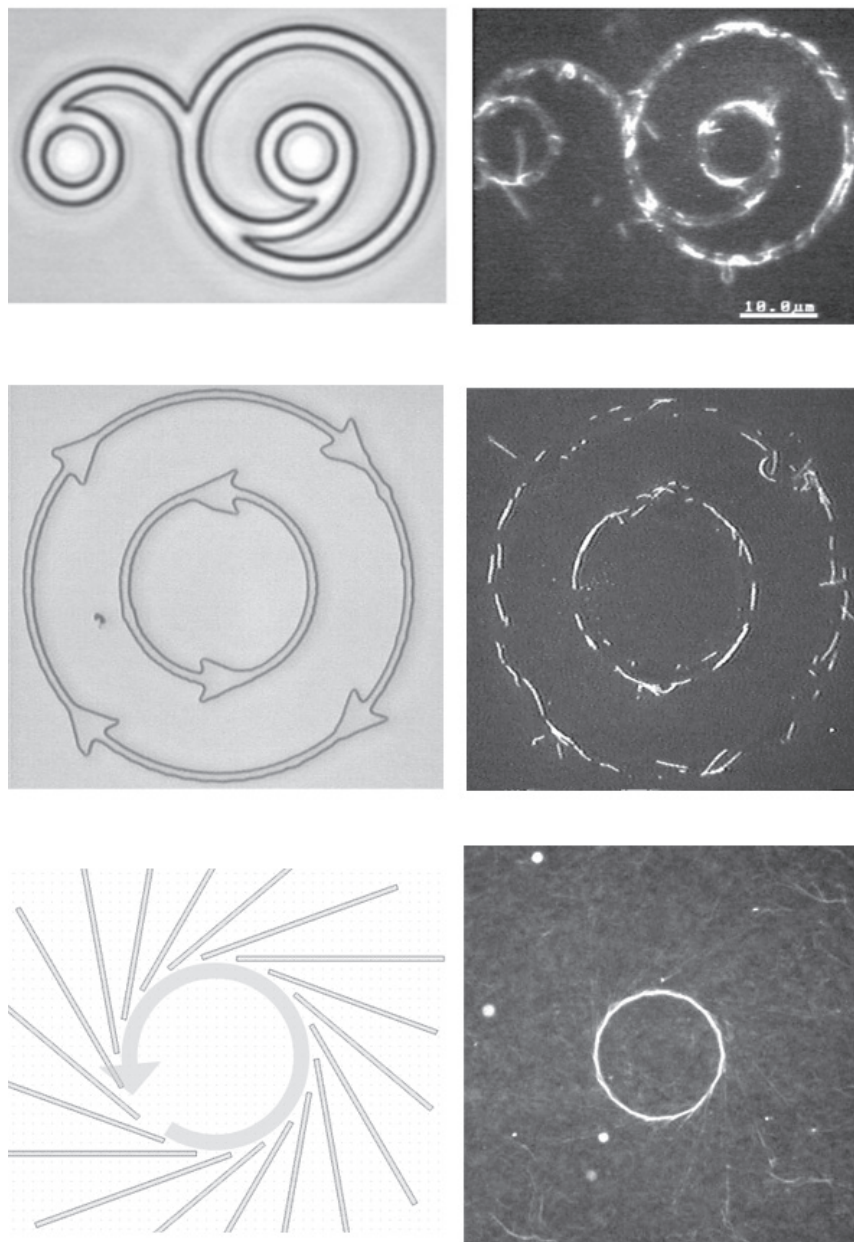In our latter approach, we combined a variety of

Fig. 8 Controlling the movement of molecular protein motors

From the top: (1) Controlling the trajectory (2) Controlling the direction of movement (3) Example of an assay of building a functional device. The column on the left shows the substrate design, and the column on the right shows the fluorescence images of microtubules whose movement has been controlled.

nanostructure building technologies with biomolecule handling technologies to successfully control the movement of molecular protein motors, and we have accumulated knowledge that will lead to the building of micromachines based on these protein motors.

As the result of our research into these technologies, our understanding of the functions and structures of protein motors has taken a giant leap forward. The assessment technology developed and results obtained through our research have extended beyond the narrow field of research into protein motors, and they are now proving themselves to be highly useful in gaining an understanding of the phenomenon of life at the molecular level.

The development of life science is always backed by some form of technological innovation. Bio-ICT research delves into information communication, a field which attracts researchers of all backgrounds, while also delving into life science. We believe these conditions make it ideal for giving rise to innovative assessment technologies.

We hope that by gaining an understanding of the subtleties of biological functions and their blueprints,

which organisms have been making use of and taken for granted for billions of years, we will be able to gain a foothold in developing new technology in the future.

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Hiroaki KOJIMA, Ph.D.

Director of Bio ICT Laboratory, Advanced ICT Research Institute
Biophysics
kojima@nict.go.jp