A Cell Organelle "Flagellum": Structures and Motile Mechanisms

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One of our research subjects is the organelle "flagella of eukaryotes" which are equipped by large extent of organisms from euglena of protozoa to mankind of vertebrate and are used when cells are propelled in the liquid. The flagellum has mechanisms for autonomously generating flagellar waveforms which are harmonious in spatial and time. We believe that we can extract the applicable new technologies in ICT technology by elucidate those clever mechanisms. Here, I will describe the research efforts of Bio ICT Laboratory about motion mechanisms and structures of flagella.

1 Introduction

1.1 Cell organelles

Organisms on Earth have advanced systems for supporting life from nanometer sized functional molecules such as DNA and proteins, complexes consisting of such molecules, to cells that are the smallest units of life ranging in size from one to several dozen micrometers, and on an even higher level, multicellular systems with advanced functions. Cell organelles are life supporting units that are one level below cells. They are found inside the cells of eukaryotes that possess intracellular nuclei and belong in human beings, too (reference material[1], Fig. 1). Cell organelles are about the same size as those of organisms known as prokaryotes (one to several micrometers) such as *Escherichia coli*, but prokaryotes do not posses organelles.

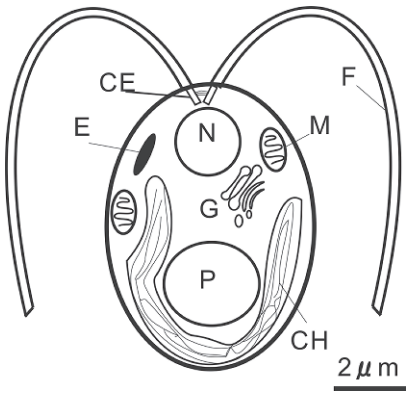


Fig. 1 The cell organelles of *Chlamydomonas*, a unicellular green algae used as a model organism in research into the flagellum
Chlamydomonas is also used in research into photosynthesis. N: Nucleus, CE: Centrosome (including the base of the flagella), CH: Chloroplast (cup-shaped in the case of *Chlamydomonas*), E: Eyespot (chromatophore unique to unicellular green algae, with a photoreceptor nearby), F: Flagellum, G: Golgi body, M: Mitochondrion, N: Nucleus, P: Pyrenoid (stores starch).

Each cell organelle carries out a specialized and vital function in accordance with the activities of the cell. For example, a typical organelle is the mitochondria, which plays the vital role of producing ATP, the source of energy for organisms. Or the chloroplast plays the role of carrying out photosynthesis within the cells of plants. The Golgi apparatus is involved in the transportation of secretory vesicles. Of all the cell organelles, mitochondria and chloroplasts are enveloped in double membranes, and they maintain isolated environments within them to function autonomously. The host cells regulate activities of these organelles from outside using signal transmitter molecules. Mitochondria and chloroplasts have their own unique DNAs, and multiply through division. According to the theory regarded to be the most likely explanation for their origin, these organelles were originally bacteria or photosynthetic bacteria such as cyanobacteria, which were taken up by the ancestral cells of eukaryotes to form symbiotic relationships with them[2].

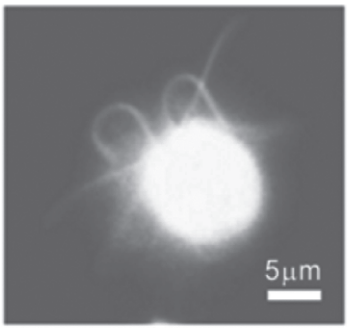
Flagella and cilia are organelles that exert a force on liquid surrounding the cells to provide propulsion[3]. Flagella and cilia show wave-like motion that is temporally and spatially controlled. If many of these organelles are found on the cell, they are called cilia. If only one or two are found, they are called flagella, but they are basically the same in structure. Based on the fact that they are all similar in shape, and the fact that there are organisms that have formed symbiotic relationships with cells, a hypothesis was

put forth that flagella, like mitochondria, originated from a different organism known as a spirochete. However, the general consensus now is that this is unlikely. In the human body, cilia are responsible for creating the flow of body fluids within the trachea, oviduct, cerebral ventricle, etc., and flagella play a vital role in the movement of sperm. In addition, recent research attracting a lot of attention has revealed that cilia play important roles in chemical detection and information processing in cells, and they have vital functions in the formation of many systems[4][5].

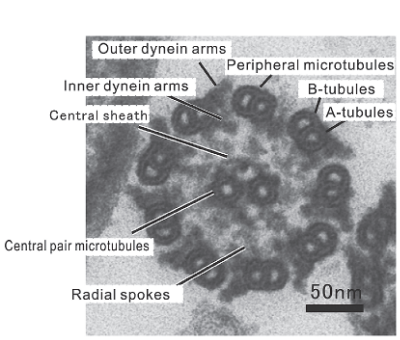
1.2 The "flagellum," the cell organelle that moves autonomously

Examination of a cross-section of a flagellum reveals a structure in which central pair microtubules are surrounded by nine peripheral microtubules (Fig. 2B). We call this structure the "9+2," and it is a complex structure consisting of approximately 200 varieties of proteins. This structure is basically the same among eukaryotes with cilia and flagella, from *Euglena* to human beings. Two types of dynein arms extend out from each peripheral microtubule to neighboring peripheral microtubules. Dyneins, which are protein motors and make up dynein arms, interact with adjacent B-tubules and generate sliding motion between the neighboring peripheral microtubules by using energy obtained from the hydrolysis of ATP. This is the elementary process of flagellar movement (general explanatory document[3]).

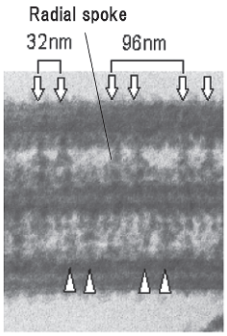
When ATP is applied to sea urchin sperm, *Chlamydomonas* cells, or flagella detached from *Chlamydomonas* cells which have had their cell membranes removed with a surfactant to expose their flagellar axoneme (the shaft of the flagellum), the flagellar axoneme begins to undulate in a way similar to its movement in the living organism. This demonstrates that the flagellum has an autonomous system that generates temporally and spatially orderly, wave-like movement without the need for a command from the cell to "bend it" or "stretch it out." Organisms sense chemicals, light, etc., in the environment around their cells, and they move toward the conditions that suit them, or control the flow of liquid around their cells. In accordance with their needs, they raise and lower their intracellular calcium ion concentration or use their system of intracellular communication such as the phosphorylation cascade to control their level of flagellar movement or the form of their undulations. In other words, they use molecular communication to regulate their flagellar movement. We have focused our research on cell



A: Flagellar movement in *Chlamydomonas* (strobe light photography)



B: Electron micrograph of cross section of a flagellum showing its internal structure



C: Longitudinal section The flagellum shows a cyclic pattern along the major axis that repeats itself every 96 nm as indic00y the arrows Arrowheads: Radial spoke positions

Fig. 2 The flagella of *Chlamydomonas*, a unicellular green algae

organelles and the mechanism behind the generation of flagellar movement. Organisms use the flagellum, an intricately assembled structure, to autonomously generate flagellar movement in accordance with their needs, and they also have free control over its movement through information transmitters. Studying the mechanism behind flagellar movement as a model case of a molecular network, there is an extremely high likelihood it will yield clues for new technology, which is the reason we have focused on this research as part of our mission.

The protein, dynein, responsible for generating movement in flagella is known for its ability to sense resistance and adjust its output accordingly. This is believed to contribute greatly to their autonomous movement. We therefore analyzed the structural changes that take place when dynein generates a force, and the characteristics of its movement, with the aim of clarifying the mechanism at the root of dynein based flagellar movement. The spatial positioning of the structural components of the flagellum are also thought to be

important for generating flagellar movement, based on the fact that the structure of the flagellum has remained little changed in all species. We therefore decided to analyze the spatial positioning of dynein within the flagellum.

2 The results

2.1 The 3-dimensional positioning of dynein within the flagellum

Microtubules such as peripheral microtubules, central pair microtubules, etc., are tubular fibers made through the polymerization of a protein known as tubulin. They form the major framework of the flagellum. The components making up the flagellum are positioned along its major axis based on a cycle of the integral multiple of the size of one tubulin molecule of 8 nm. Structures on peripheral microtubules such as radial spokes are positioned every 96 nm (Fig. 2C). Dynein is found on peripheral microtubules arranged cylindrically around the flagellum (Fig. 2B). It is obvious that if all the dynein on the peripheral microtubules exerted an equal force, the flagellum will not be able to create planar, even undulations. It is thought that dynein molecules are arranged intricately and 3-dimensionally on each peripheral microtubule, and their positions relative to adjacent microtubules change in accordance with the bending of the flagellum to compensate for the mutual action and cause the flagellum to undulate. Until recently however, it was extremely difficult to confirm details of the 3-dimensional internal structure of the flagellum. Recent advances in electron tomography technology are beginning to shed light on the structure of cilia, flagella, and in particular the 3-dimensional positioning of peripheral microtubules. The principle behind electron tomography is the same as that of a CT (computed tomography) scan. It is technology in which a 3-dimensional model is reconstructed based on a series of slant views of transmission electron microscope images taken within a field ranging from around -70 to 70 degrees upon tilting the sample at 1 to 2 degree increments, then intensity correcting the images in the Fourier space[6].

We collaborated with Dr. ISHIKAWA's group at ETH Zurich to clarify the 3-dimensional positioning of dynein molecules on the peripheral microtubules of *Chlamydomonas* (Fig. 1), a model organism used in flagella research, down to a resolution of around 4 nm (Fig. 3). Then by comparing the structures of the flagella between a mutant strain that has no dynein and a natural strain, we

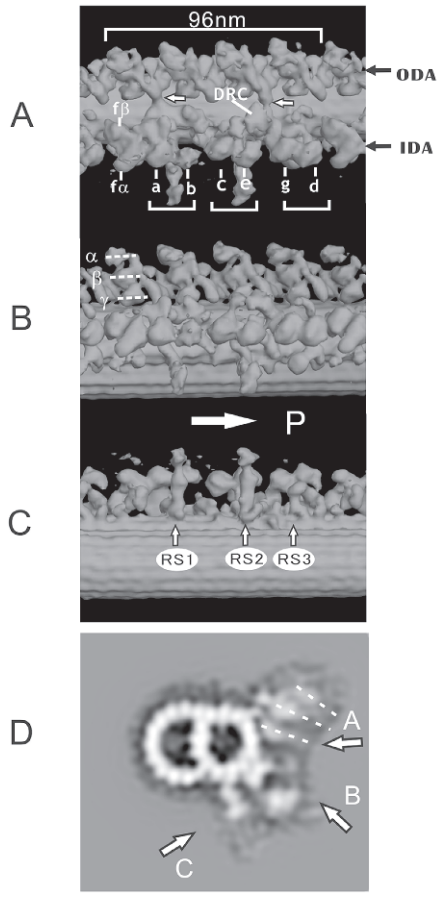


Fig. 3 Electron beam tomogram of structures on a peripheral microtubule

Data from Bui et al., (2008)[8] was turned into diagrams. The tip of the flagellum is to the right. The radial spoke head is not included in the data. Images taken from the side, A, B and C. D: A cross sectional tomographic image of a location where three outer-arm dynein heads overlap. Arrows in image D indicate the different perspectives of images A, B and C. The positions of the heads of inner-arm dynein (a-f) that have already been identified are indicated in image A. Arrows indicate the dynein outer-arm, inner-arm dynein f and the connection with DRC. ODA: Outer dynein arm. IDA: Inner dynein arm. DRC: Dynein regulatory complex. B: View from the dynein inner arm side. Each dynein tail can be seen bent toward the tip of the flagellum. The positions of heads of heavy chains of outer-arm dynein α, β and γ are indicated.

The orientation of the outer-arm dynein head rings have been indicated with dotted lines in B and D. RS1 and 2 in image C: Radial spokes 1 and 2. RS3: A projection at the location of radial spoke 3 found, for example, in the sperm of sea urchins.

determined the 3-dimensional positioning of 7 out of 11 types of dynein molecules found on peripheral microtubules[7][8]. Dr. ISHIKAWA's group has since determined the positioning of the remaining dynein molecules[9]. The dynein outer arm of the flagellum consists of four heterotrimeric (made of a heavy chain) outer-arm dynein molecules of one type placed at intervals of 96 nm. We will discuss this in greater detail in the next paragraph, but the head of the dynein molecule at the center of motor

activity has an asymmetrical ring-shaped structure[10][11]. On the peripheral microtubule, the ring structure of the head of the outer-arm dynein was observed piled up parallel to the A microtubules[7] (Fig. 3B: The dotted lines indicate the position of the outer arm). It is thought that the head ring of dynein rotates relative to its lower portion, and the microtubule attached to the end of the stalk, which protrudes from the ring running parallel to the surface of the ring at the head of the molecule, is moved by pulling on it (refer to following Subsection)[12][13]. Taking this into consideration, the positioning of the outer-arm dynein seems strange at first. But an examination of a cross section of the axoneme reveals that the B microtubule of an adjacent peripheral microtubule is positioned along the plane of each ring (Fig. 3D), and the heads stacked up on top of the A microtubules are in an advantageous position to interact with adjacent peripheral microtubules. On the other hand, the positioning of the dynein inner arm was completely different from that of the dynein outer arm. In the case of *Chlamydomonas*, an arrangement consisting of six varieties of single headed inner-arm dynein molecules (a, b, c, d, e, g) and one type of heterodimer (f, also known as I1) were observed within the 96 nm cycle of repetition (Fig. 3A)[8]. With the exception of the head of the β heavy chain of dynein f, seven heads of the inner-arm dynein heavy chains were seen arranged 3-dimensionally in a single line. It is known from previous biochemical research that the six varieties of single headed dynein molecules can be divided into those with a protein known as centrin bound to the tail as a light chain subunit (I2: b, e, g), and those with a protein known as P28 (I3: a, c, d), of which there are exactly three varieties each[14]. Determining their 3-dimensional positioning allowed us for the first time to discover that the heads of the two types of single headed dynein, I2 and I3, form three pairs that line themselves closely, side-by-side (Fig. 3A). Each pair was positioned so that they sandwiched the radial spoke at the location of the RS1 to RS3 radial spokes. This indicates that single headed dynein may function as a dimer within the living organism[8].

Three-dimensional observation of the detailed structures has clarified that the structural units within the axoneme are interconnected. These connections are believed to contribute to the generation of ciliary and flagellar movement when the structural units function in cooperation with one another. Looking at the dynein outer arm from the A microtubule, the uppermost α head and the middle β head are connected with a rod-like structure, and this rod extends toward the inner arm[7]. The tip of the rod of one of the four outer arms within the 96 nm cycle of repetition is connected to dynein f [8] (the arrow in Fig. 3A). The tip of the rod in the second outer arm from that is connected to a structure known as the dynein regulatory complex (DRC), and the DRC is in turn connected to dynein e and the base of dynein g located close to SP3 [15][16]. Furthermore, the base of the single headed inner-arm dynein (a, c, d) with P28 as its subunit, has been reported to be connected to a structure that anchors the radial spokes[17].

2.2 Structural changes in dynein at the time of force exertion

To understand the mechanism of autonomous flagellar movement, elucidation of mechanochemical properties and elementary process of dynein function are important.

Dynein is categorized as one of a family of proteins known as AAA+ (reference material[8]). The "AAA" is an abbreviation of ATPases Associated with diverse cellular Activities, and proteins of this group are differentiated by the variety of functions they perform by using energy from the breakdown of ATP. Many of them are characterized by their hexamer, ring-shaped structure formed by AAA subunits each with an ability to metabolize ATP. Dynein is a huge protein molecule made of approximately 4,500 amino acid residues. One-third of this molecule is the N-terminal called the "tail," and it is where light and medium chains bond (Fig. 4A). The amino acid arrangement in this portion of the molecule differs greatly between the types of dynein, and they function to bond their respective dynein molecules to the substrate. The remaining two-thirds of the molecule is the motor area responsible for generating the sliding motion of the microtubules. The motor area has six AAA domains (AAA1 to AAA6) making up a ring structure. The structure connecting the tail to the first AAA1 is called the linker, and it crosses this AAA ring. A projection of around 20 nm in length known as the stalk protrudes from the C-terminal of AAA4. The end of this stalk functions in synchrony with the movement of the dynein to interact with the protein rail and microtubules elucidation of mechanochemical properties and elementary process of dynein function are important (Fig. 4A).

In collaboration with Dr. BURGESS of the University of Leeds in 2003, we used negative staining electron microscopy and single-particle image processing to compare the structure of inner-arm dynein c of

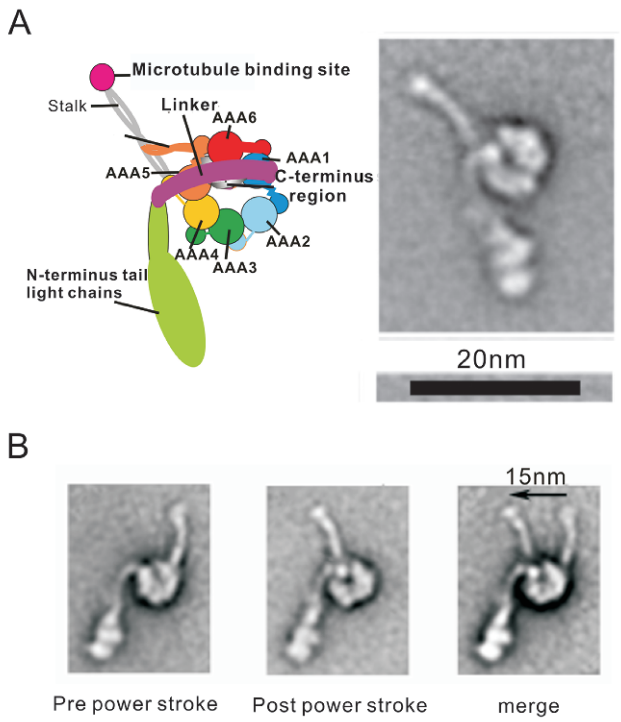


Fig. 4 Structural changes in inner-arm dynein c at time of power stroke

A: A schematic diagram of the structure of inner-arm dynein c[10][11]. Comparing molecular images obtained through negative staining electron microscopy and single-particle image processing. The electron micrograph is a transmission image so the linker above the head overlaps with other structures making it difficult to see.

B: The rotation of the dynein head elucidated through negative staining electron microscopy and single-particle image processing (electron micrographs in the reference document[12] were modified for these images).

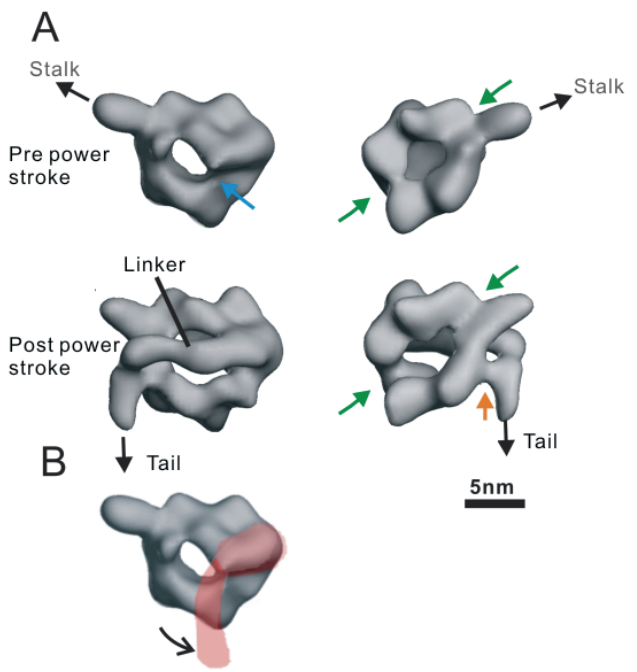


Fig. 5 3-D reconstitution of the structure of the inner-arm dynein c head

A: The structure of the dynein c head after exertion of force

B: The position of the linker before exertion of force ascertained through image dispersion
The linker can be seen to move in a swinging motion upon exertion of a force (data quoted from reference material[18])

Chlamydomonas fixed in states before and after exertion of force. We clarified that the dynein head revolves approximately 26 degrees in relation to its tail around AAA1 at the time dynein exerts a force[12] (Fig. 4B).

Negative staining electron microscopy dyes protein with a heavy metal resulting in a high contrast image of molecules. However, a drawback is that it may cause deformation of the sample as the result of dyeing and drying. Cryo-electron microscopy is used to observe samples at liquid nitrogen temperatures after lightly embedding them in ice to examine structures in a more natural state. However, the method uses the difference in electron density between water and protein to examine samples, so the signal-to-noise ratio is low and it is necessary to average numerous images. Over 10 thousand images were collected, and the projection angle was calculated for each image from the relative positions of the structures to recreate them in three dimensions. This allowed us to successfully recreate the 3-dimensional structure of dynein c with a resolution of approximately 2 nm[19] (Fig. 5).

The position of the linker is not always the same before the exertion of force, so it did not show up clearly on the average image. However, examination of the area where the linker is most likely to be found reveals that it is bent near AAA1 and is seen moving its tip massively (Fig. 5B). After exerting a force, we clearly observed the linker moving across the AAA ring. The image taken after exertion of force suggested that the area around the joint between the linker and the tail, and the AAA4 domain are bonded (Fig. 5: Orange arrow). This kind of bonding could not be seen before the exertion of force, but instead the images suggested interaction between the base of the linker and the AAA2 domain (Fig. 5: Blue arrow), with this interaction between the linker and AAA ring shifting at the time a force is exerted. We also observed a change in the shape of the AAA ring. Compared to the image after exertion of force, the molecule before exertion of force is compact, and the two gaps between AAA1 and AAA2, and AAA5 and AAA6, observed after exertion of force, were not seen before (Fig. 5: Green arrow). These were the kinds of 3-dimensional changes we observed in the shape of the dynein head at the time of force exertion. The linker in

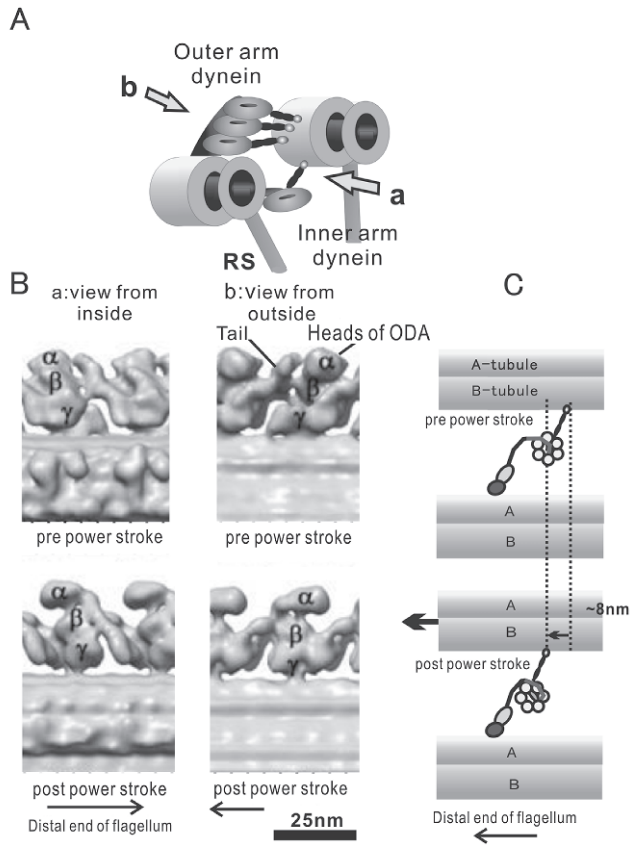


Fig. 6 The structural changes in dynein within the flagellum
A: The perspectives.
B: The 3-dimensional structure of outer-arm dynein before and after exertion of force.
C: A schematic diagram of the movement of the dynein head.

particular contributes greatly to the generation of force, so we believe that the clarification of its movement is a highly significant result.

So how does dynein actually change shape within the flagellum? We observed this under electron beam tomography at the time of force exertion (Fig. 6). Like dynein purified in isolation, it was confirmed that the dynein head rotates relative to its tail inside the flagellum, too. However, the kind of change involving the swinging of the stalk could not be observed, and the molecules were found to move approximately 8 nm in parallel along the microtubule as they exert a force (Fig. 6B, C). In conclusion, it was discovered that dynein inside the flagellum moves its head in a way as if rotating to wind in the stalk, just like a winch[13] (Fig. 6C).

2.3 The mechanical properties of flagellar dynein

Apart from flagellar movement, dynein plays important roles in a variety of intracellular movements including axonal transport in nerve cells, substance transport within the Golgi body in the cytoplasm, etc. Cytoplasmic dynein has been shown to move along the microtubule by the two dynein molecules alternately bonding and grasping firmly onto it as if they were two hands moving along a rope[20]. This pattern of movement is believed to be the result of a few molecules of dynein bound to follicles evolving to move along the microtubule over long distances without letting it go.

Compared to cytoplasmic dynein, the relative portion of time spent in a state of bonding firmly with the microtubule and exerting a force during the cycle of generating movement is small in flagellar dynein. This characteristic is thought to reduce internal friction enabling the microtubule to slide quickly in order to generate rapid sliding between peripheral microtubules of the flagellum with the cooperation of many molecules. The skeletal muscle, myosin, is an example of a motor that generates fast sliding action with the cooperation of many molecules. The mechanical properties of flagellar dynein were originally thought to be the same as those of the skeletal muscle myosin, from its characteristic of numerous molecules being involved in generating the sliding motion. However, an examination of the mechanical properties of flagellar dynein allowed us to discover a property not found in the skeletal muscle, myosin[21][22] (Fig. 7). This was that flagellar dynein, or the inner-arm dynein in particular, demonstrates high processivity in which the protein motor does not detach from the rail made of actin, microtubules, etc., and moves in multiple steps. A single molecule of *Chlamydomonas* inner-arm dynein c, which demonstrates high processivity, was attached to a microscopic bead and trapped by optical tweezers to observe its movement. This revealed that it moves along the microtubule in steps as shown in Fig. 5B. When the size of steps were measured, they were found to be exactly the same as the size of tubulin at 8 nm. It has been suggested that dynein repeatedly bonds and detaches itself as it feels its way along the latticework of microtubules. Dynein c shows backward movement in which it moves 2 or 3 steps back, and the frequency of this phenomenon increases as the load is increased. This load-dependent reaction is unique to dynein, and perhaps it contributes in some way to feedback in flagellar movement.

As it can be seen in Fig. 3, numerous varieties of dynein coexist within the flagellum. It has been reported that the motor activity of each type of dynein differs. *In vitro* motion assays show that the different types of dynein generate microtubule sliding at different speeds. The coexistence of flagellar dynein with different motor

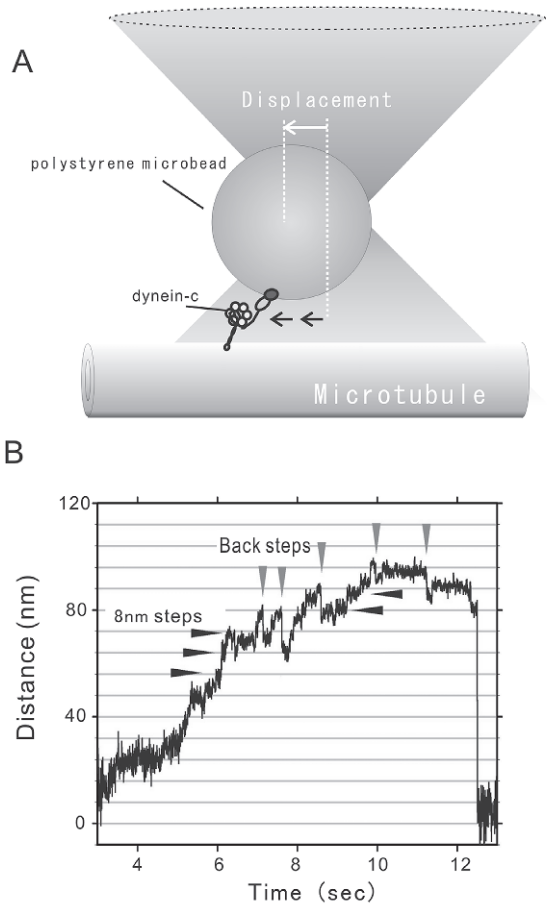


Fig. 7 Measuring the movement of a single molecule of inner-arm dynein c

A: A schematic diagram of the experiment. A small number of dynein molecules are bonded to a microscopic polystyrene bead, and optical tweezers are used to trap the bead. They are then interacted with microtubules in the presence of ATP.

B: Tracking changes in the bead (modification of diagram in reference material[21]). The data revealed 8 nm steps (horizontal arrows) and a backward motion (downward pointing arrows).

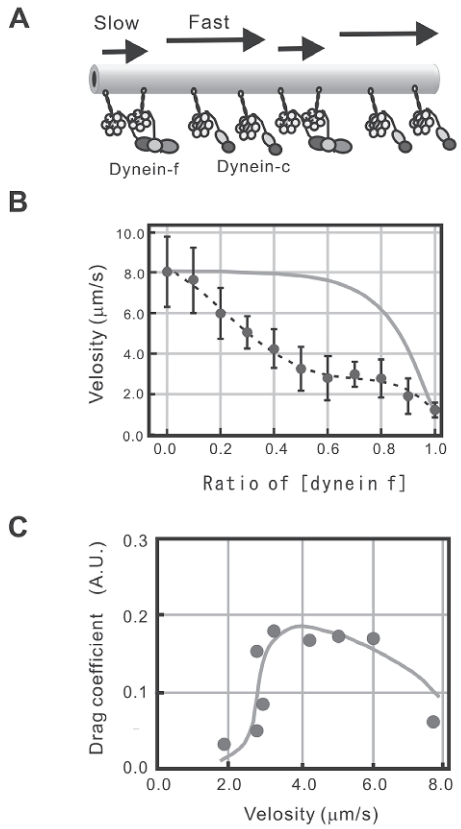


Fig. 8 The gliding speed of microtubules moving across the surface of glass on which inner-arm dynein c and f were mixed in a variety of ratios in *in vitro* assays

A: A schematic diagram of the experiment. Multiple dynein molecules were made to interact with one microtubule.

B: The changes in gliding speed of microtubules relative to the ratio of dynein c to f. The solid gray line indicates changes in the gliding speed of the microtubule when only dynein c is used, but its concentration is changed. The difference between this and the results obtained from experiments in which dynein c was mixed with dynein f is due to resistance.

C: The coefficient of viscous resistance per molecule of dynein f was calculated and plotted against the sliding speed of the microtubule. Calculations were made using the relationship between dynein c and speed (B and C are modifications of graphs in reference material[22]).

activities in a single peripheral microtubule may at first seem extremely inefficient. Slow sliding dynein will only slow down those that slide microtubules quickly. Mixing fast sliding dynein c with slow sliding dynein f on a glass substrate and making them interact with microtubules to recreate sliding movement reveals that dynein f actually works as a resistance to the movement of dynein c (Fig. 8B). However, when the force acting on dynein f becomes strong and it exceeds a certain threshold level, the dynein f suddenly detaches from the microtubule and stops resisting its movement[22] (Fig. 8C). From these observations it is believed that in flagellar movement, the numerous varieties of dynein have internal mechanisms for detaching themselves from the microtubules so that they do not slow down the movement of other dynein molecules when they are not needed. This is because of the difference, for example, in dynein molecules used in slow, large microtubule movements, and those used in less powerful, but faster movement. It could also be that the presence of dynein that resists the sliding motion assists in the bending of the flagellum.

2.4 Structural change in the dynein tail in the adjustment of flagellar movement

Ca^{2+} works as an adjustment factor in a variety of functions within the cell, and it works as a signal to change the undulating motion of flagella and cilia. In

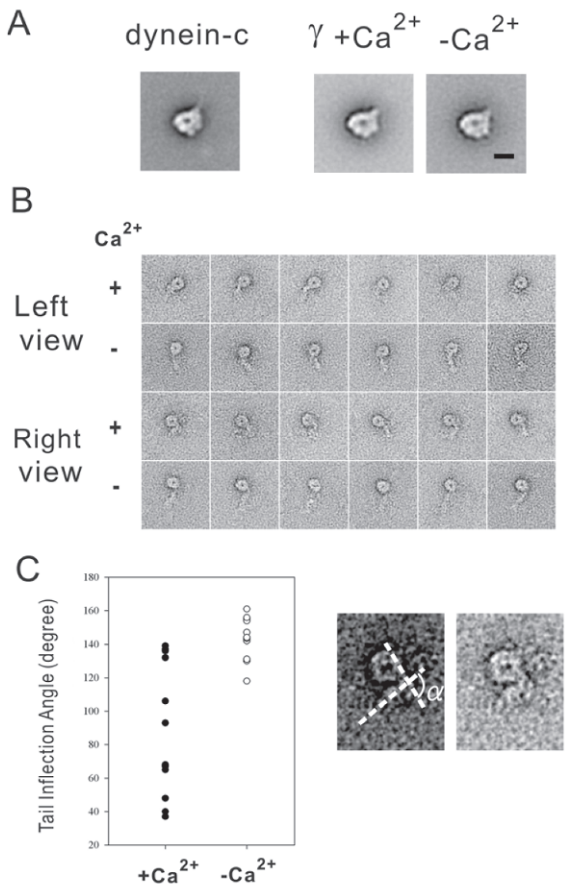


Fig. 9 Changes to the angle of the outer-arm dynein *γ* tail by calcium ions

A: Comparison of the molecular shape of the head in the presence/absence of calcium. The molecular shape of the head of the outer-arm dynein γ changed little even in the presence of calcium, and its shape was similar to the shape of other dynein molecules. Based on the image of the head, the images of molecules were aligned, and classified into groups with similar shapes. Images within the same group were then averaged. The images shown here are of dynein molecules with the tails and stalks positioned to the right of the head.

B: Average images classified into groups based on the shape of their tails. Some were seen with tails bent greatly in the presence of calcium.

C: Measuring the angle of the bend in the tail (modified and quoted from reference material[23]).

Chlamydomonas, a high Ca^{2+} concentration (pCa of less than 5) changes the beat of the flagellum from an asymmetric one to a symmetric one, causing the cell to retreat. The outer-arm dynein *γ* heavy chain has the mobility of a *γ* heavy chain deficient strain, and it is bonded with a light chain similar to calmodulin, suggesting its relationship to changes in undulation patterns by Ca^{2+}. We used negative staining electron microscopy and single-particle image processing to examine the effects of Ca^{2+} ions on the molecular configuration of the outer-arm dynein *γ* heavy chain. As a result, no changes were observed in the head structure upon increasing the concentration of Ca^{2+}. But a bend in the tail was slight at low Ca^{2+} concentrations, while it became more pronounced at high Ca^{2+} concentrations[23] (Fig. 9). These results of observations indicate the possibility that the change in shape of the dynein tail is generally used in adjusting the movement of the flagellum. The results also show the importance of the spatial positioning of the dynein heads within the flagellum in flagellar movement.

Future prospects for the research

The crystalline structure of cytoplasmic dynein in the state after exertion of force has recently been determined. This has enabled discussions on structural changes in dynein at the atomic coordinate level. The 3-dimensional structure of the inside of the flagellum is also on the verge of being elucidated. However, a lot has yet to be elucidated on how this structure changes, how the molecules are joined, and how they move during actual flagellar movement. Great expectations are held for further technological development in these areas.

Advances in biotechnology, nanofabrication and other nanotechnology are making it possible to manipulate cell organelles such as flagella, or build model experimental systems that mimic biological systems. We are on the verge of further building on this technology to find practical applications for them.

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