Review

Tubulin-dynein system in flagellar and ciliary movement

By Hideo MOHRI,[*1,*2,†] Kazuo INABA,[*3] Sumio ISHIJIMA[*4] and Shoji A. BABA[*5]

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Abstract: Eukaryotic flagella and cilia have attracted the attention of many researchers over the last century, since they are highly arranged organelles and show sophisticated bending movements. Two important cytoskeletal and motor proteins, tubulin and dynein, were first found and described in flagella and cilia. Half a century has passed since the discovery of these two proteins, and much information has been accumulated on their molecular structures and their roles in the mechanism of microtubule sliding, as well as on the architecture, the mechanism of bending movement and the regulation and signal transduction in flagella and cilia. Historical background and the recent advance in this field are described.

Keywords: tubulin, dynein, flagella, cilia, sperm motility, microtubule

Historical background

A half century ago, the actomyosin (actin-myosin) system was considered responsible for all forms of motility shown by eukaryotes, including muscle contraction, amoeboid movement, cytoplasmic streaming, cell division, flagellar and ciliary movement, and axonal flow, and there was no knowledge of any other systems involved in these functions. In 1945 Engelhardt described a myosin-like ATPase protein, named "spermosin," from bull sperm flagella,[1),*] although his group had failed to obtain actin. In the years following this report, many researchers examined the presence of myosin and actin in flagella and cilia. Active ATPase activity was indeed observed in these materials, although the ATPase properties of isolated flagella from marine spermatozoa were somewhat different from those of muscle myosin.[3),4)] Nobody, however, had succeeded in isolating actin, myosin or actomyosin from flagella or cilia or even in extracting the ATPase protein from these materials until the early 1960s. As for the detailed ultrastructure of flagella and cilia, the present image of a so-called "9+2" structure of the flagellar and ciliary axoneme was first revealed in 1959 by Afzelius, who described "arms" and "spokes" attaching to the outer doublet microtubules and also proposed a numbering system for the outer doublets[5)] (See Fig. 1[6)]).

There was a breakthrough in this field in 1963, when Gibbons isolated the ATPase protein from *Tetrahymena* cilia after removal of the ciliary membrane and indicated that the protein constitutes the arms.[7)] Since the ATPase showed properties different from myosin and more like the properties of isolated sperm flagella, he gave the name "dynein" to this new protein.[8)] In subsequent studies, the notion that dynein made up the arms was confirmed by using an anti-dynein antibody which inhibited its ATPase activity as well as the reactivation of demembranated sperm models and microtubule sliding.[9),10)] It was found that dynein molecule, especially from the outer arms, of protists consists of three heavy chains with three corresponding "heads",[11)] while that of animals consists of only two heavy chains with two corresponding heads,[12)] together with several intermediate chains and light chains. As will be described presently, the inner arms are not identical with the outer arms as postulated earlier and consist of various dynein molecules with

[*1]   Emeritus Professor, University of Tokyo, Tokyo, Japan.
[*2]   Emeritus Professor, National Institute for Basic Biology, Aichi, Japan.
[*3]   Shimoda Marine Research Center, University of Tsukuba, Shizuoka, Japan.
[*4]   Tokyo Institute of Technology, Tokyo, Japan.
[*5]   Emeritus Professor, Ochanomizu University, Tokyo, Japan.
[†]   Correspondence should be addressed: H. Mohri, Emeritus Professor, University of Tokyo, 2-22-18 Sakuradai, Nerima-ku, Tokyo 176-0002, Japan (e-mail: mohrih@jt6.so-net.ne.jp).
*   The name "spermosin" is now used for a sperm acrosin-like enzyme.[2)]
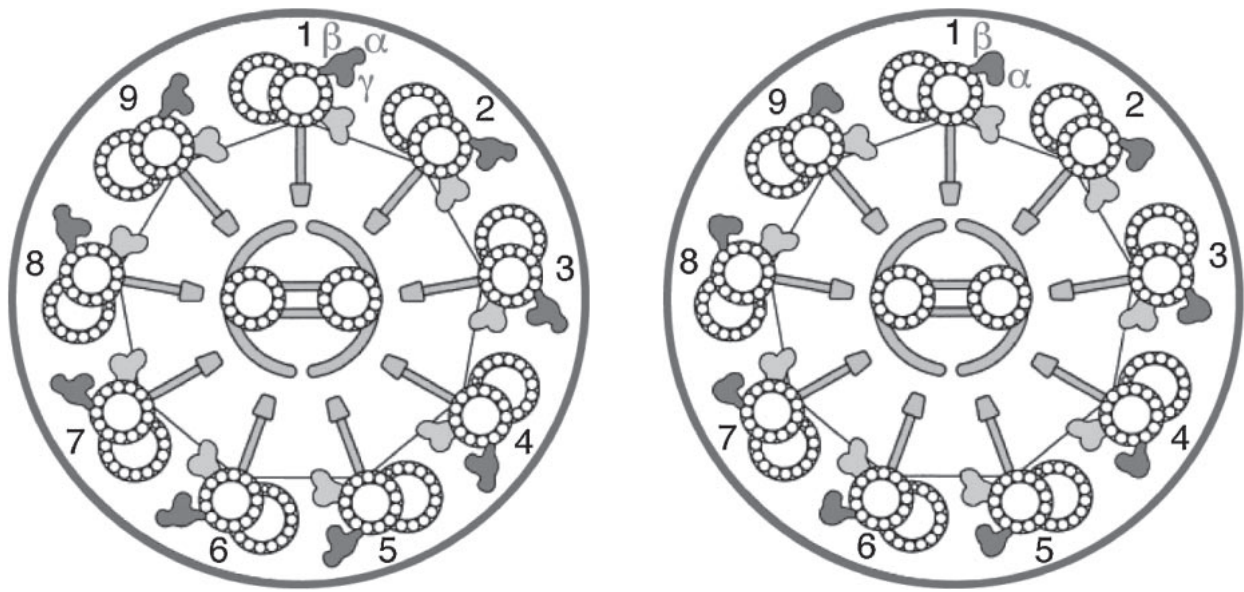
Fig. 1.   Schematic cross-sections of flagellum and cilium in protists and lower plants (left) and in choanoflagellates and animals (right), modified from Ref. 6.

one or two heads. The amino acid sequence of the dynein heavy chain was first determined by Ogawa and by Gibbons' group independently with the β-chain of the outer arm dynein from sea urchin.[13),14)]

Then a question arose as to which protein would be the counterpart of dynein in the way that actin was the counterpart to myosin in muscle. Among the components of the axoneme "9+2" microtubules would be a plausible candidate for the structure corresponding to actin filaments in muscle. After various analyses and a comparison between actin and the main constituent of microtubules, Mohri concluded that the sought protein should be a novel protein and named it "tubulin" in 1968.[15)–17)] The tubulin molecule is a heterodimer of α- and β-tubulin, binds to GTP or GDP and is post-translationally modified by phosphorylation, tyrosination, acetylation, glycosylation, glycylation, etc. (See Ref. 18). Because it binds to colchicine and other antimitotic drugs, it was once called colchicine-binding protein.[19)] Reconstitution of microtubules from tubulin dimers was accomplished by Weisenberg in 1972,[20)] which facilitated the purification and further analyses of this protein. The whole sequences of α- and β-tubulin were decoded in 1981.[21),22)] Thus in flagella and cilia, the pair of dynein, a motor protein, and tubulin, a rail or cytoskeletal protein, replaces the pair of myosin and actin in muscle and other motile systems. Later still another motor protein, "kinesin," was found to be involved in microtubule-dependent motility.[23)]

Concerning the mechanism of flagellar and ciliary movement, the idea that it was accomplished by sliding of the outer doublet microtubules with the aid of the arms had already been suggested when the ultrastructures of flagella and cilia were first revealed.[5)] The experimental evidence was obtained by electron microscopical observation of the tips of the mussel gill cilia beating metachronally[24)] and more directly by observing the extrusion of doublet microtubules from the trypsin-treated demembranated axonemes of sea urchin spermatozoa on the addition of ATP.[25)] The reactivation of sperm models by ATP was first achieved by Hoffman-Berling with glycerol-extracted locust sperm in 1955.[26)] Subsequently, the replacement of glycerol with Triton X-100 greatly facilitated the reactivation of flagellar and ciliary models.[27),28)] A local application of ATP to the demembranated sea urchin sperm by iontophoresis revealed that local active sliding of outer doublets could be converted into bending.[29)] In addition, a useful motility assay was introduced by observing the sliding of microtubules on dynein molecules absorbed on a glass surface, as in the case of kinesin or heavy meromyosin.[30)]

Flagellar and ciliary components

As described in the previous section, the sperm model demembranated with Triton X-100 reproduced normal flagellar bend propagation, indicating that the cytoskeletal element in flagella, the
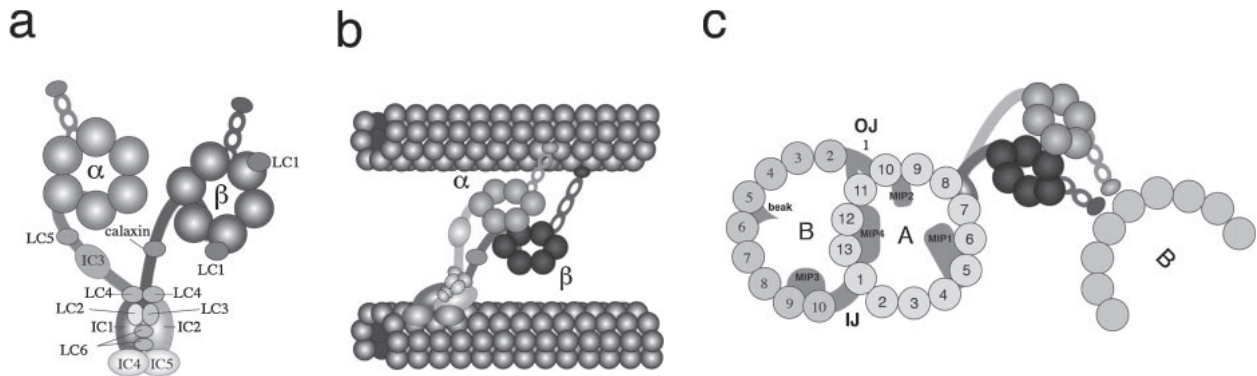
Fig. 2. Structure and *in situ* configuration of outer arm dynein. (a) Schematic subunit structure of outer arm dynein from *Ciona* sperm flagella. The structure is divided into three parts; head, stalk and stem. Outer arm dynein is composed of two heavy chains (α and β), five intermediate chains (ICs) and six light chains (LCs). (b) Longitudinal configuration of outer arm dynein bound to doublet microtubules. (c) Structure of the outer doublet microtubule and *in situ* configuration of outer arm dynein. The outer and inner junction (OJ and IJ) connect the A- and B-tubules. Microtubule inner proteins (MIP) and the "beak" structure are associated with the internal wall of the A- or B-tubule. Note that the *Ciona* α or β heavy chain corresponds to the sea urchin β or α and *Chlamydomonas* β/α or γ, respectively.

axonemes (Fig. 1), is responsible for the elemental property of flagellar motility. The motive force of flagellar motility is exerted by axonemal dyneins, which are phylogenetically distinct from cytoplasmic dyneins. Studies on the molecular structure of outer arm dynein have been more advanced than those on the inner arm dynein because the former can be easily purified from the axonemes[31] and is present as a single molecular species in flagella and cilia. The molecular structures of outer arm dyneins have been extensively studied using sperm flagella from marine invertebrates, because in these flagella the outer arm dynein is selectively extracted from the axonemes by a high salt solution and can be purified simply by sucrose density gradient centrifugation.[31),32] On the other hand, dyneins from flagella and cilia in several protists, such as *Chlamydomonas*, *Tetrahymena* and *Paramecium*, have also been used. In particular, studies on dyneins, as well as other axonemal components, have been extensively carried out using *Chlamydomonas* flagella,[33] because several mutants of this species lacking axonemal components have become available.[34),35] Here we comparatively describe the molecular structures of dyneins and other axonemal components in flagella from sperm and *Chlamydomonas*.

Dyneins.   The dynein arms are observed as projections from A-tubules of outer doublet microtubules. Based on their positions, they are called outer and inner arms. The outer arms are considered to be single molecular species arranged on each doublet microtubule with 24 nm intervals, whereas the inner arms contain multiple molecular species. The outer arm dynein of metazoan sperm is comprised of two heavy chains (HC; α, β, $\sim$500 kDa), three to five intermediate chains (IC; 60–120 kDa), and six light chains (LC; 8–30 kDa)[36),37] (Fig. 2a). *Chlamydomonas* outer arm dynein contains three heavy chains (α, β, γ), two intermediate chains (WD-repeat) and ten light chains.[38] Dynein heavy chains are members of the ATPase associated with diverse cellular activities (AAA) superfamily. Each of the six AAA domains constitutes a subdomain in the head part of dynein, forming a ring-like structure of the dynein head (Fig. 2a). The coiled-coil domain present between the fourth and fifth AAA motif forms a small stalk with a globular domain that acts as the ATP-sensitive microtubule-binding site (MTBD).[39] Recent advances in cryoEM and tomographic observation have shown that multiple AAA ring heads are stacked atop each other and the plane of each ring runs through the axis of the adjacent B-tubule (Fig. 2b and 2c).[40),41] Attempts have been made to detect the conformational changes of dynein during the mechano-chemical cycle by using biochemical methods. A study using the limited proteolytic digestion detected large conformational changes of outer arm dynein in the presence of ADP/Vi in sea urchin sperm flagella. The changes are seen not only in the vicinity of the primary ATP hydrolysis site (AAA1) but also throughout all the regions of the dynein heavy chain, including the C-terminal region.[42)–44] Advances in electron microscopy have

allowed visualization of the conformational changes in multiple head rings and in the relative position of the head and stem.[45],[46] It is known that each heavy chain of the outer arm dynein exhibits a distinct ATPase activity and distinct microtubule-sliding activity in both sea urchin sperm flagella[12],[47],[48] and *Tetrahymena* cilia.[49],[50] However, the physiological importance of the multiple heavy chains in outer arm dyneins is still unclear.

The outer arm dynein contains two ICs with WD repeats, which are involved in protein–protein interaction and may play a key role in the assembly and binding of dynein on the A-tubule. An IC with thioredoxin and nucleoside diphosphate kinase (TNDK-IC) motifs is present in metazoan sperm, but not in *Chlamydomonas* flagella.[32],[51] The structure for the assembly and anchoring of outer arm dynein to the doublet microtubules at regular intervals (24 nm) is called the outer dynein arm docking complex (ODA-DC).[52] This complex is composed of three polypeptides (DC1-DC3).[53]–[55] DC1 and DC2 are coiled-coil proteins, and likely serve in scaling regular intervals for dyneins. DC3 has four EF-hand motifs that may bind Ca^{2+} and thereby play roles in the regulation of outer arm dynein. In tunicate *Ciona*, salmonid fish and molluscs, outer arm dynein contains two or three other ICs. These additional ICs are shown to be coiled-coil proteins with sequence similarities to *Chlamydomonas* DC2.[56],[57]

Outer arm dynein LCs are thought to be involved in the assembly and regulation of dynein motor function.[57] In *Ciona* and sea urchin sperm, six distinct proteins have been identified as outer arm dynein LCs. Two of these molecules show homology to t-complex testis-expressed proteins (Tctex1 and Tctex2), which are involved in transmission ratio distortion in mice.[58],[59] Although the *Chlamydomonas* outer arm dynein contains Ca^{2+}-binding LC4, no Ca^{2+} binding protein has been identified as a subunit of outer arm dynein in metazoa. A 25-kDa protein with sequence similarity to the neuronal calcium sensor (NCS) has recently been identified in association with the *Ciona* outer arm dynein and designated calaxin. Calaxin has orthologs in metazoan species, including mice and human, and is thought to participate in the regulation of flagellar wave asymmetry.[60]

Inner arm dyneins include multiple molecular species with one or two heavy chains. In *Chlamydomonas* they contain at least seven species (named a–g dyneins).[61] The f inner arm dynein (also called I1)

contains IC140, IC138, and IC97, the 14-kDa Tctex1 LC, and an 8-kDa LC.[62],[63] Another type of inner arm dynein also contains p28, actin, centrin, and Tctex1 and a few unidentified proteins as LCs. *Chlamydomonas* IC138 is phosphorylated/dephosphorylated through a kinase/phosphatase system present in the radial spoke and central pair in response to changes in motility.[64],[65] A homolog of IC140 (Ci-IC116) was identified in *Ciona* sperm flagella. Ci-IC116 is dephosphorylated at activation of sperm motility.[66]

Tubulins and microtubules. Microtubules are tubular cytoskeletal elements polymerized from α- and β-tubulin heterodimers. The doublet microtubule is a microtubule form uniquely seen in flagella and cilia. Although the mechanism for the formation of doublet microtubules is still unclear, their structural and biochemical characteristics have been partly clarified. The doublet consists of A- and B-tubules: the A-tubules contain 13 tubulin protofilaments (A1–A13) and the B-tubules are incomplete tubules of 10 protofilaments (B1–B10).[67] Several structures were observed inside the A- and B-tubules. Extraction of doublet microtubules with the detergent Sarkosyl solubilizes tubulins and results in the isolation of remnant filamentous structures called "ribbons". One of the protein components of ribbons, tektins, were first isolated from the flagella of sea urchin sperm.[68] Other components of ribbons include some Ca^{2+}-binding proteins.[69] Cryo-electron microscopy observation has recently revealed the detailed structure of doublet microtubules. The B-tubules contain the inner junction and the outer junction between the A- and B-tubules.[70] Microtubule inner proteins (MIPs) are observed on the inside of the A- and B-tubules. According to a recent observation, MIP1, MIP2 and MIP4 are associated with the A-tubules, and MIP3 is associated with the B-tubules, with a certain longitudinal periodicity. A projection called a "beak" structure is observed within the B-tubules of *Chlamydomonas* doublet microtubules 1, 5 and 6 (Fig. 2c).[71]

The presence of multiple molecular species of both α- and β-tubulin was first demonstrated in marine sperm flagella.[72] The heterogeneity of α- and β-tubulin is important for the construction and function of outer doublet microtubules in the axonemes. It first arose from multiple genes of α- and β-tubulin. Vertebrate genomes encode six to seven genes for both α- and β-tubulin.[73] Second, axonemal tubulins undergo several types of post-translational modifications, including acetylation of

α-tubulin and polyglutamylation and polyglycylation of both α- and β-tubulin.[74),75)] Recent research on the tubulin tyrosine ligase like (TTLL) family of proteins has revealed their significant role in the assembly and regulation of axonemes.[76)] For example, knockdown of TTLL3 tubulin glycin ligase results in shortening of cilia in *Tetrahymena*.[77)] Knockout of TTLL1 (PGs3) results in a shortening of and asymmetry abnormalities of mouse airway cilia.[78)]

Radial spokes, central pair and other axonemal structures. Radial spokes (RS) and the central pair (CP) are the structures inside the outer rows of doublet microtubules and appear to modulate dynein activities. Several lines of evidence indicate that the CP/RS system is not principally required for flagellar bend formation and propagation. Rather, CP/RS are involved in the signaling for controlling the properties of bend and for modifying motility in response to specific signals.[79),80)] The regulation of dynein arms by CP/RS involves protein phosphorylation/dephosphorylation. Approximately 23 proteins ($1-350$ kDa) comprise RS in *Chlamydomonas* flagella. These proteins contain those for Ca^{2+}/CaM-dependent or cAMP-dependent signaling pathway, such as a protein with the A-kinase anchor protein (AKAP) domain PRS3.[80)] Similarly to RS, CP also contains domains for molecular assembly and signaling, including CaM, CaM-binding protein Pcdp1, AKAP-binding protein AAT-1, and a protein with the adenylate kinase domain Cpc1.[81)]

The dynein regulatory complex (DRC) is the structure at the junction between the radial spokes and inner arm dynein. DRC is thought to be important in anchoring inner arm dynein and for connecting the inner arms to the radial spokes. Seven polypeptides ($29-192$ kDa) have been proposed as DRC components in *Chlamydomonas*,[82)] but neither molecular characterization nor the presence of homologs in metazoan sperm have been reported.

Nexin links or interdoublet links contribute to the elastic resistance involved in the conversion of microtubule sliding into axonemal bending. It has been proposed that the interdoublet link is the major part of the DRC.[83)] Actually, recent analysis by electron tomography revealed that one end of the *Chlamydomonas* interdoublet link formed a bifurcated attachment to the B tubule; the other was attached to the A tubule close to the base of DRC. Link structures are also present between outer arms and between outer- and inner-arm dyneins.[84)] These link structures suggest a mechanism for coordinating inner and outer dynein arm activities.

Sliding of outer doublet microtubules

The idea of the sliding of outer doublet microtubules was first proposed by Afzelius[5)] based on the analogy of the arms projecting from the doublet microtubules with the cross-bridges between the myofilaments of the muscle cells. This idea was experimentally tested and it was confirmed that the microtubules themselves did not shorten during beating, and thus that sliding of the outer doublet microtubules occurred.[24),85)] Extrusion of the doublet microtubules from sea urchin sperm axonemes was demonstrated using segments of the sperm axoneme that were treated with trypsin and then exposed to ATP.[25)] Using this method, the sliding direction of the doublet microtubules was determined; that is, the dynein arms on tubule A of a doublet microtubule push the B tubule of the adjacent doublet microtubule toward the tip of the cilium.[86)] However, the opposite direction of sliding was later proposed in sea urchin spermatozoa at low levels of calcium/calmodulin[87)] and in mammalian spermatozoa.[88)]

The sliding of doublet microtubules was influenced by various factors, including the number of dynein arms on the doublet microtubule, ATP, Ca^{2+} and cAMP. When the dynein arms were removed from the axoneme, the velocity of the microtubule sliding was proportional to the total number of dynein arms present.[89)–91)] The sliding velocity was also directly related to $MgATP^{2-}$,[90)–92)] and its maximum value was approximately $14\,\mu m/sec$. The force produced by sliding was measured and found to be approximately $1\,pN$ per dynein arm.[93)] However, the force developed by sliding did not change with the sliding velocity.[94)] This insensitivity of force to the sliding velocity was previously pointed out by Hiramoto,[95)] and its load dependency was explained by the relation of the sliding velocity largely to stiffness of the sperm flagella, *e.g.*, those having different diameters.[96),97)] Ca^{2+} decreased the sliding velocity.[98),99)] Furthermore, Ca^{2+} affected the pattern of sliding of the doublet microtubules extruding from the axoneme; that is, at low Ca^{2+} concentrations, most of the axonemes separated into individual doublet microtubules, but at high Ca^{2+} concentrations, most of them separated into two microtubule bundles.[87),99)] The former pattern has never been seen before.[100)] The Ca^{2+} sensitivity is removed by trypsin treatment from the basic sliding machinery in *Paramecium* axonemes.[101)] The effect of pH, inhibitory agents and cAMP on the microtubule sliding has been investigated using mammalian

spermatozoa.[102]–[104] Strangely enough, the outer doublet microtubules activated by Ca^{2+} were different between the sea urchin and mammalian spermatozoa[105] because the doublet microtubules of 6–7, 7–8 and 8–9 were activated by Ca^{2+} in the sea urchin[99] while those of 2–3, 3–4 and 4–5 were slid by Ca^{2+} in rat spermatozoa.[102] It should be noted here that the principal bend defined by Woolley[106] in hamster sperm flagella corresponds to the reverse bend in sea urchin and starfish sperm flagella and *vice versa.*[105],[107] The conversion of sliding of the doublet microtubules to bending was demonstrated if there was some resistance to the microtubule sliding.[29] This sliding-to-bending conversion due to resistance has been confirmed using a fluorescent reagent that inhibits the microtubule sliding.[108]

Regulation of microtubule sliding during flagellar and ciliary beating

The sliding between the outer doublet microtubules of the axoneme must be modulated to generate the various patterns of the flagellar and ciliary beating. To clarify this regulatory mechanism, several approaches have been taken, including the computer simulation of the beating pattern in order to test the hypothesis of the regulatory mechanism and estimation of microtubule sliding from the flagellar and ciliary beating. By reconstructing the beating pattern of *Paramecium* cilium using a computer, Sugino and Naitoh[109] estimated the behavior of the microtubule sliding; that is, the sliding between the doublets 1, 2, 3, and 4 on one side of the cilium is active during the effective stroke while the activity switches to the doublets 6, 7, 8, and 9 on the other side during the recovery stroke. Furthermore, various hypotheses to explain the planar beating of sperm flagella have been tested by computer simulation, including the "curvature control" hypothesis, "geometric clutch" hypothesis, and "sliding control" hypothesis.[110],[111] However, none of these hypotheses has been widely accepted,[111],[112] possibly because the flagellar beating of spermatozoa is fundamentally three-dimensional (see below).

Hiramoto and Baba[113] developed a method for quantitatively evaluating the tangential angle (which is also referred to as the shear angle or bend angle) at any point on the flagellum from a photograph of the flagellar wave, and found that this angle of the flagellum at any distance from the base is expressed by a sine function of time plus a constant. To explain this fact, they proposed a model in which the active force required to generate sliding is propagated along and around the flagellar axoneme. This model has been strongly supported by experimental evidence obtained from the analyses of the rotational movements of spermatozoa.[114],[115] Furthermore, the helical waves were converted from the planar waves in the tunicate and sea urchin sperm flagella under certain high viscosity conditions.[116],[117] These findings suggest that the flagellar beating of the tunicate and sea urchin spermatozoa is fundamentally three-dimensional due to the intrinsic asymmetrical geometry of the sperm axoneme, and that the almost planar beating usually observed in these sperm flagella results from the additional regulation.[117] One of the most plausible candidates for this role is the central complex, because the absence of this structure from the axoneme is associated with helical beating[118] and the stiffness of sea urchin sperm flagella significantly differs between the direction parallel to the beating plane and that perpendicular to it.[119],[120]

A digital image analysis, with accuracy enhanced by Bohboh software,[121],[122] of the flagellar beating of hyperactivated mammalian spermatozoa, which are characterized by large bends at the midpiece and slow beating, revealed that the sliding velocity remained constant before and after hyperactivation, although it varied with the stiffness of the sperm flagella in several species.[96],[97] This fact suggests that the mechanisms regulating the beat frequency and flagellar waveform are tightly dependent on each other. The sliding velocity is kept constant, since it is proportional to the product of the beat frequency and tangential angle.[123] In a wide range of working seen before and after hyperactivation, the beating mode switches from a constant-sliding-extent mode, in which the frequency varies to a greater degree, to a constant-frequency mode, in which the sliding extent (maximum shear angle) varies widely (Fig. 3).[87],[123]

Initiation and activation of movement

Spermatozoa remain immotile or virtually immotile in the testis, undiluted semen or the male reproductive tract. They initiate flagellar movement at spawning or ejaculation into an aquatic environment such as seawater, fresh water or the seminal plasma and the fluid of the female reproductive tract. Furthermore, for instance in mammals, they are activated to show progressive forward movement and then are hyperactivated to exhibit vigorous whiplash-like movement in the female reproductive tract. In addition, spermatozoa of many species, including
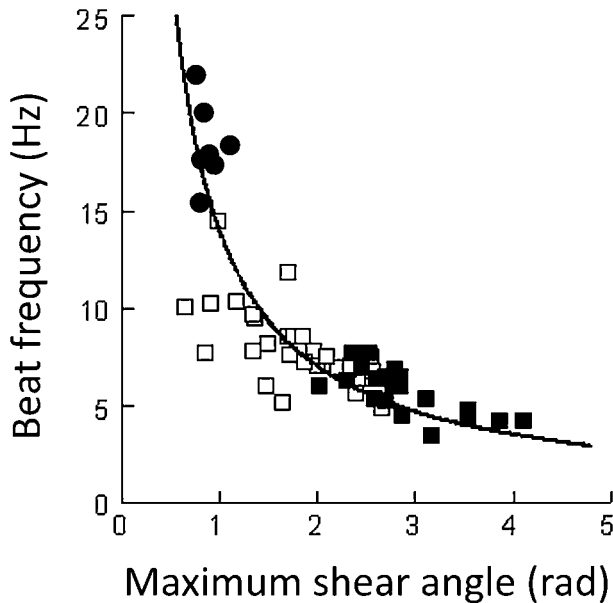
Fig. 3. Relationship between beat frequency and maximum shear angle. The flagellar beating of activated (●), transient (□) and hyperactivated (■) golden hamster spermatozoa is shown. The line is a least squares regression line, given by $f = 14/\theta_{max}$, where f is the beat frequency and θ_{max} is the maximum shear angle. Since the sliding velocity is proportional to $f \cdot \theta_{max}$, this equation clearly shows the constant rate of microtubule sliding before and after hyperactivation.

plants such as ferns and mosses, show chemotactic behavior in response to a specific substance from the egg or its surroundings. Thus spermatozoa are one of the best materials to elucidate the mechanisms underlying the initiation and such modifications of flagellar movement.

Initiation of flagellar movement has been studied with various spermatozoa that are immotile in the testis, such as those in marine invertebrates and fishes, or in the cauda epididymis, as in mammals before ejaculation. Various factors induce their motility among different animals, although there would be common cascade(s) which lead to final activation of the tubulin-dynein system.[124]−[126]

Dilution of sea urchin semen ("dry sperm") with seawater evokes the initial burst of sperm respiration and vigorous flagellar movement. Since CO_2 inhibits both respiration and motility of the spermatozoa,[127] it is plausible that release from the high P_{CO_2} in the testis or in dry sperm by dilution with seawater causes an increase in intracellular pH (pH_i) which in turn activates dynein ATPase activity.[128] In fact, a temporary decrease in the content of ATP as well as that of phosphagen has been observed immediately after dilution.[129] The initial high respiration rate would be needed for recovering high-energy phosphate compounds by oxidative phosphorylation. The phosphocreatine shuttle is considered to supply ATP produced in mitochondria situated at the proximal end of the flagellum to its distal end.[130] In flatfish species, CO_2 clearly shows a strong inhibitory effect on sperm motility. This inhibition is reversible and can be reproduced with demembranated sperm by bicarbonate ion. Inhibition by CO_2 is mediated by a carbonic anhydrase, which catalizes the conversion between CO_2 and bicarbonate.[131] An increase in pH_i is also caused by the efflux of H^+ through a Na^+/H^+ exchange, resulting in the activation of dyneins.[132] Full activation of dyneins appears to require cyclic AMP-dependent phosphorylation of specific proteins at the initiation and activation of sperm motility.[133]

In teleost fishes, Morisawa's group found that high osmotic pressure for marine teleost sperm and, conversely, low osmotic pressure for fresh water teleost sperm induce the initiation of flagellar motility, while lowering of the high K^+ concentration in the seminal plasma initiates the motility of salmonid fishes.[126),134),135] In salmonid fishes, the efflux of K^+ induces hyperpolarization of the plasma membrane, which would increase intracellular Ca^{2+} from both the intracellular Ca^{2+} store and the environment. Since cAMP is required for the initiation of motility of the demembranated spermatozoa, increased Ca^{2+} would activate adenyl cyclase, resulting in an increase of cAMP, which in turn would activate A kinase (PKA).[124] PKA then activates a tyrosine kinase (PTK) which phosphorylates the tyrosine residue of a 15K protein (the motility initiating phosphoprotein (MOPP)). MOPP resides in the basal region of the flagellum and is involved in the initial formation of the flagellar wave.[122] It is also known that at the motility initiation of salmon, a Tctex2-related light chain of the outer arm dynein is phosphorylated by PKA, and PKA is regulated by proteasomes localizing near the outer arms.[59),136] PKA also shows a similar localization.[137] Herring sperm are activated by a herring sperm activating peptide (HSAP) through proryl endopeptidase.[126] In the cases of other teleosts, changes in external osmolality induce Ca^{2+} influx by opening of the Ca^{2+} channel or the release of Ca^{2+} from the intracellular storage and trigger a Ca^{2+}-dependent, cAMP-independent signal cascade leading to the activation of the flagellar axonemes.[138]

Recently, K$^+$-independent and osmolality-dependent activation was reported in salmonid fish spermatozoa.[139),140)] Since the motility initiation is tightly associated with the transient increase of intracellular Ca^{2+} concentration, it is plausible that a Ca^{2+}-dependent mechanism might be common to the motility activation in teleost sperm.

In mammals, it has been shown that cAMP is an indispensable factor for the initiation of sperm motility.[141)–143)] HCO$_3^-$ in the seminal plasma stimulates both adenylyl cyclase (soluble adenylyl cyclase) and sperm motility and has been postulated to be a factor inducing the motility.[144),145)] Although the roles of both soluble and transmembrane adenylyl cyclases in the activation of sperm motility are controversial, it seems that two types of adenylyl cyclases play distinct roles in the motility activation or chemotaxis.[146)] A major soluble 55–56K protein in mammalian sperm phosphorylated by PKA at the initiation of motility was named axokinin and identified as the type II regulatory subunit of PKA (RII).[141)] ATP synthase and pyruvate dehydrogenase are also included in the proteins phosphorylated at the initiation or activation of motility. As PKA is a serine/threonine kinase, most of the proteins are phosphorylated at serine/threonine residues.

In general, at the initiation and activation of sperm motility, the proteins constituting the motile apparatus and those involved with the production of ATP are the main targets of phosphorylation. Although not yet thoroughly examined, dynein heavy chains are also reported to be phosphorylated.[147),148)] A proteomic approach has identified proteins that are phosphorylated/dephosphorylated upon the activation of sperm motility in *Ciona*[66)] and mammals.[149)] These proteins include not only axonemal proteins but also proteins, such as VCP97 and 14-3-3 proteins, that are potentially important for signal transduction from the plasma membrane to activate axonemes.

Modulation of movement as responces to stimuli

Upon stimulation, such as by chemicals, lights, gravity or mechanical force, protists and sperm often show changes in swimming direction. These changes accompany the symmetry-asymmetry conversion of the flagellar or ciliary waveform. For example, sperm move to the chemoattractants released from an egg of the female genital tract.[129)] From a series of studies using the ascidian *Ciona intestinalis*, it is known that sperm changes motility in response to the gradient of chemoattractants called SAAF.[126)] The sperm movement directed toward the egg is achieved by transient changes of intracellular Ca^{2+} concentration, followed by changes in the waveform asymmetry of sperm flagella.[150),151)] The demembranated sperm model shows an asymmetric waveform in the presence of a higher concentration of Ca^{2+}.[152),153)] The regulation of the flagellar waveform is carried out by Ca^{2+}-binding proteins that likely regulate axonemal dynein. Calaxin binds to the dynein heavy chain in the presence of Ca^{2+} and therefore has been postulated to be responsible for waveform asymmetry during chemotaxis.[60)]

Chlamydomonas shows two behaviors in response to light: phototaxis and photophobic or avoidance response. These responses require Ca^{2+}-mediated modulation of the flagellar waveform.[154)] Phototaxis of *Chlamydomonas* is initiated by sensing through a single eyespot. The relative position of the eyespot to two flagella is always the same, but changes of the position relative to the light cause the difference in the movement of two flagella. The difference in the flagellar movement is caused by Ca^{2+}-dependent modulation of flagellar asymmetry and by activation of flagellar motility. It has been shown that the Ca^{2+}-dependent regulation of the flagellar waveform is conducted by outer arm dynein.[155)–157)] On the other hand, the activation of flagellar beating is regulated by dephosphorylation of a 138 kDa intermediate chain of inner arm dynein.[64),65)]

Maturation and hyperactivation of mammalian spermatozoa

In mammals, the spermatozoa are not yet fully matured either morphologically or functionally even after spermiation from the testis. They gradually acquire motility and fertilizing capacity during the transit through the epididymis. Although the flagella of early spermatids, which are not yet surrounded with a mitochondrial sheath, outer coarse fibers and a fibrous sheath, show an active wave-like motion, the spermatids differentiating to the testicular spermatozoa progressively become immotile.[158)] In the caput epididymis, the spermatozoa are still virtually immotile even after dilution with saline solutions, while in the cauda epididymis they are ready for active progressive movement after ejaculation. In this case, vigorous motility requires extracellular factors such as Ca^{2+} and bicarbonate.[159)]

Furthermore, mammalian sperm are "capacitated" in the female reproductive tract before fertil-
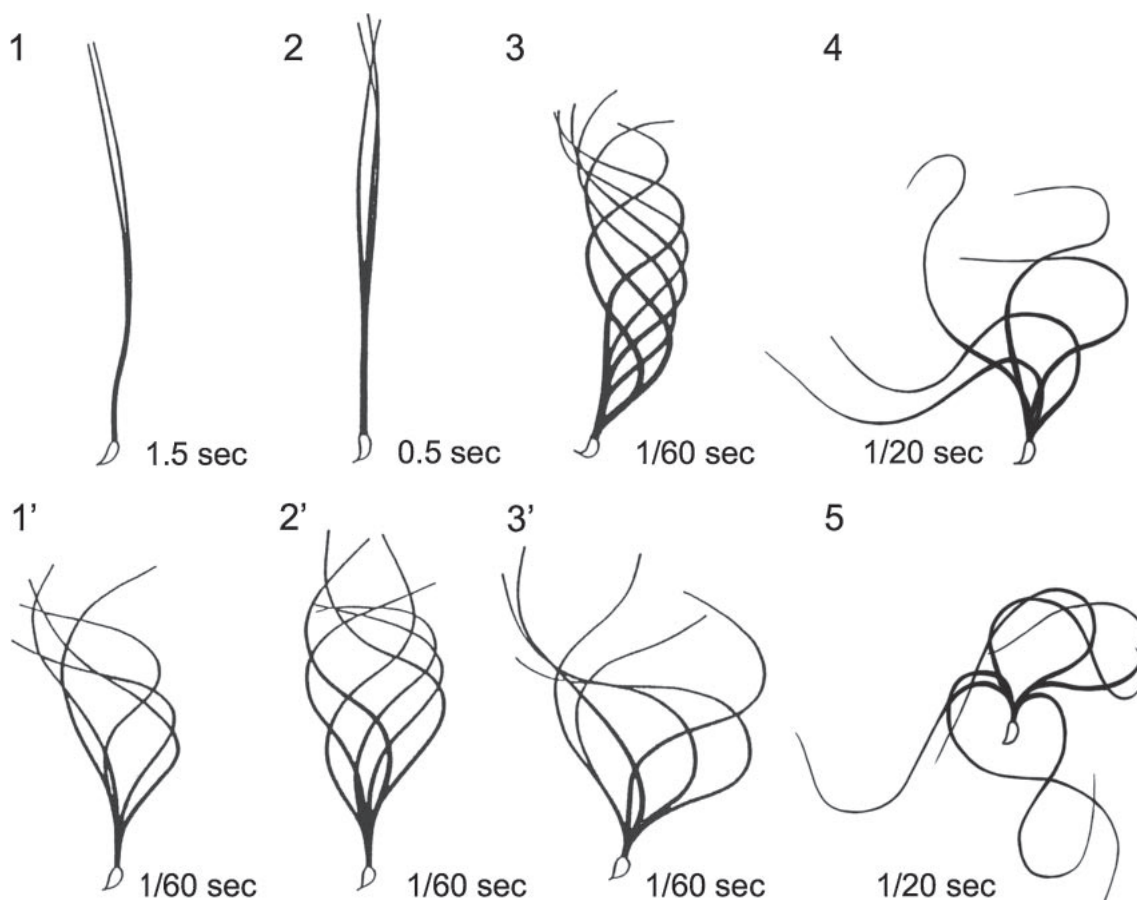
Fig. 4.　Maturation and capacitation of mammalian spermatozoa. The flagellar movement of intact (1–3) and demembranated and ATP-reactivated (1′–3′) testicular, caput epididymal and cauda epididymal golden hamster spermatozoa are shown, respectively. Panels 4 and 5 represent the intact hyperactivated and the intact hyperactivated and acrosome-reacted spermatozoa, respectively. The time indicates the intervals between successive tracings.

izing the egg successfully. The capacitated spermatozoa exhibit a very vigorous movement called "hyperactivation" as well as the acrosome reaction, although capacitation and hyperactivation could occur independently from each other. Hyperactivation is characterized by high amplitude and asymmetrical flagellar bending (see Fig. 4) and is required for passing through the cumulus and zona pellucida and for release of sperm from the oviductal storage reservoir, both of which are prerequisite for fertilization *in vivo*.[160)–162)] In the hyperactivated and acrosome-reacted spermatozoa, a further increase in the curvature of bends, especially at the proximal midpiece, and reduction in beat frequency are observed.[163)]

In our previous computation, the thrust and hydrodynamic power output developed by the flagellum in hyperactivated hamster sperm were twice and 2.5 times those of the flagellum in activated cauda epididymal sperm showing the progressive movement, respectively.[105)] Although the large thrust generated by the flagellum was considered to enable sperm to penetrate through the zona pellucida mechanically,[161),164)] the role of the thrust during the zona penetration is not yet definitely settled. A recent re-examination of this problem with monkey spermatozoa has revealed that there was no difference in the propulsive force, parallel to the longitudinal sperm axis, between the activated and hyperactivated sperm, but that the transverse force, perpendicular to the axis, of the hyperactivated sperm was 2.5 times the propulsive force. Because the beat frequency decreased remarkably during hyperactivation, the slowly oscillating transverse force seems to be most effective for the zona penetration.[165)]

To elucidate the mechanism of such modulations of movement in mammalian sperm, we demembranated hamster sperm of various stages during maturation and capacitation and examined their movement after reactivation by the addition of ATP and other factors.[165),166)] The results revealed that the demembranated models of testicular and caput epididymal sperm showed a movement almost identical with that of intact cauda epididymal and ejaculated spermatozoa, indicating that the motile apparatus, the tubulin-dynein system, is already assembled in the former, while the movement of the models of cauda epididymal or ejaculated sperm was quite similar to that of hyperactivated spermatozoa (Fig. 4).

The flagellar movement of mammalian sperm has at least three steps, initiation, activation and hyperactivation, in contrast to the one-step activation observed in marine invertebrate and fish sperm.[167)] The acquisition of sperm motility during the transit through the epididymis correlates with an increase in cAMP level and pH_i.[168)] As already mentioned, these factors together with Ca^{2+} are considered to evoke signal cascades which would cause the modulation of flagellar movement through phosphorylation and/or dephosphorylation of specific proteins. Such an acquisition of sperm motility as spermatozoa pass from the testis to the sperm duct was also reported in salmonid fishes along with a description of the roles played by cAMP, pH and HCO_3^- in this process.[169)]

It is known that hyperactivation is triggered by Ca^{2+}.[160),161),170)] For instance, both demembranated bull and monkey sperm reactivated in the presence of low concentrations of Ca^{2+} begin to hyperactivate with increasing concentrations of this ion, indicating rather direct effects as the primary second messenger on the 9+2 axoneme, outer coarse fibers and fibrous sheath.[171),172)] High levels of cAMP in the presence of low levels of Ca^{2+} increase the curvature of both the principal and the reverse bend of the flagellum, while Ca^{2+} increases only that of the principal bend, at least in monkey sperm. Hyperactivation requires a higher level of ATP and higher pH than those required for activation.[171)] Although the sperm mitochondria have the ability of oxidative phosphorylation,[173)] glycolysis is reported to mainly supply ATP for flagellar movement in mouse sperm.[174)] Because of the absence of the phosphocreatine shuttle in mammalian sperm, it is postulated that glycolytic machinery would provide ATP throughout the flagellum. In addition, ATP produced by glycolysis

is assumed to be essential for sperm functions, including capacitation and tyrosine phosphorylation.[175),176)] It has been shown that glycolytic enzymes are tightly bound to the fibrous sheath of mouse sperm.[177)]

It is known that increased tyrosine phosphorylation of flagellar proteins is associated with hyperactivated motility in mammalian sperm.[178),179)] Although the sperm PTK is not cAMP-dependent, it is regulated by PKA.[170)] One such tyrosine phosphoprotein is the A-kinase anchoring protein (AKAP), which assembles into the fibrous sheath.[180)] Glycolytic enzymes such as glycerol-3-phosphate dehydrogenase are also targets of tyrosine phosphorylation at capacitation and hyperactivation.[181)] Successive phosphorylation of members of the MAP kinase cascade (RAF/MEK/ERK) has been reported. AKAP is situated in the region downstream of ERK.[182)]

Most of the experiments concerning capacitation and hyperactivation have been done *in vitro* and the physiological factors that trigger hyperactivation *in vivo* have remained to be elucidated for many years. Recently, progesterone was identified as the ligand which causes non-genomic regulation of hyperactivation.[170)] Progesterone enhances CatSper, a Ca^{2+} channel in the plasma membrane of the principal piece of the sperm flagellum, which is synergistically activated by elevation of intracellular pH.[183)] CatSper-deficient mouse sperm cannot exhibit hyperactivation and are infertile.[162)] Progesterone would also bind to its receptor on the acrosome region of the sperm head and cause mobilization of intracellular Ca^{2+} stores.[170)] In both cases the resulting increase of intracellular Ca^{2+} would lead to tyrosine phosphorylation of the proteins necessary for hyperactivation through CAMK and PTK. In addition, it is of interest that estrogen suppresses the hyperactivation enhanced by progesterone and tyrosine phosphorylation.[184)] Melatonin, which binds to its receptor on the sperm mid-piece, controls the level of reactive oxygen species (ROS), including NO produced by the mitochondria. Low concentrations of ROS positively regulate hyperactivation, possibly through the MAPK cascade and tyrosine phosphorylation.[182),185)]

Phylogenetic considerations

Microtubule and tubulin are found in eukaryotes including protists, plants and animals, although it has been revealed that FtsZ, a homolog of tubulin, is involved in the cell division of prokaryotes.[186)]

The same is true with flagella and cilia: these complex organelles are widely distributed among eukaryotes and would be acquired at the very early phase of their evolution.

As mentioned above, the outer arm dynein molecule consists of three heavy chains (three-headed) in protists such as *Tetrahymena* and *Chlamydomonas*, while that of animals such as sea urchins, fish and mammals consists of only two heavy chains (two-headed). All these animals, however, belong to Deuterostomia. It was found that the outer arm dynein purified biochemically from oyster, as a representative of Protostomia,[187] and that of sea anemone (Cnidaria)[6] situated at the root of two main branches of the phylogenetic tree also consist of two heavy chains. Furthermore, the analysis of a *Chlamydomonas* mutant lacking one of the heavy chains of the dynein has made it possible to identify the number of heavy chains by observing the cross-sections of flagella or cilia electron-microscopically[188] (see also Fig. 1). By this means it was revealed that all the flagella and cilia in animals including sponges, Mesozoa and choanoflagellates showed the two-headed outer arm profile.[189] On the other hand, the above-named protists as well as *Paramecium* and green algae such as *Bryopsis* had the three-headed profile.

The sea urchin sperm α heavy chain (OAD α family) and β heavy chain (OAD β family) correspond to the *Chlamydomonas* γ heavy chain (ODAγ) and α heavy chain (ODAα), respectively, while *Chlamydomonas* α and β (ODAβ) arose from a single gene by duplication at a certain point of evolution (both belong to the OAD β family). Such duplication also occurred in *Tetrahymena* and *Paramecium*, etc.[190] An amitochondriate protist, Lambl flagellate (*Giardia intestinalis*), had once been considered to be the most primitive eukaryote because mitochondria seem to be included in proto-eukaryotic cells through endosymbiotic event. Examination of the outer arms of this species indicated the two-headed structure which was supported by an image analysis. Among the *Giardia* genome, however, there is a short ORF corresponding to the *Chlamydomonas* β gene in addition to the genes corresponding to α and γ. As in the case of mitochondria which had once been acquired but then had secondarily been lost, one of the once duplicated genes of the outer arm dynein would be degenerated into a pseudogene owing to parasitism in *Giardia*.[191]

Loss of all or some of the dynein family members, including outer arm, inner arm and cytoplasmic dyneins, is a feature of the dynein history in eukaryotes.[190] In fact, the flagella of moss and fern spermatozoids as well as the cilia of *Ginkgo* sperm (See Ref. 192) lack the outer arm dyneins both morphologically and genomically. Even among animals *Caenorhabdatis elegans* does not have any arm dyneins, leaving only cytoplasmic dyneins. A recent survey of axonemal proteins in plant genomes revealed that the genes for axonemal dyneins, radial spoke proteins and central pair proteins have been completely lost, although a set of flagellar proteins is conserved and expressed in pollen cells, suggesting their role in the formation of male gametes.[193]

As far as the outer arm dynein is concerned, the dimeric, two-headed form found in Opisthokonta, including animals, choanoflagellates and fungi, would be the prototype. On the other hand, in another group consisting of kingdoms such as Archaeplastida, including plants and *Chlamydomonas*, and Alveolata, including *Tetrahymena* and *Paramecium*, the duplication of the OADβ family gene took place at a certain point of evolution and brought about the three-headed outer arms. The findings in *Giardia* would support the idea that Excavata or more definitely Diplomonadida, including this protist, would not be a primitive eukaryote but would also belong to the latter group corresponding to the bikonts. In other words, only Opisthokonta kept the prototype of the outer arm dynein after its establishment in the ancestral eukaryote, while in another group or other kingdoms the duplication of the OADβ family gene occurred once in a common ancestor or took place in various kingdoms in parallel.[190]

Perspectives

Axonemes in flagella and cilia are supramolecular complex with ~250 proteins, which are arranged so as to exert bending motion by dyneins and microtubules. Recent studies with cryoelectron tomography explore fine substructures and conformational changes of dyneins and axonemes. These lines of studies, as well as analyses by X-ray diffraction and nuclear magnetic resonance, are expected to join single molecular imaging technique and reveal structure-function relationship in dyneins and between dynein and tubulin. Ultimately it might become possible to answer the questions why dyneins are so big, why tubulins undergo a variety of post-translational modifications and why multiple dynein heavy chains are needed for flagellar and ciliary motility. Furthermore, discovery of small inhibitors

for dynein or tubulin based on these structural studies would accelerate the researches not only on diverse function of cilia but also on drug discovery in the medical therapy against human ciliopathies. Challenging studies are expected to be carried out in the near future on *in vitro* assembly of doublet microtubules and the axonemes, which would lead to artificial 'cilia implantation' to a cell. In addition, the detailed mechanism underlying the initiation of flagellar and ciliary wave at their base should be explored.

Recent findings that primary cilia participate in various critical signaling pathways such as those conducted by Sonic hedgehog and Wnt elicit a general function of cilia in signaling for proliferation and differentiation of cells and the morphogenesis in the establishment of body plans.[194] The importance of cilia is emphasized in some hypotheses proposed for driving force of metazoan or vertebrate body plan.[195] However, the roles of cilia as 'cell antenna' in multicellular organisms are not surprising because the properties of flagella and cilia that change the motility in response to physical or chemical stimuli are already acquired in protists. In fact, motile protists or sperm (free cells released from multi-cellular organisms) achieved peculiar innovation in the diversification of flagellar and ciliary structures and functions. Because flagella and cilia have been conserved to adapt in aquatic environments during evolution, the diversification in these structures and regulation might give an important insight for the adaptation of organisms to several environments. In the near future, we anticipate studies on the regulation of sperm motility in several organisms with different fertilization environments, structures of multicilia or macrocilia seen in several protists and metazoa, and degenerated structures of cilia seen in some sensory organs in metazoa.

Although the question why flagella and cilia as well as centrioles represent nine-fold radial symmetry is still unanswered, it is considered that ancient axonemes already showed motile 9+2 structures and their variants in structures and motility are evolved during evolution.[196] The 9+2 structures are generally seen in protists and also conserved well in metazoa. Then, two general questions become emerged; what is the origin of flagella and cilia and how they are diverged. The endosymbiont hypothesis was previously proposed,[197] but basic cytoskeletal structure of the possible symbiont is far from eukaryotic axonemes. Recent knowledge on the connection of cilia with the regulation of cell cycle

and intracellular vesicular transport supports autogenous emergence of cilia through functional transition of microtubule and motor proteins.[198] Further investigation on the diversity of flagella and cilia in the future using well-known species or those newly found, such as loss of motile cilia in plants or a certain group of protostomes and compound cilia or macrocilia in metazoa, should shed new light on the biology of dynein and tubulin.

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Profile

Hideo Mohri was born in 1930. After graduating from the Zoological Institute, the Faculty of Science at the University of Tokyo in 1953, he started his academic carrier with studies on respiration of sea urchin spermatozoa at the Misaki Marine Biological Station. It was shown that sea urchin spermatozoa utilize phospholipids as the main substrate for respiration. From 1962 he spent two years at the Stazione Zoologica, Naples, the Wenner-Gren Institute, University of Stockholm and the Marine Biological Laboratory, Woods Hole. In Stockholm he demonstrated oxidative phosphorylation in mitochondria isolated from mammalian spermatozoa. After this stay abroad his interest was concentrated in "contractile proteins" in flagella and cilia at the Komaba Campus of the University of Tokyo, although he had already described ATPase activity of the flagella isolated from sea urchin spermatozoa in 1958, and in 1968 he discovered "tubulin" as the counterpart of dynein ATPase in flagellar and ciliary movement. Then up till now, various contributions were made together with his students to elucidate the mechanism of flagellar and ciliary movement. Some work was also done on male contraceptives and on separation of X- and Y-bearing spermatozoa. He promoted to Professor in 1976 and was elected Dean of the College of General Education, the University of Tokyo, in 1987. He organized "spermatologists", investigators in sperm cell research, both in Japan and in the world. Later he much contributed to the development of reproductive biology in this country. He was awarded the Prize of the Zoological Society of Japan in 1974 and the Purple Ribbon Medal in 1996. After having served as a Vice-President of the University of the Air for three years, he was Director-General of the National Institute for Basic Biology between 1995 to 2001 and was President of the Okazaki National Research Institutes during the following two years.