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[REVIEW]

Functional Diversity of Axonemal Dyneins As Assessed by In Vitro and In Vivo Motility Assays of *Chlamydomonas* Mutants

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This review outlines the current knowledge of the functional diversity of axonemal dyneins, as revealed by studies with the model organism *Chlamydomonas*. Axonemal dyneins, which comprise outer and inner dynein arms, power cilia and flagella beating by producing sliding movements between adjacent outer-doublet microtubules. Outer- and inner-arm dyneins have traditionally been considered similar in structure and function. However, recent evidence suggests that they differ rather strikingly in subunit composition, axonemal arrangement, and molecular motor properties. We posit that these arms make up two largely independent motile systems; whereas outer-arm dynein can generate axonemal beating by itself under certain conditions, inner-arm dynein can generate beating only in cooperation with the central pair/radial spokes. This conclusion is supported by genome analyses of various organisms. Outer-arm dynein appears to be particularly important for nodal cilia of mammalian embryos that function for determination of left-right body asymmetry.

Key words: cilia, flagella, central pair, radial spokes, dynein heavy chain, outer-doublet microtubules, in vitro motility assay

INTRODUCTION

Cilia and flagella are cell organelles that perform a variety of motility and signaling functions. This review addresses their motility mechanisms, focusing on the properties of axonemal dyneins, the motor proteins that generate force (King, 2011), as studied in the model organism *Chlamydomonas* (Witman, 2009). Most data presented herein may be relevant to motile cilia and flagella of other organisms as well, as the structure and protein composition of the axoneme (internal structure of cilia/flagella) are extremely well conserved among many organisms ranging from protozoa to humans. More specific topics regarding metazoan axonemal dyneins can be found elsewhere (Inaba, 2007, 2011). Because cilia and flagella are essentially identical in structure and function, the term “cilia” will be used to describe both cilia and flagella, while the term “flagella” will only be used when its distinction from cilia is necessary.

Cilia are peculiar organelles that generate bending

waves, which produce cell movements or fluid flow over tissue surfaces. This movement is somewhat similar to the undulating motion of eels or snakes, which is generated by neuro-muscular circuits. How this subcellular structure produces such elaborate movements is a long-standing puzzle. In nearly all species, cilia possess a unique “9+2” structure, containing nine microtubules (termed outer-doublet microtubules) surrounding a pair of central microtubules (Fig. 1). Despite extensive studies, the origin of this particular structure and its functional advantages are not yet understood. Another striking feature of motile cilia is the presence of multiple motor protein species (dyneins) within a single cilium. Functional difference between different dyneins will be discussed in detail in this review.

The beating of cilia is generated by dynein-driven sliding between adjacent outer-doublet microtubules (Satir, 1968; Summers and Gibbons, 1971). The basic process of ciliary beating is therefore the same as in other cell motility phenomena, such as muscle contraction and vesicle transport,

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ABBREVIATIONS

DHC, dynein heavy chain; IC, intermediate chain; LC, light chain; DRC, dynein regulatory complex; CP, central pair; RS, radial spoke; OID, outer-inner dynein; ODA-DC, outer dynein arm-docking complex.

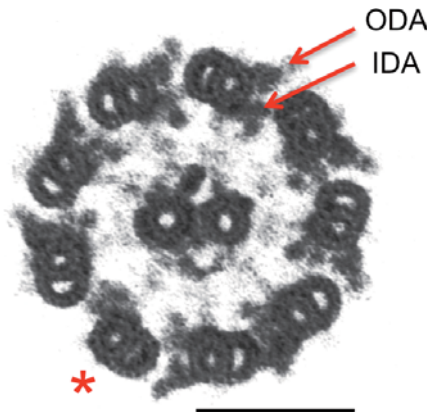


Fig. 1. *Chlamydomonas* axoneme. Flagella are demembrated by detergent treatment and observed by thin-section electron microscopy. This photo shows a view from the flagellar bottom to the tip. Nine outer-doublet microtubules (outer-doublets) surround a pair of singlet microtubules (the central pair) at the center, forming the so-called “9+2” structure. ODA, outer dynein arm; IDA, inner dynein arm. * denotes doublet #1, which lacks ODA. All other outer-doublets are numbered #2–#9 in the clockwise direction. Scale bar, 100 nm.

which are also based on a sliding motion between cytoskeletal filaments and motor proteins. However, the microtubule sliding in cilia has several distinct features not found in other systems. Most importantly, ciliary sliding velocity periodically varies along the length of the nine outer-doublet microtubules. The variability of sliding velocity is the basis for bend formation.

Unlike other cell motility systems, which typically involve only one kind of motor protein, the axoneme uses multiple types. Axonemal dyneins are largely classified as outer- or inner-arm. Biochemical and genetic studies have established that outer-arm dynein comprises a single species containing two (in metazoan and some unicellular organisms) or three (in many unicellular organisms) force-generating dynein heavy chains (DHCs). On the other hand, inner-arm dynein exists as several species that can be further classified into two subtypes: one with two DHCs and the other comprised of multiple species, each having a single DHC (Fig. 2). As a number of *in vitro* studies have shown, different dyneins differ greatly in properties. Most notably, the different dynein types undergo microtubule translocation at different velocities. The presence of multiple dyneins having distinct properties may be essential for the mechanism that generates ciliary beating. Here, we will consider various previous studies that reveal functional differences among axonemal dyneins. Due to space limitations, we have omitted several important experiments from this review [for more detailed experimental accounts, see Kamiya (2002); King and Kamiya (2009)].

AXONEMAL DYNEIN VARIETIES

Identification of diverse dynein species

Axonemes of most motile cilia have both outer and inner dynein arms. Early studies with *Tetrahymena* cilia and sea urchin sperm flagella revealed the presence of multiple ATP-hydrolyzing enzymes (dyneins) in axonemal extracts (Gibbons, 1963; Bell and Gibbons, 1982). However, the true

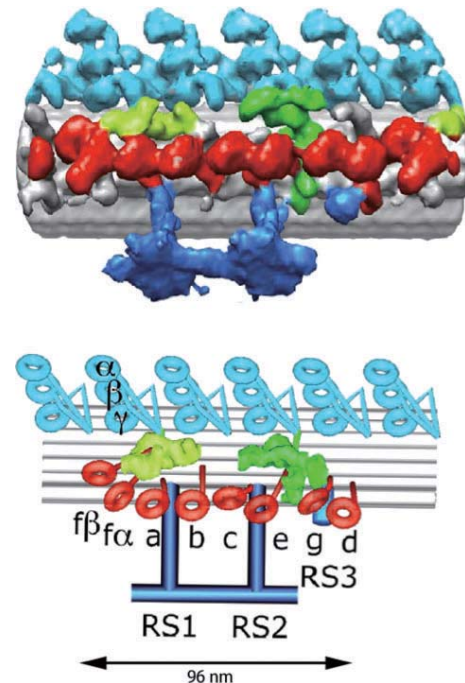


Fig. 2. Cryoelectron tomography image (upper panel) and its interpretation (lower panel) of an outer doublet. These images represent outer doublets #2–#8; doublets #1 and #9 have slightly different structures (see text). Outer-arm dynein is shown in pale blue, inner arm dynein in red, radial spokes (RS) in dark blue, the basal portion of inner-arm dynein in yellow-green, and the dynein regulatory complex (DRC) in green. In the lower panel, the motor domain (head portion) of each dynein heavy chain is depicted as a circle. Outer-arm dynein contains three heavy chains (α , β , γ), inner-arm dynein f/1 contains two heavy chains, α and β , while all other inner-arm dyneins contain a single heavy chain. The *Chlamydomonas* axoneme has two complete RSs (1 and 2) and a remnant structure (RS3); this arrangement of components repeats every 96 nm. Adapted from Bui et al. (2012).

diversity of axonemal dyneins was not clearly recognized until 1979, when dynein-deficient mutants were isolated from *Chlamydomonas* and analyzed by high-resolution SDS-PAGE (Huang et al., 1979). Piperno and Luck (1979a; 1981) established that inner-arm dyneins consist of multiple distinct species, while outer-arm dyneins consist of a single species that contain three force-generating DHCs and various other subunits. Table 1 lists the subunits of outer-arm dynein in *Chlamydomonas* (King and Kamiya, 2008) and the tunicate *Ciona* (Inaba, 2007).

Dynein composition was further analyzed in various *Chlamydomonas* mutants lacking outer-arm dynein or inner-arm dynein subsets by ion-exchange chromatography using a Mono-Q column (Kagami and Kamiya, 1992). High-salt extracts from mutant axonemes lacking outer-arm dynein were separated into seven discrete peak fractions, each containing a single major dynein species subdivided among “subspecies a” (or dynein a) to “subspecies g” (or dynein g). SDS-PAGE patterns obtained from these fractions clearly showed that subspecies f [identical to dynein I1 reported by Piperno et al. (1990)] contained two DHCs, while all others contained only one. Electron microscopy further characterized dynein f (hereafter referred to as dynein f/1) as a two-headed

Table 1. Subunit composition of outer-arm dyneins in *Chlamydomonas* and *Ciona*.

<i>Chlamydomonas</i>			<i>Ciona</i>		
Subunits	Mass (kD)	Comments	Subunits	Mass (kD)	Comments
α HC	504	Motor protein	α HC		Motor protein
β HC	520	Motor protein	β HC		Motor protein
γ HC	513	Motor protein			
IC1	76	WD repeat	IC1	68	WD repeat
IC2	63	WD repeat	IC2	78	WD repeat
			IC3	73	TRX/NDK domains
LC1	22	γ HC motor-binding LRR	LC1	21	LRR
LC2	16	Tctex2	LC2	19	Tctex2
LC3	17	Thioredoxin (TRX)	LC3	13	Tctex1
LC4	18	Ca ²⁺ binding	LC4	15	Cr LC8-like
LC5	14	Thioredoxin (TRX)	LC5	11	Roadblock
LC6	14	LC8-like	LC6	10	Cr LC8-like
LC7a	12	Roadblock			
LC7b	11	Roadblock	IC4	63	Docking complex (?)
LC8	10	Dimerization core?	IC5	63	Docking complex
LC9	13	Tctex1	66 k	66	Docking complex
LC10	12	LC8-like			
DC1	83	Docking complex (DC)	Calaxin		Ca ²⁺ binding
DC2	62	DC			
DC3	21	DC: Ca ²⁺ binding			

Chlamydomonas and *Ciona* data are from King and Kamiya (2009) and Inaba (2007), respectively. LRR, Leucine-rich repeat; Cr, *Chlamydomonas reinhardtii*

Table 2. Subunit composition of *Chlamydomonas* inner-arm dyneins.

Type	2-headed					1-headed					
Subspecies	f/I1	a	b	c	d	e	g	Minor dyneins			
DHC gene	1, 10	6	5	9	2	8	7	3	4	11	12*
IC/LC	IC140										
	IC138										
	IC97										
		Actin	Actin	Actin	p44 Actin	Actin	Actin	(Actin)	(Actin)	(Actin)	(Actin)
		p28		p28	p38 p28					(p28)	(p28)
			Centrin			Centrin	Centrin	(Centrin)	(Centrin)		
	Tctex1										
	Tctex2b										
	LC7a, b										
	LC8										

Adapted from King and Kamiya (2009) and Yagi et al. (2009).

*Toshiki Yagi, unpublished

IC, intermediate chain; LC, light chain

Proteins in parentheses are those determined in preliminary experiments.

dynein, while all others were single-headed (Goodenough and Heuser, 1985).

Biochemical analyses of dyneins from wild-type and mutant *Chlamydomonas* axonemes lacking specific inner-arm dyneins (see below) clarified the subunit composition of each species. The two-headed dynein f/I1 has a much more complex subunit composition than single-headed dyneins (Table 2). Furthermore, although the six single-headed

dyneins have different DHCs, they are similar to each other in subunit composition; all contain an actin (Piperno and Luck, 1979b) and either a 28 kD protein (p28) or centrin (Piperno et al., 1992). Additionally, dynein d also contains a 38 kD protein (p38) and a 44 kD protein (p44) (Yamamoto et al., 2006, 2008). Homologs of p28, p38, and p44, as well as actin and centrin, have also been found in other organisms.

All DHCs, both axonemal and cytoplasmic, share a common structure consisting of a ring-shaped head domain and a tail (King, 2011). The head domain is composed of six tandemly repeated AAA+ domains (termed AAAs #1–6) that form a ring and a coiled-coil stalk protruding from the boundary between AAAs #4 and #5; the tip of the stalk is the microtubule binding site. AAAs #1–4 each contain a nucleotide binding site, and AAA #1, located adjacent to the tail, catalyzes ATP hydrolysis. Nucleotide binding to AAAs #2–4 is thought to play a regulatory function. Extensive studies on cytoplasmic dynein, especially the determination of its three-dimensional atomic structure, have allowed us to consider the molecular mechanism of force generation by DHCs (Kon et al., 2012; Roberts et al., 2013; Carter, 2013). Force generation involves a swinging motion of the DHC tail in the plane of the head domain ring, which changes the angle between the tail and stalk (Burgess et al., 2003). It is likely that the DHCs of all axonemal dyneins produce force in this way.

Minor dyneins

Identification of one species of two-headed inner-arm and six species of single-headed inner-arm dyneins in the *Chlamydomonas* axoneme is consistent with electron microscopy results, which show eight DHCs within the 96 nm structural repeat length of the axoneme (see below). Unexpectedly, however, the *Chlamydomonas* genome contains four more DHC genes associated with inner-arm dyneins (Wickstead and Gull, 2007; King, 2011). Studies using specific antibodies to these extra dyneins demonstrate that they are only present in small amounts, and at least three of them exclusively localize to the proximal portion of the axoneme (Yagi et al., 2009; Bui et al., 2012). The *Chlamydomonas* flagella thus contain 15 DHCs altogether: three for the outer-arm, eight for the major inner-arm species, and four for the minor inner-arm dyneins.

The presence of multiple axonemal dyneins was confirmed by genome analyses of other organisms, including humans (Fig. 3). Despite the overall similarity of DHCs in all species of cytoplasmic and axonemal dyneins, their amino acid sequences differ in agreement with biochemical analyses classifying dyneins. In fact, the DHCs of outer-arm dynein, two-headed inner-arm dynein, and single-headed inner-arm dynein form three separate groups in the DHC sequence (Morris et al., 2006; Wickstead and Gull, 2007). Based on sequence characteristics, the DHCs of single-headed dyneins are further classified into three subgroups (Fig. 3), although the functional characteristics of each group are not known.

DYNEIN ARRANGEMENT ON THE OUTER-DOUBLET

Striking difference between outer- and inner-arm arrangements

The arrangement of various types of dyneins in the axoneme has been studied using a variety of electron microscopic methods, including cryo-electron tomography, which enables three-dimensional observation of flash-frozen specimens without chemical fixation or staining (Nicastro et al., 2006; Bui et al., 2008). Use of this method for various dynein-deficient axonemes from *Chlamydomonas* enabled, for the first time, visualization of the complex arrangement

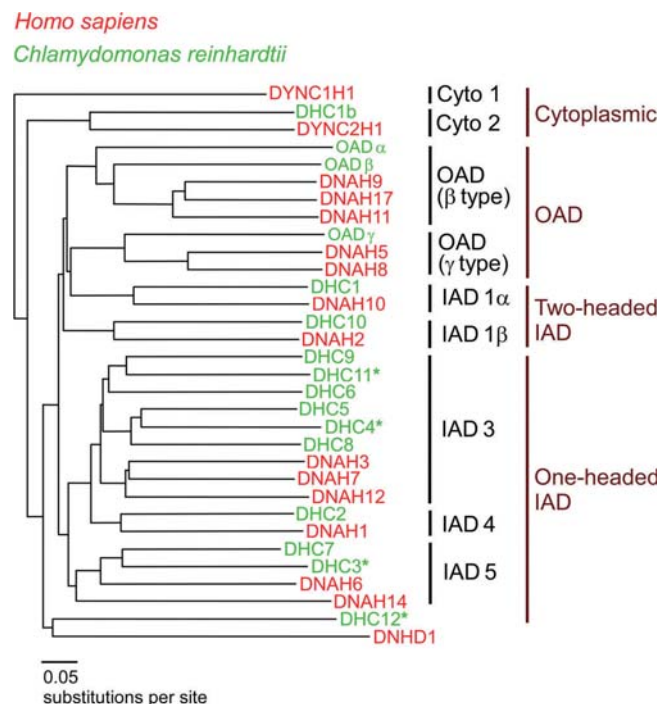


Fig. 3. Phylogenetic tree of dynein heavy chains (DHCs) in human (red) and *Chlamydomonas* (green). The classification IAD3, IAD4, etc. follows that of Morris et al. (2006). Dynein types are shown on the right. Cyto1 is the cytoplasmic DHC that functions in the cellular cytoplasm, while Cyto2 is the DHC specific for intra-flagellar transport. *Chlamydomonas* has no cytoplasmic dynein that functions in the cell body. Asterisks indicate inner-arm DHCs that are present only in minute amounts in the axoneme. OAD, outer-arm dynein; IAD, inner-arm dynein.

of axonemal dyneins (Fig. 2). As recognized in early dynein studies, outer dynein arms are linearly arranged every 24 nm. In contrast, inner dynein arms consisting of multiple dynein species have a complex arrangement within a 96 nm unit repeat length (Goodenough and Heuser, 1985; Mastroratte et al., 1992). In the main part of the axoneme, the 96 nm repeat unit contains seven major inner-arm dyneins (a–g), as well as four outer-arms and two radial spokes (RS) (Bui et al., 2008). In the ~2 μ m proximal portion, minor dynein species replace some major dyneins, although their exact localization remains unknown (Bui et al., 2009, 2012). The proximally localized dyneins may be engaged in functions specific to the proximal portion of the axoneme, such as bend initiation.

Rather surprisingly, the dynein arrangement found within the axoneme is not uniform among all nine microtubule doublets. While seven of these doublets share the dynein arrangement outlined above, doublet #1 (positioned closest to the *Chlamydomonas* cell central axis) and doublet #9 (adjacent to doublet #1) display different arrangements (the nine outer doublets are numbered 1–9 in the clockwise direction as viewed from axoneme base to tip). Doublet #1 lacks outer- and some inner-arm dyneins and has extensions that connect to doublet #2 (Hoops and Witman, 1983; Bui et al., 2012; Lin et al., 2012). Moreover, doublet #9 lacks one particular species of inner-arm dynein (Bui et al., 2009).

Such an asymmetrical dynein arrangement is likely important for planar *Chlamydomonas* flagella beating.

Other structures on the outer-doublet

Other than dyneins and RS, several other distinct structures are present on the outer-doublet microtubules that are most likely intimately involved in regulation of dynein function (Figs. 1, 2). The base of dynein f/I1 forms a large regulatory complex composed of its own intermediate and light chains, as well as other proteins involved in the phosphorylation of its 138 kDa intermediate chain (IC138) (Porter and Sale, 2000; VanderWaal et al., 2011). Apart from this complex, there is another large protein complex called the dynein regulatory complex (DRC). It was first found as a group of proteins that regulate dynein activities, but cryo-electron tomography revealed that it constitutes the nexin link (Heuser et al., 2009), a structure that bridges adjacent outer-doublets (Stephens, 1970). Additionally, another protein complex, the MIA (modifier of inner arms) complex, is present between dynein f/I1 and the DRC and may regulate f/I1 and other dyneins (Yamamoto et al., 2012). Lastly, there are links between outer- and some inner-arm dyneins, termed OID (outer-inner dynein) linkers which may function to coordinate outer and inner arm activities (Nicastro et al., 2006; Bui et al., 2012). An intermediate chain of outer-arm dynein has been suggested to form part of the OID linker (Oda et al., 2013).

ASSESSMENT OF AXONEMAL DYNEIN FUNCTION BY MUTANT ANALYSIS

Inferred functions

Simple comparisons between the motility of cilia lacking a particular dynein species and cilia with a full set have provided important insights into the functional diversity of dyneins. Gibbons and Gibbons (1973) were the first to show that outer-arm dynein functions to increase the frequency of axonemal beating; when axoneme motility in demembrated sea urchin sperm was reactivated by addition of ATP, axonemes deprived of outer-arm dynein by salt-extraction beat at half the frequency of intact axonemes but maintained normal waveforms.

Further assessment of dynein function in the axoneme was performed by the use of *Chlamydomonas* mutants lacking defined sets of dynein species. Mutants shown in Table 3 are among those extensively used in dynein functional studies. Although the first *Chlamydomonas* dynein-deficient mutants isolated by Luck's group were non-motile (Huang et al., 1979), mutants isolated later on were motile despite the absence of all outer-arm (Kamiya and Okamoto, 1985; Mitchell and Rosenbaum, 1985; Kamiya, 1988) or some inner-arm dyneins (Kamiya et al., 1991; Kato-Minoura et al., 1997). Discrepancies between the results obtained by Luck's group and those reported thereafter may have resulted from different mutant selection methods; Luck's group isolated dynein mutants from non-motile cells, whereas others looked for slow swimming mutants.

Consistent with observations in sea urchin sperm axonemes, *Chlamydomonas* mutants lacking outer-arm dynein display flagellar beating at about half the wild-type beat frequency, without significant changes in waveform. In contrast, mutants lacking inner-arm dynein f/I1 or those lack-

Table 3. Representative mutants lacking various dynein components.

Affected protein	Mutated gene	Mutant	Missing structure
Outer arm			
α DHC	<i>DHC13</i>	<i>oda11</i>	α DHC
β DHC	<i>DHC14</i>	<i>oda4, sup-pf-1</i>	OAD
γ DHC	<i>DHC15</i>	<i>oda2, pf28</i>	OAD
IC1	<i>DIC1</i>	<i>oda9</i>	OAD
IC2	<i>DIC2</i>	<i>oda6</i>	OAD
DC1	<i>DCC1</i>	<i>oda3</i>	OAD, ODA-DC
DC2	<i>DCC2</i>	<i>oda1</i>	OAD, ODA-DC
Inner arm			
DHC1	<i>DHC1</i>	<i>ida1, pf9</i>	dynein f/I1
DHC10	<i>DHC10</i>	<i>ida2</i>	dynein f/I1
IC140	<i>DIC3</i>	<i>ida7</i>	dynein f/I1
DHC9	<i>DHC9</i>	<i>ida9</i>	dynein c
p28	<i>DII1</i>	<i>ida4</i>	dynein a, c, d
actin	<i>DII4 (ACT1)</i>	<i>ida5</i>	dynein a, c, d, e
MOT48	<i>DAP2</i>	<i>ida10</i>	dynein b, c, d, e (reduced)

Adapted from King and Kamiya (2009).

Data for *ida10* are from Yamamoto et al. (2010); Gene names are after Hom et al. (2011).

ODA-DC, outer-arm docking complex.

ing some subunits of the DRC display flagellar beating frequencies comparable to wild-type, but with significantly reduced bend angles. In other words, inner-arm dynein f/I1 and the DRC apparently function to produce normal waveforms (Brokaw and Kamiya, 1987; Bayly et al., 2010). Although no extensive analysis has been performed on mutants lacking inner-arm dyneins other than dynein f/I1, they also appear to be important for waveform determination rather than producing high beat frequency because most mutants display a decrease in swimming velocity but not flagellar beat frequency (Kamiya et al., 1991; Kato-Minoura et al., 1997).

Different types of dyneins are functionally interdependent

Based on the phenotypes described above, mutants lacking any one species of dynein are motile, albeit less vigorously than wild-type (Fig. 4), suggesting that there is no "master dynein" that dictates the motility of the entire flagella. However, double mutants between two of the three types of dynein-deficient mutants (i.e., mutants lacking outer-arm, two-headed inner-arm, and single-headed inner-arm dyneins) are completely non-motile under physiological conditions (Kamiya, 1995). Therefore, the functions of the three types of dyneins appear to be interdependent, and axonemal beating, which involves microtubule sliding at varying speed and force, may require the function of at least two types of functionally different dyneins.

However, we have also found that axonemes are able to beat only with outer-arm dyneins under certain non-physiological conditions (see below for details). Thus, the above-mentioned requirement of at least two different types of dyneins for motility appears to hold only for axonemes under physiological conditions. The ability of axonemes to

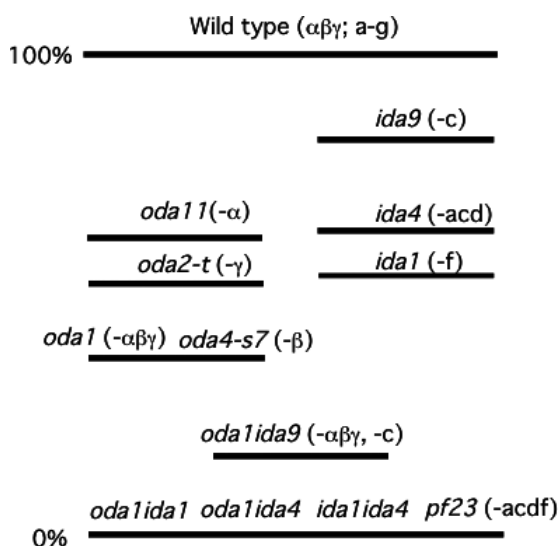


Fig. 4. Comparison of swimming velocities in wild-type and various dynein-deficient mutants. α , β , and γ denote the three outer-arm heavy chains, while a–g denote inner-arm subspecies. “ $\alpha\beta\gamma$ ” indicates a mutant that lacks the three heavy chains of outer-arm dynein (i.e., those lacking the entire outer-arm assembly). “ $\alpha\beta\gamma f$ ” indicates a double mutant lacking both the entire outer-arm and inner-arm dynein f/11. Note that mutants with modest dynein deficiency display motility, while those with serious deficiencies, such as the mutant lacking inner-arm dyneins a, c, d, and f, are non-motile.

beat with only outer arm dynein under certain non-physiological conditions may be due to the presence of three distinct force-generating heavy chains in a single outer arm.

Microtubule sliding in disintegrating axonemes

The primary function of dynein is to produce microtubule sliding. Most in vitro motility assays carried out thus far have measured the velocity of microtubule translocation, although a full evaluation of dynein activity requires other parameters, particularly the force generated. In fact, force measurements have been performed in several studies, and have provided important information (Kamimura and Takahashi, 1981; Shingyoji et al., 1998; Sakakibara et al., 1999); however, it is not yet possible to characterize various axonemal dyneins by their force generation properties.

Microtubule sliding generated by axonemal dyneins can be observed either in disintegrating axonemes or in microtubules translocating on a dynein-coated glass surface. Sliding disintegration of axonemes was first demonstrated using sea urchin sperm flagella. Axonemal fragments treated with protease undergo disintegration through inter-doublet sliding, a process that can be observed by dark-field light microscopy (Summers and Gibbons, 1971). Sliding velocity depends on the ATP concentration in a manner consistent with Michaelis-Menten kinetics (Yano and Miki-Noumura, 1980; Okagaki and Kamiya, 1986). Using wild-type and mutant axonemes lacking several kinds of dyneins, we have shown that loss of outer-arm dynein greatly reduces the sliding velocity (Okagaki and Kamiya, 1986), whereas loss of inner-arm dyneins does not (Kurimoto and Kamiya, 1991). These experiments suggest that outer- and inner-arm

dynein are molecular motors functioning at intrinsically different speeds.

Sliding disintegration experiments have thus yielded important qualitative information. However, there are certain limitations to these types of experiments. Most significantly, the measured sliding velocities apparently represent simultaneous functioning of multiple dyneins. Another inevitable problem is protease treatment of axonemes. Rather unexpectedly, more extensive protease treatments of axonemes tend to result in faster sliding velocity (Yano and Miki-Noumura, 1980; Kikushima, 2009). Therefore, the measured sliding velocity cannot be taken as the maximal velocity expected for axonemes under load-free conditions.

In vitro motility assays

In vitro motility assays measuring actin or microtubule translocation velocity on a motor-protein-coated glass surface, initially developed for myosin and kinesin, were first applied to axonemal dyneins by Paschal et al. (1987) and Vale and Toyoshima (1988). We have also used this method for fractionated inner-arm and mutant outer-arm dyneins lacking specific DHCs. As shown above, major inner-arm dyneins in *Chlamydomonas* flagella can be separated by ion-exchange chromatography into seven subspecies (a–g). The velocities of microtubule translocation displayed by these dyneins greatly vary from one subspecies to another (Kagami and Kamiya, 1992). At 0.1 mM ATP, the velocity varies from 0 $\mu\text{m/s}$ in dynein f/11 to 6 $\mu\text{m/s}$ in dynein c. Although other studies have reported slightly different velocities (Kotani et al., 2007; Toba et al., 2011; Sakakibara et al., 1999), it seems clear that different inner-arm dyneins have significantly different intrinsic sliding velocity.

Outer-arm dynein of *Chlamydomonas* displays somewhat unexpected microtubule translocation velocities in vitro. Outer-arm dynein of sea urchin sperm flagella (Paschal et al., 1987) or *Tetrahymena* cilia (Vale and Toyoshima, 1988) display fairly high maximal translocation velocities ($\sim 10 \mu\text{m/s}$), which is similar to the velocity of axonemal sliding disintegration. However, saturating concentrations of *Chlamydomonas* outer-arm dynein exhibit a low maximal velocity of $\sim 5 \mu\text{m/s}$ (Furuta et al., 2009). In systematic measurements using mutant outer-arm dyneins lacking a specific DHC, a dynein without the γ DHC (Liu et al., 2008) displayed faster translocation velocity (6.8 $\mu\text{m/s}$), while a dynein without the α DHC (Sakakibara et al., 1991) exhibited low velocity (0.8 $\mu\text{m/s}$). Although these results are thought-provoking, they cannot be readily explained, as wild-type cells swim much faster than these mutants and the α DHC mutant displays better motility than the γ DHC mutant. Thus, in vitro motility assays using a dynein-adsorbed glass surface do not faithfully reflect the properties of dyneins within the axoneme.

Fast sliding in regularly arranged microtubule bundles

The slow microtubule translocation velocity observed in vitro may well be due to the dynein arrangement on the glass surface; adsorbed dynein molecules probably lie randomly, with some potentially upside down. In contrast, outer-arm dynein molecules in the axoneme are regularly arranged every 24 nm, facing to the same direction. To examine microtubule translocation by regularly arranged outer-arm dynein molecules, some studies have used

dyneins regularly attached to cytoplasmic microtubules (Mimori and Miki-Noumura, 1994, 1995; Aoyama and Kamiya, 2010). Microtubules form bundles when outer-arm dynein is added in the absence of ATP (Haimo et al., 1979). Aoyama and Kamiya (2010) measured the microtubule sliding velocity protruding from the bundle upon quick application of ATP by photolysis of caged ATP. The results were surprising; sliding velocity was as high as 30 $\mu\text{m/s}$, about 1.5 times the maximal sliding velocity observed in disintegrating axonemes. Faster sliding may indicate that microtubule bundles formed in vitro impose much less friction on dynein movement than outer-doublets in protease-treated axonemes. Another remarkable observation was that all mutant outer-arm dyneins lacking any of the three DHCs produced sliding at similarly high velocities. These observations are consistent with the idea that each DHC of outer-arm dynein can produce high velocity microtubule sliding (30 $\mu\text{m/s}$) when the molecules are regularly aligned and drag force is small. Whether inner-arm dyneins can also display higher microtubule sliding in vitro under certain conditions is an important issue for future investigation.

REGULATION OF DYNEIN ACTIVITIES

Direct DHC regulation

The activity of axonemal dyneins is regulated by various factors. First of all, dynein activity must be periodically turned on and off in order to produce oscillatory movements. Such regulation may well involve mechanical feedback on dynein-microtubule interactions. A simple oscillatory phenomenon has been observed in a pair of outer-doublets in frayed axonemes (Kamiya and Okagaki, 1986; Aoyama and Kamiya, 2005) and analyzed by computer simulation (Brokaw, 2009, 2014). This oscillation has been interpreted by a feedback mechanism that involves mechanical tearing of dynein-microtubule cross-bridges. In such cases, "regulation" means a simple mechanical switching of dynein-microtubule interaction. Being generators of oscillatory movements, axonemal dyneins may well be particularly sensitive to external force compared to other motor proteins.

Secondly, the activity of dynein can be modulated by chemical factors that directly act on DHCs. A clear example is the acceleration of microtubule translocation by ADP, in the presence of ATP (Yagi, 2000); indirect evidence suggests this acceleration is due to ADP binding to one or more of the three non-catalytic nucleotide binding sites on DHCs (Kikushima et al., 2004; Inoue and Shingyoji, 2007). Although it is unknown whether nucleotide-dependent regulation bears physiological relevance in vivo, axonemal dyneins clearly have built-in regulatory sites. Another possible regulatory mechanism involving DHCs is phosphorylation, as several DHCs have been found to be phosphoproteins (Piperno and Luck, 1981; Boesger et al., 2009). However, the mechanism and regulation of phosphorylation, as well as its physiological significance, are currently unknown.

Regulation through dynein subunits

In addition to DHCs, various subunits of axonemal dyneins may function as targets for dynein regulation. Several dynein subunits are known to bind Ca^{2+} , which is responsible for the change in waveform or beat frequency in

cilia/flagella of many organisms. Examples include the LC4 light chain of outer-arm dynein (King and Patel-King, 1995), the DC3 subunit of the outer dynein arm-docking complex (ODA-DC) (Casey et al., 2003), and centrin contained in some inner-arm dyneins (Piperno et al., 1992). Proteins sensitive to redox poise, another motility regulator, have been identified among the light chains of outer-arm dynein and the ODA-DC (Wakabayashi and King, 2006). Phosphorylation of IC138 of inner-arm dynein f/11 is crucial for waveform regulation underlying phototactic behavior of *Chlamydomonas* (King and Dutcher, 1997).

Each of these regulatory sites may control the activity of specific sets of axonemal dyneins and modulate flagellar waveform or beat frequency. However, these factors do not necessarily affect dynein motor activity. Despite the marked Ca^{2+} -dependence of *Chlamydomonas* flagellar movement (Hyams and Borisy, 1978; Bessen et al., 1980; Kamiya and Witman, 1984), in vitro dynein motility assays have so far failed to show clear Ca^{2+} dependence in microtubule sliding velocity. Likewise, IC138 phosphorylation apparently does not affect the motor activity of dynein f/11; rather, phosphorylation changes its overall structure (Hitoshi Sakakibara, personal communication).

Regulation by changes in inter-microtubule spacing

Changes in axonemal structure, such as inter-doublet distance, may also sensitively affect the activity of various dyneins in situ, as postulated by the "geometric clutch" model of axonemal beating (Lindemann and Kanous, 1995; Lindemann, 2011). In particular, structures that connect the two adjacent outer-doublet microtubules and those bridging the central pair (CP) and outer-doublets are likely important. It has been suggested that adjacent outer-doublets are connected by the nexin link/DRC (Stephens, 1970; Gibbons, 1981; Heuser et al., 2009). The CP and outer-doublets are connected by RSs. RSs are involved in phosphorylation-based regulation of inner-arm dynein f/11 (Porter and Sale, 2000) and also likely engaged in mechanical interactions between microtubules (Witman et al., 1978; Oda et al., 2014). It is interesting to note that the nexin link/DRC, the CP/RS, and dyneins all contain proteins that bind Ca^{2+} or calmodulin. Thus, Ca^{2+} may indirectly regulate dynein activities by binding to multiple microtubule-bridging structures and modulating inter-microtubule spacing.

Functional interdependence of microtubule-bridging structures

An important feature of microtubule-bridging structures is that their functions are interdependent. This is possibly not surprising considering that axonemal structure is determined by the force balance between multiple components. A clear example of interdependence is the function of the nexin link/DRC; this structure was originally identified as a multi-protein complex whose defects restore motility in non-motile mutants lacking the CP or RS (Huang et al., 1982). The mutations that restore motility was termed suppressors. At the same time, the CP and RS are thought to regulate dynein motor activity. RS-modulation of dynein activity was most clearly demonstrated by Smith and Sale (1992), who found a significant reduction in microtubule sliding velocity in the absence of RS. Later studies demonstrated that applica-

tion of protein kinase inhibitors, which was known to increase axonemal motility in vitro (Hasegawa et al., 1987), blocks the reduction of sliding velocity in RS-deficient mutants (Howard et al., 1994; Habermacher et al., 1996; 1997). From these and other results, RSs are thought to function in cooperation with CPs to regulate the phosphorylation state of IC138. This regulatory process has been shown to involve several conserved proteins located on the outer-doublers (VanderWaal et al., 2011; Yamamoto et al., 2013). These proteins and the CP/RS may interact with each other physically, as well as chemically.

The DRC is not the only axonemal component that restores motility in mutants lacking CP or RS. Suppressor mutants isolated by Huang et al. (1982) include, in addition to DRC mutants, mutants of outer-arm dynein with a slight structural alteration. Similarly, a lack of functional dynein f/11 also exhibits suppressor activity (Porter et al., 1992). Therefore, both the absence of the DRC and certain dynein changes can override the inhibition of axonemal motility by CP/RS loss.

Regulation by covalent modification of tubulin

Tubulin in the outer-doublet microtubules of cilia and flagella undergoes various post-translational modifications, including tyrosination, acetylation, polyglycylation, and polyglutamylation (Janke and Bullinski, 2011). Polyglycylation and polyglutamylation have been shown to be particularly important for motility regulation. While polyglycylation has not been detected in *Chlamydomonas* (Tomohiro Kubo, unpublished), polyglutamylation appears to critically affect the function of inner-arm dynein. This was previously demonstrated in mutants lacking a particular polyglutamylating enzyme (TTLL9) and displaying a lack of tubulin with a long polyglutamate side-chain (but not tubulin with a short polyglutamate side-chains) in the axoneme; this mutation completely inhibited motility in mutants lacking outer-arm dynein, but did not significantly affect wild-type motility (Kubo et al., 2010). Furthermore, mutant analyses suggest that long-chain tubulin polyglutamylation specifically affects the function of dynein e, as well as the DRC (Kubo et al., 2012). Although the mechanism has not yet been established, we speculate that tubulin with highly acidic polyglutamate side-chains elicit strong attractive forces on dynein e and the DRC. Interestingly, the dynein e DHC has the most basic microtubule-binding site among all DHCs, and it is physically associated with the DRC (Piperno et al., 1992; Bui et al., 2012). If tubulin polyglutamylation critically affects the interaction of outer-doublers with a specific dynein and the DRC, then tubulin polyglutamylation could be regarded as a regulatory system based on inter-doublet separation. It should be noted that while the regulation may directly act on a single species of dynein and DRC, changes in inter-doublet separation would affect the function of other nearby dyneins, since all dyneins are attached to the same microtubule.

DISTINCT FUNCTIONS OF INNER- AND OUTER-ARM DYNEINS

Cilia/flagella beating without CP/RS

So far we have discussed dynein regulation by various mechanisms, but have not examined which species are under the control of which regulatory system, with exception

of dynein e. Our studies have indicated that the manner of regulation differs greatly, at least between outer- and inner-arm dyneins. *Chlamydomonas* axonemes (i.e., detergent-extracted flagella) can be made to beat by addition of ATP in vitro and qualitatively reflects movement in vivo; axonemes from mutants that display aberrant flagellar motility usually display a similarly aberrant movement, and those from non-motile mutants do not move in the presence of ATP. However, axonemes from non-motile mutants lacking the CP or RS have been shown to display beating in the presence of very low concentrations (20–50 μ M) of ATP or in the simultaneous presence of physiological concentrations (~1 mM) of ATP and a few mM ADP (Omoto et al., 1996). In another line of experiments, flagellar beating was induced by application of mechanical force in live cells of CP or RS mutants (Hayashibe et al., 1997). Thus, flagella without the CP/RS can also beat in vivo.

The movement of CP- or RS-deficient axonemes under non-physiological conditions, such as low ATP concentrations, is somewhat abnormal in waveform or beat frequency. However, it should be noted that the beating is rhythmic and undergoes Ca^{2+} -dependent waveform changes. Like the wild-type axoneme (Hyams and Borisy, 1978; Bessen et al., 1980), the CP or RS mutants display asymmetrical waveforms at lower Ca^{2+} concentrations ($< 10^{-6}$ M), whereas they have fairly symmetrical waveforms at $\geq 10^{-4}$ M Ca^{2+} (Fig. 5) (Wakabayashi et al., 1997). Therefore, it appears that the mechanism that produces waveform conversion does not entirely depend on the CP/RS. Although it is possible that Ca^{2+} regulates axonemal movement partially through the CP/RS, it must also act on other components apart from the CP/RS.

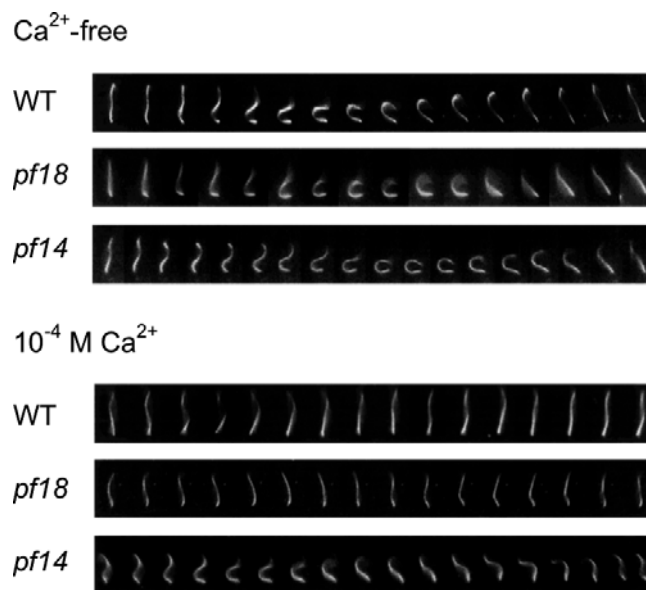


Fig. 5. Ca^{2+} -dependent waveform conversion in mutant axonemes lacking the central pair (*pf18*) or radial spokes (*pf14*). Axonemes were reactivated with 20 μ M ATP in the absence or presence of Ca^{2+} ; dark-field micrographs were taken every 1/60 s. The axonemes shown were attached to the glass surface by the proximal end (the end nearer to the bottom of the page). Scale bar (right corner), 10 μ m. Adapted from Wakabayashi et al. (1997).

Necessity of outer-arm dynein for CP- or RS-deficient axoneme beating

An important feature observed in studies described in previous sections is that outer-arm dynein is absolutely necessary for the induction of axonemal beating; CP- or RS-deficient mutants do not display any oscillatory movements if combined with a mutation that causes loss of outer-arm dynein. In contrast, axonemes from a non-motile double mutant *ida2ida4*, which lacks two-headed inner-arm dynein (f/I1) and a subset of single-headed inner-arm dyneins (a, c, and d), can also be made to beat in the simultaneous presence of ATP and ADP. In addition, beating can be induced in axonemes lacking the CP, RS, or inner-arm dyneins under a variety of conditions, such as the presence of high concentrations of MgSO₄ or sucrose, together with ATP (Yagi and Kamiya, 2000). These observations suggest that outer-arm dynein can produce bending waves in the axoneme under various conditions, and that this activity is largely independent of the CP/RS and inner-arm dyneins. Since *Chlamydomonas* mutants lacking outer-arm dynein are motile, this conclusion suggests that the axoneme uses two largely independent systems to generate beating.

Implications from diverse organisms

The above conclusion has gained support from genome analysis of various organisms; among various ciliated organisms, the diatom *Thalassiosira* possesses genes encoding outer-arm dynein subunits, but lacks those encoding the CP, RS, and inner-arm dynein subunits, whereas the moss *Physcomitrella* has no outer-arm dynein genes but possesses genes encoding the CP, RS and inner-arm dynein subunits (Wickstead and Gull, 2007; Merchant et al., 2007). These findings support the view that outer- and inner-arm dyneins function independently, and that only inner-arm dyneins require the CP and RS to function (Fig. 6).

If the function of CP and RS is to specifically regulate inner-arm dyneins, then cilia and flagella naturally devoid of CP/RS (called the “9+0” type) might also lack inner-arm dynein. However, eel sperm flagella, which lack the CP/RS and outer-arm dynein, vigorously beat with a helical waveform, indicating that their flagellar beating is produced only by inner-arm dynein (Gibbons et al., 1983). The structural organization of inner-arm dyneins in eel sperm appears similar to that of *Chlamydomonas* (Woolley, 1997), which brings into question how the flagellar movement of eel sperm can be produced and regulated in the absence of the CP/RS. We posit that some inner-arm dynein(s) in this organism may behave like an outer-arm dynein in *Chlamydomonas*.

In contrast to eel sperm, some other examples of “9+0” type cilia do seem to lack both the CP/RS and inner-arm dynein. Specifically, mouse nodal cilia, which lack the CP and RS (Nonaka et al., 1998), also display a three-dimensional beating pattern. This is most likely due to the absence of the CP and RS, which are thought to function in determining the beating plane in metazoan cilia. The three-dimensional beating of nodal cilia is important for generation of nodal flow underlying the determination of left-right body asymmetry. It is interesting to note that, although mouse nodal cilia have been reported to be equipped with both inner-arm and outer-arm dyneins (Takeda et al., 1999), the images of inner-arm dynein are much less clear than those

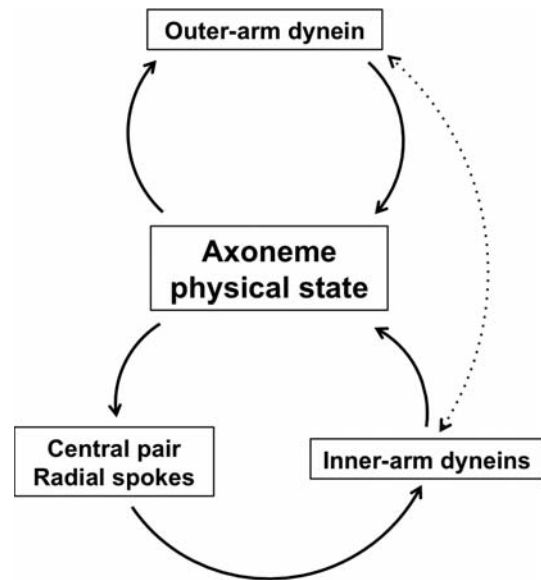


Fig. 6. Two dynein systems for axonemal beating. One system involves outer-arm dynein, while the other involves inner-arm dyneins and the central pair/radial spokes. They constitute mostly independent mechanical feedback loops necessary for production of axonemal beating. The arrows indicate the direction in which one component affects another. Under physiological conditions, the outer-arm loop can function only in the presence of the inner-arm loop. The dotted line indicates a possible direct interaction between outer-arm and inner-arm dyneins. Figure based on Hayashibe et al. (1997) and Kamiya (2002).

of outer-arm dynein in published electron micrographs of nodal cilia (Takeda et al., 1999; Hirokawa et al., 2006). Furthermore, human congenital syndromes with aberrant left-right body asymmetry have frequently been attributed to defects in genes related to outer-arm dynein, but never to genes related to inner-arm dyneins (Sutherland and Ware, 2009). Thus, outer-arm dynein seems particularly important for nodal cilia function in the absence of CP and RS.

CONCLUSIONS AND PERSPECTIVES

In this review, we have summarized the current state of knowledge of the diversity of axonemal dyneins based on previous studies of *Chlamydomonas*. An important observation is the strikingly large difference in structure and organization between outer- and inner-arm dynein. Outer and inner dynein arms were regarded as similar, if not identical, entities for a relatively long period of time following the discovery of axonemal dynein (Gibbons, 1963). However, detailed biochemical and electron microscopic analyses have revealed completely different structural organization (Huang et al., 1979; Goodenough and Heuser, 1985; Mastronarde et al., 1992; Nicastro et al., 2006; Bui et al., 2008). Likewise, our studies have shown that the two types of dyneins greatly differ in function.

In our view, the axoneme is equipped with two qualitatively different, largely independent systems for beating: one consisting of outer-arm dynein and the other involving inner-arm dyneins and the CP/RS (Fig. 6). Overall, the function of inner-arm dyneins appears to be more sophisticated, controlled by multiple axonemal structures. How do these two

different systems coordinate in a single axoneme to produce beating with a high frequency and a correct waveform? One possibility is that they spontaneously coordinate simply because they are attached to the same outer-doublet and put in the same mechanical state in a beating axoneme. In addition, there may be special mechanisms that ensure effective cooperation. Linker structures (OID-linkers) found between outer- and inner-arm dyneins (Nicastro et al., 2006) and the MIA complex (which links inner-arm dynein f/11, the DRC, and outer-arm dynein) (Yamamoto et al., 2012) may have such functions. Supporting the importance of the OID linker, a change in outer-arm intermediate chain was shown to cause apparently defective inner-arm dynein function (Oda et al., 2013).

We have focused this discussion mainly on phenomenological properties of the axonemal dynein system. At present, we can only speculate which dynein property or properties are crucial for the production of axonemal beating in a controlled manner. In future studies, clearly we must explore the details of motor properties and regulatory mechanisms of each species of dynein. Those studies should include single-molecule analysis of individual dynein species, as well as clarification of the collective properties of multiple dyneins incorporated in ordered structures. Exploring the functional properties of individual dyneins will be a challenging task because all axonemal dyneins function at specific positions in the rapidly bending axoneme. However, such intricacies will likely make functional studies of cilia and flagella motility more attractive; therefore, we can expect a number of new studies will provide novel clues to the mechanism of dynein function in the axoneme.

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