Structural mechanism of the dynein power stroke

Jianfeng Lin¹, Kyoko Okada¹, Milen Raytchev³, Maria C. Smith¹ and Daniela Nicastro¹,²

Dyneins are large microtubule motor proteins required for mitosis, intracellular transport and ciliary and flagellar motility¹,². They generate force through a power-stroke mechanism, which is an ATP-consuming cycle of pre- and post-power-stroke conformational changes that cause relative motion between different dynein domains³–⁵. However, key structural details of dynein’s force generation remain elusive. Here, using cryo-electron tomography of intact, active (that is, beating), rapidly frozen sea urchin sperm flagella, we determined the in situ three-dimensional structures of all domains of both pre- and post-power-stroke dynein, including the previously unresolved linker and stalk of pre-power-stroke dynein. Our results reveal that the rotation of the head relative to the linker is the key action in dynein movement, and that there are at least two distinct pre-power-stroke conformations: pre-I (microtubule-detached) and pre-II (microtubule-bound). We provide three-dimensional reconstructions of native dyneins in three conformational states, in situ, allowing us to propose a molecular model of the structural cycle underlying dynein movement.

Dyneins are minus-end-directed motors that use conformation-dependent changes associated with ATP binding and hydrolysis to attach to and walk along microtubules¹–⁵. Dyneins are categorized into two functional classes: axonal and cytoplasmic¹. Axonemal dyneins are arranged in a repeating pattern along the microtubular cytoskeleton of cilia and flagella, and power the sliding motions between outer doublet microtubules to drive ciliary and flagellar motility⁶–⁷ (Fig. 1); cytoplasmic dyneins function as individual homodimers and transport diverse intracellular cargoes along microtubules¹. Dysfunction of dynein causes devastating conditions in humans, including ciliopathies and neurodegenerative diseases⁸,⁹.

The structural mechanisms that underlie dynein movement, and therefore function, are not completely understood. Dynein motility is generated by a conserved motor domain that contains a ring-shaped head with six AAA domains, a coiled-coil stalk with a microtubule-binding domain (MTBD) at its tip, and the linker, which connects on its carboxy-terminal end with the cargo-binding tail of the motor domain (Fig. 1a). To move cargo along microtubule tracks, dyneins generate force through an ATP-consuming power stroke, which is preceded and followed by pre- and post-power-stroke conformational changes, respectively³–⁵. Previous two-dimensional (2D) negative-stain electron microscopy studies of isolated dynein, in the absence of microtubules, visualized two conformational states (primed and unprimed), which are thought to represent the pre- and post-power-stroke forms¹⁵,¹⁶. Subsequent cryo-electron microscopy and tomography studies determined 3D structures of dynein⁻¹⁷,¹⁸, but did not resolve key motor subdomains, for example, the stalk and linker, in the pre-power-stroke state(s). Recent studies revealed the crystal structure of the motor domain of truncated dynein in the post-power-stroke state¹⁰–¹². However, a complete understanding of dynein’s motility mechanism requires 3D visualization of the different conformational states adopted by native dyneins throughout their mechanochemical cycle, under physiological conditions and in their cellular context with both microtubule tracks and cargo present. Here we present the most complete set of structures, so far, of the conformational cycle of native dyneins in their cellular context.

The characteristic, oscillatory beating of cilia and flagella is driven by the activity of thousands of precisely, spatio-temporally regulated dyneins. It has previously been shown that cryo-electron tomography and subtomogram averaging of inactive flagella can provide 3D structures of macromolecular complexes, including dyneins in their post-power-stroke (inactive) state, with 3–5 nm resolution¹⁹. Flagellar dyneins are organized into two rows, the outer and inner dynein arms (ODA and IDA) that repeat periodically along the nine doublet microtubules that form the axonemal core of cilia and flagella; each doublet microtubule is composed of 96-nm-long axonemal repeat units¹²,¹⁰ (Fig. 1b–d). To visualize these dyneins, not only in their post-power-stroke state, but in different conformations throughout their mechanochemical cycle, we used cryo-electron tomography to image intact flagella from sea urchin (Strongylocentrotus purpuratus) sperm that had been rapidly frozen while actively beating (Supplementary Video 1), which trapped the dyneins (within milliseconds) in different conformations along the sinusoidally bent flagella. A powerful technique to improve the

¹Biology Department and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02454-9110, USA.
²Correspondence should be addressed to D.N. (e-mail: nicastro@brandeis.edu)

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resolution of the 3D reconstructions of axonemal complexes is subtomogram averaging of the 96 nm axonemal repeats\(^{22}\); the repeat units were extracted from the raw tomograms, aligned to one another in 3D and averaged. However, to preserve biologically meaningful structural differences between dyneins in different conformations it was important to use classification tools\(^{22}\) that separated the many 3D maps of individual complexes into homogeneous (same conformation) subgroups that were then combined into class averages. Mapping the class assignment of each repeat unit back to its location in the raw tomogram showed a highly specific distribution of classes along the flagellar axonemes (Supplementary Fig. 1); this is consistent with regulated dynein activity driving flagellar motility and suggests that the automated classification resulted in a meaningful separation of specific functional dynein states. Class averages that clearly visualized all main domains of dynein (resolutions between 3.0 and 4.8 nm; Supplementary Table 1) were subsequently used to determine the key structural differences between different dynein conformations (Figs 3 and 4). As both a control and to reinvestigate the structure of post-power-stroke dynein at high resolution, we also imaged flagella from sea urchin sperm that were either inactivated using ATPase inhibitor\(^{22}\) (Supplementary Video 2) or demembranated (nucleotide-free) axonemes from the unicellular algae _Chlamydomonas reinhardtii_ were also analysed to compare the post-power-stroke axonemal dyneins and conservation of structural features among evolutionarily distant species (Supplementary Fig. 2).

To determine the interactions of the linker with other regions of the motor domain, we docked the recently published post-power-stroke crystal structures\(^{12}\) into our 3D class averages and identified the position of the linker relative to the six AAA domains in different conformations (Figs 3h,j and 4b,d). In most post-power-stroke dyneins, the N terminus of the linker (distant from AAA1) latched onto the AAA4 and AAA5 domains close to the base of the stalk (Figs 2, 3a–c,g,h and 4a,b), as expected from previous studies\(^{11,12,15,17}\). In addition to confirming results from previous studies, we also identified previously undetected small, but conserved (from algae to sea urchin) differences between ODA and IDA structures (Fig. 2 and Supplementary Fig. 2). Specifically, the head and stalk of IDAs were rotated slightly more anticlockwise relative to the linker as compared with ODAs, such that the IDA linker-neck region is even closer to the stalk base (Figs 2, 3a–g,h and 4a,b). The differences observed in the post-power-stroke conformations of different dynein isoforms may be due to spatial constraints between the complexes in the axoneme or to intrinsically different functions of the dyneins. A similar positional difference in the linker was observed between the crystal

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**Figure 1** Dynein and its arrangement in sea urchin sperm flagella. (a) Sequence diagram and schematic of domain organization of one dynein heavy chain. (b) Diagrams of a sea urchin sperm cell (top) and its flagellum viewed in cross-section from the proximal side (bottom). (c) Diagram of an outer doublet microtubule (DMT) viewed in cross-section. (d) Diagram of a 96-nm-long axonemal unit that repeats along the doublet microtubule; each repeat unit contains: four outer dynein arms (ODAs), six single-headed inner dynein arms (IDAs: a, b, c, d, e and g, based on the nomenclature of _Chlamydomonas_), and one double-headed IDA (I1 or IDA f) anchored to the A-tubule (A\(_t\)) of the doublet microtubule. Other labels: B-tubule (B\(_t\)), central pair complex (CPC), nexin–dynein regulatory complex (N–DRC), radial spoke (RS), microtubule-binding domain (MTBD), microtubule polarity (+ and − end).
structures of cytoplasmic dyneins from both *Saccharomyces cerevisiae* and *Dictyostelium discoideum*, and is thought to be induced by ADP as the two crystal structures were obtained in either the absence or presence of ADP (refs 11,12). As nucleotides were removed from our axoneme samples, our data suggest that distinct dynein isoforms can exhibit at least two different apo- (nucleotide-free) conformations.

Our analysis of active flagella not only determined the high-resolution, 3D structure of full-length, pre-power-stroke dynein, but also revealed structures for two different pre-power-stroke conformations, which we termed the pre-I and pre-II states (Fig. 3d–f,i,j). As for the post-power-stroke state, we also found small differences between the ODA and IDA pre-power-stroke conformations. To analyse the structural differences between the observed dynein conformations, we superimposed the cargo-binding site of the tails of all class averages and compared the positions and orientations of all main domains (Fig. 3a–f,h,j). We found that in all observed pre-power-stroke conformations the dynein head had swung towards the minus end of the microtubule-track, pivoting around a point at the C-terminal end of the linker (close to AAA1), which effectively moved the stalk several nanometres towards the microtubule minus end as compared with the post-power-stroke structure. In contrast to the head and stalk, the pre-power-stroke neck-linker region did not significantly move relative to the microtubule when compared to the post-power-stroke state (Fig. 3c,e,h,j); only the angle of the linker changed in some cases; for example, the linker of ODAs was more parallel to the microtubule axis in the pre-power-stroke state (Fig. 3e,j). The relative motion between the rotating head and the more-or-less stationary linker resembled an opening jackknife with an angular amplitude of ~95°, and altered the interactions between the linker and head domains. The C-terminal half of the ODA linker that is positioned close to domains AAA1 and AAA6 in the post-power-stroke conformation interacts with domains AAA1 and AAA2 in the pre-power-stroke state whereas the N-terminal half of the linker that is located close to the stalk base in the post-power-stroke state protrudes from the head domain in the pre-power-stroke conformation (compare Fig. 3b,h with Fig. 3e,j). This large distance between the linker and the base of the stalk in our 3D pre-power-stroke structures is consistent with previous 2D negative-stain images of isolated dynein in a nucleotide-bound, ‘primed’ state.15

Overall, the IDAs showed the same structural changes between post- and pre-power-stroke conformations with only small differences: the angle of the IDA linker remained even more
Figure 3 In situ structural changes of ODAs between post- and pre-power-stroke states. (a–f) Longitudinal tomographic slices of averaged axonemal dyneins in post- (a–c), pre-I (d,e) and pre-II (f) power-stroke states; note the difference in curvature of the stalks (orange arrowheads). Coloured dots and rings in a–f indicate interaction sites of the linker with specific AAA domains of the dynein heads: AAA1 (dark blue), AAA2 (light blue), AAA4 (yellow); in b and e, linker and tail are indicated with magenta and pink dots, respectively. (g–j) 3D isosurface renderings of DMT in cross-view (g,i) and the conformational changes of the ODAs between post-power-stroke (h) and pre-power-stroke states (j). Coloured lines (g,i) indicate the orientation of the tomographic slices (a,c,d,f); protofilaments numbered according to ref. 31. Interactions between the linker and head are shown by docking the crystal structure of S. cerevisiae cytoplasmic dynein (ribbon representation with AAA1-6 from PDB 4AKI; ref. 12) into our electron microscopy volume (insets in h,j); note the linker base that connects to AAA1. Other labels: A-tubule (At), B-tubule (Bt), doublet microtubule (DMT), nexin–dynein regulatory complex (N–DRC). Scale bars, 10 nm.

Stationary between different conformations than the ODA linker; the angular amplitude of the IDA head rotation relative to the linker, as it changed from a post- to pre-power-stroke conformation, was slightly smaller (~50°). Therefore, the interaction of the N-terminal side of the linker with the head changed from AAA5 in the post-power-stroke to AAA3 and AAA2 in the pre-power-stroke state (Fig. 4 and Supplementary Fig. 3). Our comparison of dynein conformations suggests that rotation of the ring-shaped head relative to the more...
static linker is the key action of the recovery (priming) stroke (post- to pre-power-stroke transition) and the power stroke (pre- to post-power-stroke transition), rather than major conformational changes in the linker or tail regions as previously suggested\(^{18,23}\).

Our classification revealed two different pre-power-stroke conformations of the ODAs, the pre-I and pre-II states. Although the head was rotated into the ‘primed’ forward position in both states, in pre-I, the stalk was detached with the MTBD located a few nanometres above the microtubule track (Fig. 3d; \(\alpha\)-ODA), whereas in pre-II, the stalk was microtubule-bound (Fig. 3f; \(\beta\)-ODA). For the MTBD to reach the microtubule track, the pre-II head and stalk are tilted \(~20^\circ\) anticlockwise in comparison with pre-I (Fig. 3d,f). However, the linker–head interactions seemed unchanged between the two pre-power-stroke states, because the rotation was compensated for by bending in the neck region (Fig. 3d–f). The 3D structure of the microtubule-detached pre-I conformation, including its stalk and MTBD, was remarkably well resolved. A reconstruction with this clarity would probably be unfeasible using an \textit{in vitro} system owing to diffusion of the detached head, but was made possible here through the structural scaffold provided by intact flagella; that is, the pre-I dyneins were held in place (instead of diffusing in space) by their tails anchoring to the cargo–microtubule and other domains contacting neighbouring axonemal structures.

The pre-I conformation (with primed linker and detached stalk) probably follows the post-power-stroke state, but precedes pre-II with a reattached MTBD. This is supported by different amounts of movement of the dynein heads towards the microtubule minus end in the pre- and post-power-stroke states; between the pre-I and post-power-stroke structures, the head was shifted slightly less than 8 nm towards the microtubule minus end (compare Fig. 3a and d), whereas between the pre-II and post-power-stroke states the shift was 8 nm (compare Fig. 3c and f), which is the most frequently observed step size of load-carrying dynein\(^{24,25}\). This is consistent with a previous 2D electron microscopy study of isolated sea urchin ODAs re-bound to microtubules \textit{in vitro} that measured different shifts between dynein heads\(^{23}\). In sea urchin flagella, two dynein heavy chains, \(\alpha\)- and \(\beta\)-dynein, form a dimeric ODA complex. Our classification of dyneins from active flagella revealed that the \(\alpha\)-ODA was in the pre-I and the \(\beta\)-ODA in the pre-II state in more than 90% of the classified ODA dimers. This could be due to distinct roles of different ODA isoforms in axonemal motility generation, as suggested by previous studies\(^7,26\). Nonetheless, it indicates that the \(\beta\)-ODA predominates as the leading ‘leg’ as this dimeric ODA complex walks along the microtubule track in an inchworm fashion; this is similar to cytoplasmic dynein\(^{27}\), but different from the ‘hand-over-hand’ stepping characteristic of kinesins\(^{28}\), the other molecular motors that walk along microtubules.
Previous studies have proposed a linker-swing model for dynein movement, mainly based on images of isolated dynein. Here, we directly visualized 3D structures of both the pre-power-stroke linker and various dynein conformations in the presence of microtubule track and cargo. We demonstrate that the linker remained remarkably stationary between post- and pre-power-stroke structures, whereas a rotation of the head domain, that is, a ‘head-swing mechanism’, seems to cause the major movement of dyneins relative to the microtubule (Figs 3 and 4 and Supplementary Fig. 3). Previous studies predicted that a narrow cleft in the linker might be the hinge point for the swing of the linker relative to the head. We simulated the swing around a pivot point using the published S. cerevisiae crystal structure and by cutting the linker at a proximal site close to the proposed hinge-like cleft, to then rotate the C-terminal regions of the dynein to the different positions suggested by our 3D conformational structures (Supplementary Fig. 4). The simulated maps closely resemble the reconstructed in situ conformations of flagellar dyneins, but we cannot exclude the possibility that the proximal linker region undergoes (additional) remodelling during the head swing.

The presence of microtubule tracks and cargo in the intact flagella allowed visualization of previously unresolved conformational changes, including differences in the stalk structures. Our two most-clearly resolved dynein species, β-ODA and IDA a, showed that the stalk was bent in opposite directions between the pre-II and post-power-stroke states (Figs 3c,f and 4a,c). It is possible that these structural differences are due to flexibility in the coiled-coil stalk; that is, the stalk is passively moved in different directions when it is subjected to changing forces during the mechanochemical cycle of dynein. Alternatively, these differences could correspond to distinct functional states of the stalk MTBD; that is, opposite bends of the coiled-coil stalk could cause changes in the registration between the two helices of the coiled-coil stalk and thus of the microtubule binding affinity of the MTBD (refs 4,13).

Along with a comprehensive understanding of the mechanochemical power-stroke cycle, we have visualized two previously undetected electron densities, directly attached to the dynein motor domain (Fig. 4 and Supplementary Figs 2 and 3; white arrowheads). A likely candidate for one of these densities is the lissencephaly protein, Lis1, which is the only cofactor known to directly interact with and modulate the activity of the dynein motor domain. A single-particle cryo-electron microscopy reconstruction of Lis1 bound to cytoplasmic dynein in vitro showed that Lis1 interacts with the AAA3 and AAA4 domains of dynein. We observed an extra density attached to the AAA3 domain and the neck region of the tail of the post-power-stroke α-ODA in Chlamydomonas axonemes (Supplementary Fig. 2, white arrowheads). Lis1 was previously shown to associate with α-ODA in Chlamydomonas axonemes, making it a likely candidate for the extra density detected in our study. Although this extra density was not observed in sea urchin sperm flagella, another density was observed to be specifically attached to the linker, AAA1 and AAA2 of sea urchin pre-power-stroke IDA dyneins a–c and e (Fig. 4c,d and Supplementary Fig. 3c,d, white arrowheads). As AAA1 is the essential ATP hydrolysis site for the power stroke, this extra density could be an isoform specific regulator involved in the dynein power-stroke mechanism. Further studies are required to identify and functionally characterize these densities. Nonetheless, our findings emphasize that different dynein isoforms function in unique ways by employing distinct regulatory mechanisms.

Combined with previous kinetic studies, our structural data support the following model of dynein movement (Fig. 5 and Supplementary Video 3): In the absence of nucleotides, a post-power-stroke conformation exists with the N terminus of the linker attached to AAA4 or AAA5 (depending on the dynein isoform); the stalk is tightly bound to the microtubule track with a 60°–70° angle relative to the microtubule (Fig. 5a). ATP binding to AAA1 of the head domain causes conformational changes that result in weakening of the microtubule affinity of the MTBD and its dissociation from the microtubule track. Following or concomitant with this dissociation, dynein undergoes a recovery stroke to the pre-power-stroke conformation pre-1 by rotating the head and stalk clockwise (50°–70°). This moves the dynein head towards the microtubule minus end and the dynein is now primed for a power stroke (Fig. 5b). During or after ATP hydrolysis, the motor domain transitions from
the pre-I to pre-II state (Fig. 5c) by tilting the head, together with the stalk, anticlockwise such that the MTBD can re-bind (weakly) to a new binding site on the microtubule track, closer to the minus end. On release of phosphate, dynein once again binds strongly to the microtubule. The power stroke brings the linker again close to the stalk base, like a closing jackknife, and into the post-power-stroke position, while the tail and bound cargo are pulled towards the new binding site (Fig. 5d). The stalk serves as a tether to the microtubule, while the cargo is ‘winched’ towards the microtubule minus end. On release of ADP from the dynein, a new mechanochemical cycle begins.

In summary, we highlight three key findings: the priming recovery stroke is mainly achieved by rotation of the head domain with respect to the more-or-less stationary linker, rather than shifting of the head through remodelling of the linker and/or tail; during dynein stepping, different sites of the linker interact with various AAA domains of the head; and by visualizing the 3D structure of full-length dynein in multiple conformations we can propose a mechanistic model that shows how conformational changes of different domains are coordinated and coupled with ATP hydrolysis to achieve cyclical dynein movement. Overall, our study provides insights into the structural changes that govern dynein movement under physiological conditions and establishes an improved platform on which to continue studies of dynein in situ.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.N. conceived and directed the study. J.L. performed the experiments. J.L., M.R., J.C. and D.N. analysed the data. J.L., K.O., M.C.S. and D.N. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cryo-sample preparation. Live sea urchins (Strongylocentrotus purpuratus) were purchased from Monterey Abalone. Spawning was induced by the injection of 1–2 ml of 0.5 M KCl into the perivisceral cavity\(^3\). Sperm samples were collected from adult male sea urchins and divided to prepare three different types of sample: (part A) active sperm, (part B) ATPase-inhibited sperm, and (part C) isolated axonemes. Before preparation, inclusion criteria required: live and active (beating) sea urchin sperm flagella. Sperm were diluted in artificial sea water (360 mM NaCl, 50 mM MgCl\(_2\), 10 mM CaCl\(_2\), 10 mM KCl and 30 mM HEPE, at pH 8.0), and the motility was imaged using the DIC mode of a Marianne spinning-disc confocal microscope (3I) consisting of a Zeiss Observer Z1 microscope (Carl Zeiss) equipped with a Yokogawa CSU-X1 spinning-disc confocal head (Yokogawa) and a QuantEM 512SC EMCCD camera (Photometrics). All collected cells were active and no samples were excluded. Immediately following motility confirmation, part A (active sperm that were diluted in artificial sea water, Supplementary Video 1) was rapidly frozen (as described below). Part B was diluted in artificial sea water containing the ATPase inhibitor erythror-9-[3-(2-hydroxynonyl)]-adenine (EHNA, 2 mM; ref. 22); after a five minute incubation at \(-16^\circ\)C, the sample was imaged at the light microscope to confirm that sperm were completely immotile (Supplementary Video 2), and they were then rapidly frozen. Part C was diluted in demembranation buffer (30 mM HEPE, at pH 8.0, 150 mM KCl, 4 mM MgCl\(_2\), 0.5 mM EGTA and 0.1% Triton X-100) to remove the flagellar membrane. After a 1 min incubation, the sperm were collected by centrifugation, resuspended in demembranation buffer (without Triton X-100), and rapidly frozen.

Chlamydomonas reinhardtii axonemes were isolated from a pseudo wild-type strain (pf2-4:PF2-GFP), as previously described\(^3\). The pseudo wild-type strain is a rescued mutant and is structurally and phenotypically indistinguishable from the wild type. Flagella were detached from the cells using the pH-shock method\(^4\), and purified by two centrifugation steps at 2,400 g, for 10 min, at 4 °C with a 20% sucrose cushion. Purified flagella were demembranated with 0.1% IGEPAL CA-630 (Sigma-Aldrich) and axonemes were collected by centrifugation at 35,000 g for 1 h at 4 °C. The axoneme pellet was washed, resuspended in HMEEN buffer (30 mM HEPE, at pH 7.4, 5 mM MgSO\(_4\), 1 mM EGTA, 0.1 mM EDTA, 25 mM NaCl, and 0.1 μg ml\(^{-1}\) aprotinin, leupeptin and pepstatin), and rapidly frozen.

Sperm and axoneme samples were rapidly frozen on Quantifoil holey carbon grids (Quantifoil Micro Tools) that were glow discharged, coated with 10 nm colloidal gold (Sigma-Aldrich), and loaded on a home-made plunge freezing device. Sample (3 μl) and fivefold-concentrated 10 nm colloidal gold solution (1 μl; ref. 35) were applied to the grid. The grid was blotted with filter paper for 1.5–2.5 s and immediately frozen by plunging into liquid ethane. The vitrified samples were then stored in liquid nitrogen until examined by electron microscopy.

Cryo-electron tomography. Tilt series acquisitions and tomogram reconstructions were conducted as previously described\(^3\). In brief, cryo-samples were imaged at 300 kV, with \(-6 \text{ μm} \text{ or} -8 \text{ μm} \text{ defocus, under low-dose conditions and with an energy filter} \text{(Gatan) in zero-loss mode (20 eV slit width) on a Tecnai F30 transmission electron microscope (FEI). Electron tomography data were recorded only for flagella that seemed well preserved by electron microscopy inspection. Tilt series were recorded while stepwise rotating the sample from approximately \(-65 \text{ to} +65^\circ\) with \(-2.5^\circ\) angular increments, using the SerialEM image acquisition software\(^7\). The cumulative electron dose per tilt series was limited to \(-100 \text{ e}^{-2} \text{Å}^{-2}\) to avoid radiation damage. All images were recorded on a 2k x 2k CCD (charge-coupled device) camera (Gatan) at a nominal magnification of 13,500, resulting in a pixel size of \(-1 \text{ nm}.\)

Image processing. The tilt series images were first aligned using the 10 nm gold particles as fiducial markers, and then reconstructed by weighted back-projection into tomograms using the IMOD software\(^8\). The Chlamydomonas tomograms were previously utilized for the analysis of other axonomal complexes\(^9\). Tomographic reconstructions with compressed flagella (caused by embedding in too thin ice during cryo-sample preparation) or insufficient fiducial markers for alignment were excluded from subtomogram averaging and analysis. The 96 nm axonemal repeat units along the doublet microtubules were extracted from the raw tomograms, aligned (volume size, 110 x 84 x 80 nm) and averaged (including missing wedge compensation) using the program PEET (ref. 20) to obtain sub-tomogram averages with enhanced signal-to-noise ratio and improved resolution. To obtain structures with consistent conformations, we further performed automated classification on the aligned sub-tomograms from sea urchin sample A (sperm frozen while actively beating), using a clustering approach (principle component analysis) built into the PEET program\(^10\). To focus the classification on different dynein isoforms of interest, appropriate masks were applied before classification. Subtomograms that contain the dynein of interest in the same state were grouped into the same class and were averaged using the original alignment to generate a class average; masks were used only for the classifications, but not for alignment or averaging. The resolution of the resulting averages was estimated in a (20 nm)\(^3\) sub-volume in the centre of interest using the 0.5 criterion of the Fourier shell correlation method\(^11\). The number of tomograms, and both particle numbers and resulting resolutions are reported in Supplementary Table 1, for each sample. The crystal structure fitting and 3D visualization by isosurface rendering were carried out using the UCSF Chimera program\(^12\). To generate the simulated density maps of dynein in different conformational states, the linker was cut at the proposed linker-swing hinge site from the crystal structure of S. cerevisiae (yeast) cytoplasmic dynein (PDB 4AKI A chain)\(^12\), and the N-terminal linker part (between hinge and tail) was rotated around the hinge point to the locations predicted by our cryo-electron tomography structures. The density maps were generated from these modified crystal structures using the Chimera molmap command.

Accession numbers. Cryo-electron tomography structural data have been deposited in the Electron Microscopy Data Bank (primary accessions: EMD-5757 and EMD-5758) and the corresponding fitted atomic coordinates have been deposited in the Protein Data Bank (primary accessions: 3J67 and 3J68; referenced accession used to generate 3J67 and 3J68 is 4AKI).

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Supplementary Figure 1 Distributions of different dynein conformations inside a sea urchin sperm flagellum revealed by classification analysis. Subtomogram volumes containing the 96 nm axonemal repeat were aligned and classified, focusing on β-ODA. The distributions of the different classified conformations were then mapped back to their locations in the raw tomograms of the sea urchin sperm flagella. Different conformations of β-ODA (and the o-SUB5-6 bridge structures) are indicated by different coloured dots: post-powerstroke (red), pre-II (blue), minor and low resolution classes possibly representing other dynein conformations (orange), o-SUB5-6 bridge structure (green); note that the distribution of identified dynein conformations is highly specific to particular doublets, which is consistent with the precise regulation of dynein activity to generate the flagellar waveform.
Supplementary Figure 2: \textit{In situ} structures of \textit{Chlamydomonas} axonemal dyneins in post-powerstroke state as revealed by cryo-ET. (a) Tomographic slice of an averaged doublet microtubule (DMT) viewed in cross-section from proximal, showing the \textit{in situ} arrangement of the axonemal dyneins. Coloured lines indicate locations of tomographic slices in (b-d) and (f-g). (b-h') Longitudinal (parallel to the microtubule) tomographic slices show the averaged axonemal dyneins. \textit{e}' and \textit{h}' are zoom-ins from (d) and (g), respectively. In \textit{e}' and \textit{h}', isosurface-rendered 3D images of averaged \textit{α}-ODA and IDA dynein \textit{c} were superimposed upon the corresponding tomographic slices (\textit{e}, \textit{h}). (i-k) 3D isosurface renderings show the averaged axonemal dyneins. Note that each \textit{Chlamydomonas} ODA is composed of three dynein heavy chains (\textit{α}, \textit{β}, and \textit{γ}-ODA); the innermost \textit{γ}-ODA corresponds to the sea urchin \textit{α}-ODA (compare to Fig. 2). Tail (pink), linker (magenta) and head (green) domains are clearly visible in both tomographic slices and isosurface renderings; coiled-coil stalks (orange arrowheads) are distinct in the tomographic slices (b-d, f). IDA \textit{a}, \textit{b}, \textit{c}, \textit{e}, \textit{g}, \textit{d} are also known as IDA2, \textit{x}, \textit{3}, \textit{4}, \textit{5}, \textit{6}, respectively. Other labels: A-tubule (\textit{A}'), B-tubule (\textit{B}'), nexin-dynein regulatory complex (\textit{N-DRC}), radial spoke (\textit{RS}), radial spoke 3 stand-in (\textit{RS3S}). Structure-colour coding is preserved in the isosurface renderings in all following figures. White arrowheads in \textit{e}, \textit{e}' and \textit{j} highlight an extra density that is specifically attached to the dynein head and tail domains. Scale bars: 10 nm.
Supplementary Figure 3 *In situ* structural changes of sea urchin IDAs between post- and pre-powerstroke states. (a-d) 3D isosurface rendering of averaged IDAs showing conformational changes observed for IDA dyneins c and e between post-powerstroke (a, b) and pre-powerstroke (c, d) states. In b and d, the crystal structure of *S. cerevisiae* cytoplasmic dynein (ribbon representation with AAA1-6 from PDB 4AKI)\(^{12}\) is docked into our IDA EM volume to illustrate changes in the interaction between the linker and head. White arrowheads in c and d highlight an extra density that specifically attaches to the linker and AAA1 in pre-powerstroke states.
Supplementary Figure 4 Comparison between simulated and real 3D structures of dynein in different conformational states. The averaged 3D cryo-ET structures of two different dynein isoforms (IDA dynein a and β-ODA) in two different conformational states (post- and pre-II-powerstroke states) provide structural details of the relative motion between the AAA-domain head and the linker. The first column on the left shows the x-ray crystal structure of *S. cerevisiae* cytoplasmic dynein (PDB 4AKI A chain)\(^{12}\). To interpret the relative motions between different conformational states, the crystal structure was cut at the proposed hinge site (arrow heads); the major part of the linker was then aligned (held stationary), while the dynein head and stalk were rotated around the hinge site to the locations predicted by our different cryo-ET structures; the rotation angle between the head and linker increases from top to bottom: IDA a, post-powerstroke (top); β-ODA, post-powerstroke; IDA a, pre-II state; β-ODA; pre-II state (bottom). The second column shows simulated density maps that were generated from the modified x-ray structures using the Chimera molmap command. The third column shows the 3D *in situ* structures of sea urchin axonemal dyneins (with the major part of their linker aligned) as revealed by cryo-ET. The fourth column shows the same structures as the third, but with the crystal structures from the first column docked into the cryo-ET structures. The linker and tail domains in the cryo-ET structures were coloured in magenta and pink, respectively.
**Supplementary Table 1. Summary of 3D averages of axonemal dyneins.**

| Organism     | Sample          | Dynein | State                | Number of tomograms | Averaged subtomograms | Resolution [nm] |
|--------------|-----------------|--------|----------------------|---------------------|-----------------------|-----------------|
| *Strongylocentrotus* | Inhibited intact sperm | α-ODA  | Post-powerstroke     | 9                   | 1100                  | 3.4             |
|               | Inhibited intact sperm | β-ODA  | Post-powerstroke     | 9                   | 1100                  | 4.2             |
|               | Demembranated sperm | IDA a,b,c,e | Post-powerstroke     | 7                   | 700                   | 3.9-4.8         |
|               | Active intact sperm | α-ODA  | Pre-powerstroke (pre-I) | 41               | 2800                  | 3               |
|               | Active intact sperm | β-ODA  | Pre-powerstroke (pre-II) | 41              | 2800                  | 3.3             |
|               | Active intact sperm | IDA a,b | Pre-powerstroke (pre-II) | 41              | 1400                  | 3.3-3.4         |
|               | Active intact sperm | IDA c,e | Pre-powerstroke      | 41                   | 1800                  | 3.5-3.7         |
| *Chlamydomonas*      | Isolated axoneme | α-ODA  | Post-powerstroke     | 5                   | 640                   | 3.6             |
|               | Isolated axoneme | β-ODA  | Post-powerstroke     | 5                   | 640                   | 3.4             |
|               | Isolated axoneme | γ-ODA  | Post-powerstroke     | 5                   | 640                   | 3.3             |
|               | Isolated axoneme | IDA a,b,c,e | Post-powerstroke     | 5                   | 640                   | 3.5-3.9         |
Supplementary Video legends

**Supplementary Video 1** Live cell DIC light microscopy video of beating *Strongylocentrotus* (sea urchin) sperm. This video shows sea urchin sperm, rapidly swimming due to actively beating flagella. In this preparation, freshly harvested sea urchin sperm were diluted in artificial seawater.

**Supplementary Video 2** Live cell DIC light microscopy video of inhibited *Strongylocentrotus* (sea urchin) sperm. This video shows immotile sea urchin sperm. In this preparation, freshly harvested sea urchin sperm were diluted in artificial seawater containing the ATPase (dynein) inhibitor EHNA (2mM). Visible movement of the sea urchin sperm is passive, due to buffer flow.

**Supplementary Video 3** Mechanistic model of dynein movement. This video combines real electron microscopic data and a schematic model of the powerstroke cycle of dynein. The first part of the video shows three representative tomographic slices of dynein in the post-, pre-I and pre-II powerstroke states. Initially, a schematic outline of dynein is superimposed upon each of these slices to highlight conformational changes of dynein domains. The second part of the video highlights the proposed head-rotation mechanism of dynein movement, as described in this study.