

BIOPHYSICS

Force production of human cytoplasmic dynein is limited by its processivity

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Cytoplasmic dynein is a highly complex motor protein that generates forces toward the minus end of microtubules. Using optical tweezers, we demonstrate that the low processivity (ability to take multiple steps before dissociating) of human dynein limits its force generation due to premature microtubule dissociation. Using a high trap stiffness whereby the motor achieves greater force per step, we reveal that the motor's true maximal force ("stall force") is ~2 pN. Furthermore, an average force versus trap stiffness plot yields a hyperbolic curve that plateaus at the stall force. We derive an analytical equation that accurately describes this curve, predicting both stall force and zero-load processivity. This theoretical model describes the behavior of a kinesin motor under low-processivity conditions. Our work clarifies the true stall force and processivity of human dynein and provides a new paradigm for understanding and analyzing molecular motor force generation for weakly processive motors.

INTRODUCTION

Cytoplasmic dynein 1 (hereafter referred to as dynein) is a large >1.5-MDa multiprotein complex (1). As a member of the AAA+ [adenosine triphosphatase (ATPase) associated with various cellular activities] protein family (2), mammalian dynein performs a multitude of cellular functions. These include minus end-directed microtubule (MT) transport of various cargos (3), mitosis (4), nuclear positioning (5), and cell migration (6). The dynein holoenzyme is a dimer of heavy chains (HCs); these comprise the ring-shaped motor domain (MD; containing six AAA+ domains), the MT-binding stalk, and the dimerization domain (tail) (7). The latter forms a scaffold for associated subunits, such as light chains (LCs), light intermediate chains (LICs), and intermediate chains (ICs), as well as for regulatory cofactors, such as dynactin, Lis1 (lissencephaly protein 1), NudE (nuclear distribution factor E), and NudEL (NudE-like protein) (1).

In this work, we sought to clarify longstanding uncertainties regarding mammalian dynein's single-molecule, intrinsic stall force (maximal sustained force generation in the absence of cofactors) and processivity (ability to take multiple steps before MT dissociation, as measured by run length). Over the past several decades, dynein's molecular function has been investigated both *in vivo* and *in vitro*. Until recently, most studies have focused on dyneins from *Dictyostelium discoideum* and *Saccharomyces cerevisiae* (yeast) due to their stability, ease of genetic manipulation, and established purification protocols (8, 9). However, there are notable differences between the function of yeast dynein and that of higher eukaryotes (10). Even in the absence of cofactors, yeast dynein is highly processive. The run length of isolated yeast dynein is in the range of 1 to 3 μm (8, 11), with a measured stall force of ~4 pN (12, 13) [we show here that the previously reported 7-pN stall force (14) is likely the result

of an unintended electronic low-pass filtering of the trapping data; see Supplementary Materials]. In contrast, the study of mammalian dynein has yielded variable results. A wide range of single-molecule stall forces, between ~0.8 and 7 pN (15–21), have been reported, and single-molecule processivity has ranged from immeasurable (22, 23), to over several hundred nanometers (17), to 1 μm (20, 24, 25). A number of technical issues have likely contributed to the variability of both processivity and stall force measurements for mammalian dynein. These include variations in protein purification, protein labeling, and microscopy techniques. We discuss these issues in detail below, but much of the challenge in these measurements actually arises from the relatively low intrinsic processivity of mammalian dynein. Whereas yeast dynein frequently achieves its stall force before MT dissociation (as determined by a plateau or "stalling" of its displacement versus time) (14), mammalian dynein's low processivity results more frequently in short displacements with runs terminated by detachment from the MT rather than sustained force plateaus (17, 26). Do the forces achieved represent stall force or simply "premature" MT dissociation due to low processivity? How can stalling be defined objectively? Is processivity affected by force? Answering these questions in the absence of dynein cofactors has been challenging but important for at least two reasons. First, there are reports of dynactin-independent recruitment of dynein to organelles (27, 28), and understanding dynein function under these circumstances will be greatly aided by accurately characterizing its single-molecule processivity and force generation in isolation. Second, it is essential to study dynein function in isolation to understand the effects of its various cofactors. Despite variable processivity *in vitro* (see further discussion below), mammalian dynein complexes have been shown to move over several micrometers *in vivo* (17, 18, 29). However, this appears to be mediated by the presence of dynactin and other cofactors.

Recent publications have shown that when mammalian dynein is complexed with dynactin via the coiled coil-containing cargo adaptor, Bicaudal-D2 (BicD2), dynein moves over several micrometers *in vitro* [~5 (30) and ~9 μm (31)]. This augmented processivity appears to result from relief of a low MT-affinity, autoinhibited "phi-particle" (32) conformation [in which the stalks cross each other (7, 26)] by dynactin and BicD (as well as other coiled coil-containing cargo adaptors) (7). Cofactor binding reorients the two

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MDs parallel to each other and converts mammalian dynein from a weakly processive (10) to an ultraprocessive motor (30, 31, 33). It is likely that, by virtue of increased processivity, these dynein-dynactin-BicD2 complexes exhibit more classic stalling behavior and greater stall forces in optic tweezers assays (12, 34). However, without knowing dynein's intrinsic function, it is impossible to understand the effects of its cofactors on force generation. Is the stall force actually increased or is it the same but simply easier to observe? Might cofactor association increase processivity while actually reducing stall force? Moreover, although dynein appears to be more processive when bound by these cofactors, its intrinsic processivity is still somewhat uncertain (10, 22). We clarify several of these questions here by observing both processivity and force generation in an optic tweezers assay and developing a theoretical model to describe these observations and predict both intrinsic processivity and stall force despite the difficulty of observing these attributes directly. Below, we discuss some of the technical challenges in measuring mammalian dynein single-molecule function.

Different purification strategies and protein sources [brain (16, 17, 20, 23, 25) and tissue culture (26, 35)] may have resulted in varying fractions of inactive or conformationally inhibited proteins [see, e.g., (30, 31)]. In addition, when determining dynein processivity using optical microscopy (using attachment to fluorescence tags or visible beads to track the motor), the detection limit is typically several hundred nanometers. Therefore, run lengths for single motors have remained immeasurable in some studies (22, 23). In contrast, others have reported a run length of 1.3 μm for 1% of the cofactor-free dynein molecules (30). When bound to beads, mammalian dynein generates movement over ~ 0.7 to 1 μm (20, 24, 36). Conversely, using both Qdots suited for high-precision fluorescence microscopy and bead assays, Ori-McKenney *et al.* (17) measured a run length of ~ 0.3 μm for a subfraction of the motor population. In addition to a low fraction of moving motors, diffusive bidirectional events have also often been reported instead of, or in addition to, unidirectional movement (17, 20, 22, 23, 25). A recent study by Torisawa *et al.* (26) reported bidirectional diffusion biased toward the MT minus end, with an average run length of ~ 100 nm. In this case, it was attributed to an autoinhibited conformation of dynein dimers in the absence of cofactors. However, even in the presence of dynactin/BicD2, the diffusive fraction can constitute $\sim 30\%$ of all data (30).

In the present study, we analyze native human dynein purified from human embryonic kidney (HEK) 293 cells (37, 38) using single-molecule optical tweezers. With this method, we confirm a very low fraction of stalling events (≥ 200 ms), similar to other studies (17), which complicates stall force analysis. On the basis of the reported submicroscopic run length of mammalian dynein (10), we hypothesized that the ability to observe motor stalling is directly limited by the motor's weak processivity. That is, dynein force generation is commonly observed at trap stiffnesses of 0.01 to 0.02 pN/nm, resulting in the motor having to travel 50 to 100 nm to reach a force of 1 pN. Our analysis, which included a range of trap stiffnesses, indeed reveals a novel hyperbolic trap stiffness dependence for the average detachment forces, with a force plateau of ~ 2 pN at saturating adenosine triphosphate (ATP) concentrations. This force plateau coincides with the measured stall force at high trap stiffnesses. We further derive a theoretical model that accurately describes the hyperbolic trap stiffness dependence and the experimentally measured force plateau. This equation is independent of motor compliance and

allows for determination of the maximal force generation of dynein without the observation of motor stalling. We also find that a hyperbolic increase in measured forces correlates with a hyperbolic decrease in the corresponding distance displacements. Dynein's run length at zero load can therefore be estimated from the y intercept at a trap stiffness of zero, resulting in a zero-load run length of ~ 100 nm. Application of our experimental framework to full-length yeast dynein and kinesin-1, both of which are highly processive motors, reveals that, at low ionic strength, both motors are insensitive to changes within a practical trap stiffness range, while a similar trap stiffness dependence is observed for kinesin-1 at elevated ionic strengths. Our study, therefore, provides a method for determining the force-free processivity and stall force of mammalian dynein (and possibly other cytoskeletal motors), without the need to directly measure dynein displacements at zero load or to directly measure motor stalling. Thus, our work clarifies longstanding discrepancies regarding mammalian dynein single-molecule functional properties and provides a novel framework for studying weakly processive molecular motors in general.

RESULTS

Processivity and force generation of individual native human dynein complexes

To determine the motion and force generation capabilities of human dynein, we used a native human dynein containing a multifunctional streptavidin- and green fluorescent protein (GFP)-tagged intermediate chain (mfGFP-IC) (37, 38). Tagged dynein complexes were purified from HEK293 cells using the streptavidin tag in the mfGFP-IC construct via one-step StrepTrap column purification (fig. S1A). This yielded mostly dynein HC dimers, as confirmed by blue native polyacrylamide gel electrophoresis (BN-PAGE; fig. S1B). Western blot analysis further confirmed the copurification of dynein subunits (IC, LC, and LIC) and showed trace amounts of cofactors, including Lis1, nuclear distribution element 1 (Nde1), and dynactin p150 subunit (fig. S1C). To remove inactive motors, bound cofactors, dynein aggregates, and other possible contaminants, we then conducted a second purification step using the MT-binding and release (MTBR) assay. Here, dynein was bound to MTs in the presence of the ATP analog, AMP-PNP (adenylyl-imidodiphosphate). Active motors were then released by the addition of ATP and salt (fig. S1D). After MTBR, cofactors were no longer detectable by Western blot, and when adhered to carboxyl-trapping beads, the purified dynein complex exhibited exclusively minus end-directed motility (see Supplementary Materials for additional information about the characterization of the StrepTrap- and MTBR-purified protein fractions).

To next test whether native human dynein is a weakly processive motor in isolation, we conducted optical trapping studies (Fig. 1, A and B). For these analyses, dynein was adhered to 1- μm -diameter carboxyl beads, and processive motion was measured by determining the motile bead fraction as a function of relative motor bead concentration (Fig. 1C). In contrast to single-molecule fluorescence microscopy and MT-landing rate assays that have a resolution limit of several hundred nanometers (22, 30, 31), bead displacements in the sub-100-nm range can be resolved in the optical trap (Fig. 1B). Because most publications on mammalian dyneins [see, e.g., (15, 17, 18, 20, 21)] have reported stall forces between 1 and 2 pN, we chose a trap stiffness of $k = 0.01$ pN/nm, which is expected to result in bead-trap

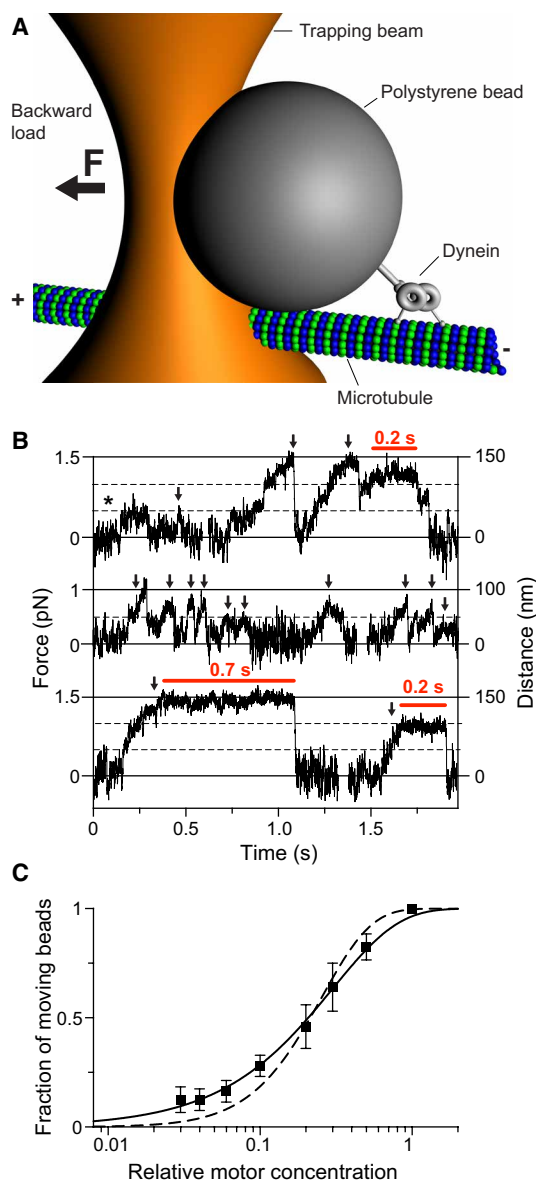


Fig. 1. Optical trapping assay to probe dynein force generation. (A) Schematic of the optical tweezers assay (not to scale). A 0.9- μm -diameter carboxyl bead is nonspecifically bound to a purified single human dynein and is trapped by a near-infrared optical trapping beam focused via a high-numerical aperture microscope objective lens. The trap holds the bead directly above an MT that is covalently linked to the glass surface of the coverslip. When the dynein binds to and moves along the MT in the presence of ATP, it pulls the attached bead with it. The trap resists this motion, exerting a force $F = -k \Delta x$ on the bead-motor complex, where k is the trap stiffness and Δx is the distance from the trap center to the center of the bead. (B) Example traces at 1 mM ATP and $k = 0.01$ pN/nm (see also fig. S2B). Stalling events (red horizontal bars) can be observed but are rare. Fast events, including large single forward-backward steps without any resolved intermediate steps (black star), are frequent. Events that are counted as force generation events are marked with black arrows. (C) Dilution curve counting beads as moving if forces equaled or exceeded 0.5 pN. Error bars were calculated assuming a binomial distribution. Twelve to 85 beads were tested for each dilution ($N_{\text{total}} = 318$). The curves are fits to equations assuming processive motors (Eq. 6, $\lambda = 3.3 \pm 0.1$, solid line, $R^2 = 0.996$) and nonprocessive motors (Eq. 7, $\lambda = 7.9 \pm 0.7$, dashed line, $R^2 = 0.949$).

separations of 100 to 200 nm. A bead was counted as moving if its displacement was ≥ 50 nm, equivalent to ≥ 0.5 pN. The dilution curve obtained from this assay was then analyzed on the basis of two models [see Materials and Methods, Supplementary Materials, and (39–41)]: one describing force generation by one or more motors (“processive” model) and the other describing force generation driven by two or more motors (“nonprocessive” model). We found that our data were best described by the processive model [$R^2 = 0.996$; Akaike’s information criterion (AIC) probability of 99.99%]. This was true even when beads displaying exclusively large (up to 50 nm) single forward-backward steps, without any resolved intermediate steps (Fig. 1B), were counted as nonmoving ($R^2 = 0.992$; AIC probability of 99.97%; fig. S2A) [we suspect that these displacements are generated by dynein motors that transition reversibly from the autoinhibitory to the open conformation (7)]. We, therefore, find that under these experimental conditions, native human dynein is a weakly processive motor (17).

The “stall force” of single human dynein complexes at saturating [ATP] was next determined by analyzing trapping data obtained at the single-molecule level [in this case, $\leq 30\%$ bead motility (39–41)]. Stalling was defined as a ≥ 200 -ms force plateau [as in (17)], excluding jump-like events to such a plateau (Fig. 1B). The resulting stall force histogram (Fig. 2A) followed a Gaussian distribution, with an average of 0.9 ± 0.3 pN ($\pm\text{SD}$; $N = 77$; $k = 0.01$ pN/nm), which is consistent with previously published stall forces for mammalian dyneins (15, 17, 18, 20, 21). However, the vast majority of runs did not result in stalling, and in most cases, dynein released prematurely from the MT (Fig. 1B, middle). Hence, the stall percentage (number of stalling events/number of all events $\times 100\%$) was extremely low (6.8%). In addition, the detachment force, defined as the average maximum force of all events, was found to be significantly lower (0.63 ± 0.01 pN, $\pm\text{SEM}$) than the stall force (0.89 ± 0.02 pN, $\pm\text{SEM}$) (Fig. 2, A and B). It has been previously noted (42) that motors with low processivity, such as kinesin-5 (Eg5), rarely show clear stalling events before dissociation. Our observations therefore suggest that the limited processivity of human dynein in isolation may limit the observed stall force in the optical tweezers assay.

Processivity limits the measured peak force of isolated human dynein

To determine whether dynein’s weak processivity did decrease its measured force generation, we repeated the optical trapping experiments at a trap stiffness of 0.03 pN/nm (such that dynein has to move only ~ 33 nm to reach a force of 1 pN). As expected, both the stall forces (Fig. 2A) and the detachment forces (Fig. 2B) increased with elevated trap stiffness (for all presented detachment force analyses, we took all events into account that were identifiable as force generation events, even when they occurred below 0.5 pN). Next, we raised the trap stiffness to 0.03 pN/nm for a given dynein-bound bead, following data acquisition at 0.01 pN/nm, to show that individual motors exhibiting one force generation behavior at a lower trap stiffness exhibit larger peak forces at higher trap stiffness. As expected, we found that force output increased as the trap stiffness was raised (Fig. 2C). We then determined whether the increased force generation at higher trap stiffnesses is reversible or whether dynein switches into a persistent high-force state, even if the trap stiffness is reduced again. To distinguish between these possibilities, we changed the trap stiffness from 0.06 to 0.01 pN/nm and back to

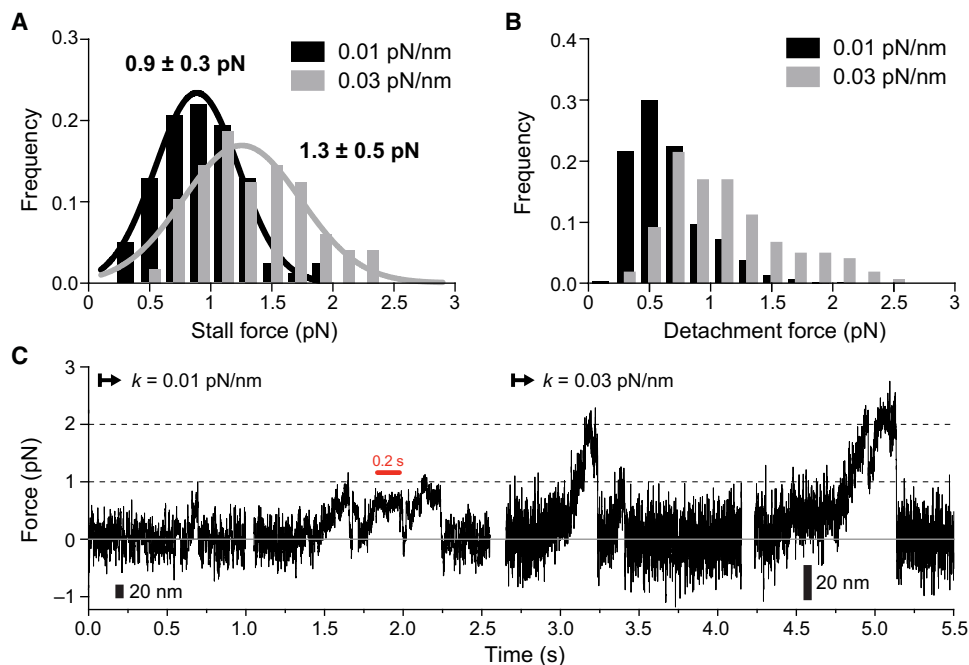


Fig. 2. Force generation of human dynein increases with increasing trap stiffness. (A) Stall force histograms of StrepTrap-purified and MT-binding released human dynein counting stalling events ≥ 200 ms and excluding jump-like behavior measured at $k = 0.01$ pN/nm (black bars) and $k = 0.03$ pN/nm (gray bars). The Gaussian distributions (solid curves) are centered at 0.9 ± 0.3 pN (\pm SD; $N = 77$) and 1.3 ± 0.5 pN (\pm SD; $N = 48$). (B) All measured forces (“detachment forces”) acquired at $k = 0.01$ pN/nm (mean force: 0.64 pN; $N = 572$) and $k = 0.03$ pN/nm (mean force: 1.1 pN; $N = 225$). (C) Example record showing force generation events of a single dynein molecule bound to trapping bead measured at 0.01 pN/nm (left) and subsequently at 0.03 pN/nm (right), demonstrating an increase in force generation with increasing trap stiffness.

0.06 pN/nm (fig. S3). From these experiments, we found that the trap stiffness-induced increase in measured force generation is reproducible.

To determine dynein’s maximal force-generating capabilities, the impact of its weak processivity must be negligible. Therefore, we adjusted the trap stiffness over a wide range of values (0.005 to 0.1 pN/nm), thereby probing the effects of run length on the measured forces. The force histograms obtained at 0.01 and 0.02 pN/nm (Fig. 2B and fig. S4) are in the range of previously reported values (15, 17, 18, 20, 21, 26). In addition, the obtained mean detachment forces are statistically indistinguishable from the mean detachment forces measured at the same trap stiffnesses for dynein motors that are attached specifically to the beads coated with anti-GFP antibodies against the GFP-tagged IC of dynein ($P < 0.97$ at 0.01 pN/nm and $P < 0.83$ at 0.02 pN/nm for nonspecific versus specific bead coupling; fig. S4), demonstrating that the measured detachment forces do not depend on the coupling strategy. When plotting the cumulative distribution functions (CDFs) of the detachment forces measured at low to high trap stiffnesses (Fig. 3A), a continuous increase in force was observed, a phenomenon that has, to our knowledge, not been reported previously. In contrast, the CDFs of the detachment distances (Fig. 3B) showed a continuous decrease with increasing trap stiffness, consistent with the trend observed for the three initially tested trap stiffnesses of 0.01 , 0.02 , and 0.03 pN/nm. This dependence on trap stiffness for both the average detachment force and displacement distance appears to follow a hyperbolic function (Fig. 3C), with the forces approaching a force plateau at infinite trap stiffness and the distances decaying to zero. We, therefore, hypothesized that this detachment force plateau corresponds to the maxi-

mal force that a single mammalian dynein motor can generate in isolation.

The average detachment force increases hyperbolically with trap stiffness

To test our hypothesis that the average detachment force increases hyperbolically as a function of the trap stiffness and that the force plateau corresponds to the maximal force that a dynein molecule can generate in isolation, we derived an analytical equation for the average detachment force. In this model, the average detachment force is defined as follows

$$\langle F \rangle = \int_0^{F_s} F p(F) dF \quad (1)$$

where F_s is the stall force, and $p(F)$ is the probability density of the detachment forces. This probability density is related to the motor’s force-dependent unbinding rate ϵ , its force-velocity relation $v(F)$, and its compliance (43); here, the force-dependent stepping behavior of dynein’s two MDs (44–46) is effectively accounted for by $v(F)$. However, because we record the trajectory of the trapped bead and not the trajectory of the moving motor (the trajectories of the bead and motor would only coincide if the motor were noncompliant), we transformed these quantities into parameters that describe the motor-powered bead movement. We, therefore, derived the probability density function as follows

$$p(F) = \frac{\epsilon(F)}{k v_b(F)} e^{-\int_0^F \frac{\epsilon(F')}{k v_b(F')} dF'} \quad (2)$$

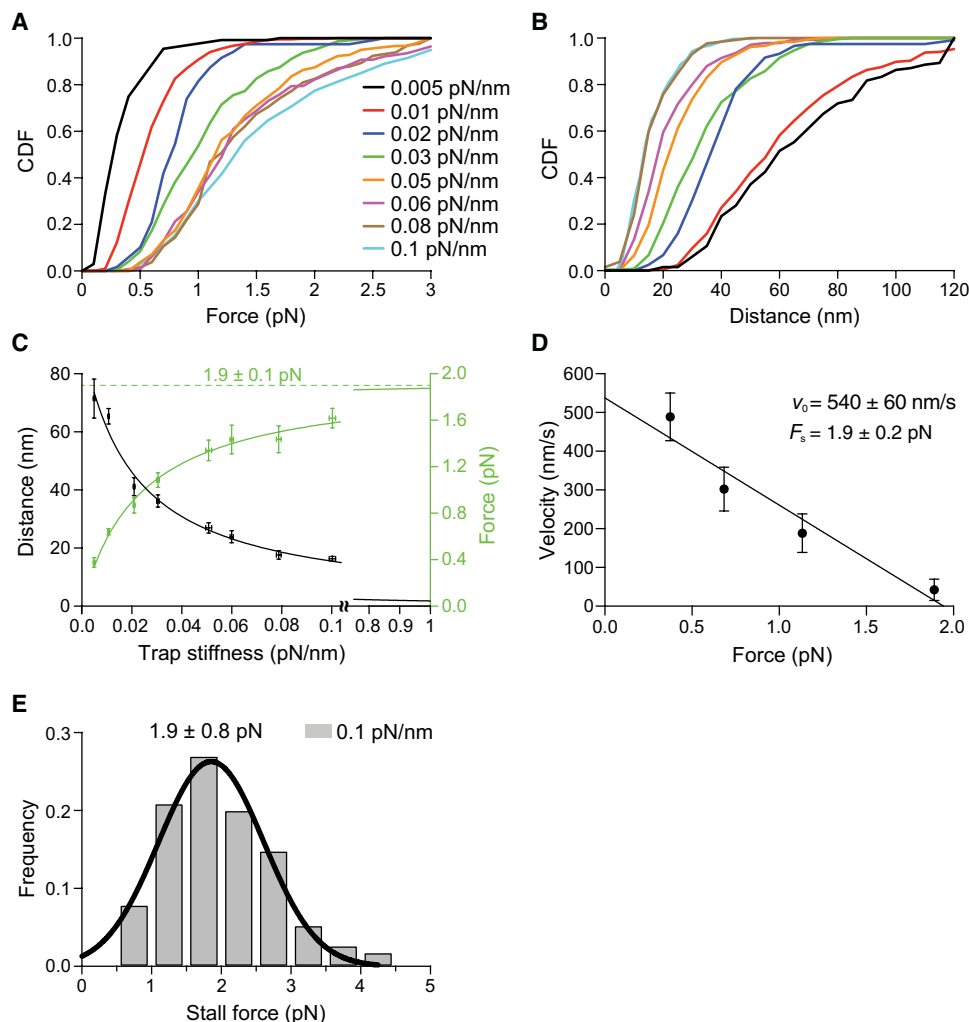


Fig. 3. Force production of human dynein is limited by its processivity. (A and B) CDFs of measured detachment forces (A) and corresponding displacements (B) acquired at various trap stiffnesses: black, $k = 0.005$ pN/nm; red, $k = 0.01$ pN/nm; blue, $k = 0.02$ pN/nm; green, $k = 0.03$ pN/nm; orange, $k = 0.05$ pN/nm; magenta, $k = 0.06$ pN/nm; brown, $k = 0.08$ pN/nm; cyan, 0.1 pN/nm. (C) Forces and distances as a function of trap stiffness [trap stiffnesses are displayed as mean \pm 95% confidence intervals (CIs)]. The average distances (left y axis, black spheres) and average forces (right y axis, green spheres) (the error bars represent the 95% CI of the mean) were fit to Eqs. 5 and 4 (black curve, $R^2 = 0.983$; green curve, $R^2 = 0.991$), resulting in the force-free run length $x_0 = 91 \pm 5$ nm (\pm SEM) and the stall force F_s depicted by the dashed line (mean \pm SEM). (D) Velocity versus force. Points are means, and error bars span 95% CIs of the mean. The solid line is a linear fit that intercepts the ordinate at 540 nm/s (± 60 nm/s, \pm SEM) and the abscissa at 1.9 pN (± 0.2 pN, \pm SEM; $N = 83$ to 84 at each force; 335 events total). (E) Stall force histogram compiling stall forces (≥ 200 ms) at $k = 0.1$ pN/nm ($N = 115$; mean \pm SD from Gaussian fit).

where $\epsilon(F)$ is the force-dependent unbinding rate, k is the trap stiffness, and $v_b(F)$ the force-velocity relation of the bead [as $\epsilon(F)$, k , and $v_b(F)$ have the units $1/s$, N/m , and m/s , $p(F)$ has the unit $1/N$]. To derive an analytical solution, we made two assumptions. First, we presumed that the force-velocity relation of the bead decreases linearly with the force, such that

$$v_b = v_0(1 - F/F_s) \quad (3)$$

where v_0 is the force-free velocity. By extracting the force-velocity relation from the recorded traces, we indeed find that this equation describes the experimental force-velocity relation reasonably well (Fig. 3D). Our second assumption is a force-independent constant unbinding rate, $\epsilon(F) = \epsilon$. While this is a rather crude approximation, we find that the resulting analytical solution fits the data well

(see below). As a further validation of our theoretical framework, we also investigated a scenario in which the unbinding rate depends exponentially on force and obtained very similar estimates for the stall force and the force-free run length (fig. S5). These results suggest that the stall force and the force-free run length are well constrained parameters, but that the accurate form of the force-dependent unbinding rate cannot be obtained using this approach (see Supplementary Materials for a detailed discussion). Therefore, the assumption of a constant unbinding rate is sufficient for our analysis, and together with the linear force-velocity relation for the bead, we can finally obtain an analytical equation describing the average detachment force

$$\langle F \rangle = \frac{F_s}{1 + \frac{F_s}{k x_0}} \quad (4)$$

where $x_0 = v_0/\epsilon$ is the force-free run length. Fitting this equation to the experimental data, we obtain a value of 1.9 ± 0.1 pN (\pm SEM) for F_s and 87 ± 6 nm (\pm SE) for the force-free run length, x_0 . If we restrict the fit to the data acquired at trap stiffnesses up to only 0.05 pN/nm, we obtain a very similar result (1.9 ± 0.1 pN and 85 ± 7 nm), demonstrating that this method yields reliable results even if we obtain data in a relatively narrow range of trap stiffnesses. In addition, at the limit for an increased trap stiffness, $\langle F \rangle \rightarrow F_s$. Providing further support that this analytical equation describes the data well enough, we find that the resulting value for F_s coincides with both the stall force value resulting from the force-velocity analysis (1.9 ± 0.2 pN, \pm SE; Fig. 3D) and with the stall forces (≥ 200 ms) measured at the largest trap stiffness of 0.1 pN/nm (1.9 ± 0.1 pN, \pm SE; Fig. 3E). In contrast, stall forces measured below 0.04 pN/nm deviate significantly from the hyperbolic plateau (Fig. 2A), suggesting that the ≥ 200 -ms force plateaus at low trap stiffnesses do not correspond to true stalling events.

Analogous to the hyperbolic relationship between the detachment force and the trap stiffness, the average detachment distance decays hyperbolically with increasing trap stiffness (Fig. 3, B and C). As is the case for the average detachment forces, the data can be fit to the theoretical average run length by the equation

$$\langle x \rangle = \langle F \rangle / k = \frac{x_0}{1 + \frac{kx_0}{F_s}} \quad (5)$$

At the limit for a decreasing trap stiffness, the average run length converges to the force-free run length, $\langle x \rangle \rightarrow x_0$.

Thus, our theoretical analysis shows that the average detachment force and the average run length depend hyperbolically on the trap stiffness. In addition, the force plateau corresponds to the stall force of the motor. This suggests that the detachment forces measured at commonly used trap stiffnesses (0.005 to 0.04 pN/nm) may be limited by dynein's processivity. We, therefore, hypothesized that this behavior should also be observed with highly processive motors under conditions where their processivity is significantly impaired. To test this possibility, we analyzed two other MT-associated molecular motors—the highly processive yeast dynein and the plus end-directed kinesin-1—as a function of the trap stiffness in buffers of varying ionic strength.

Force generation of yeast dynein is insensitive to changes within a practical trap stiffness range

Yeast cytoplasmic dynein is a highly processive molecular motor that displays run lengths between 1 and 3 μ m, with some runs up to 20 μ m (8, 11, 47), and stall forces reported as high as 7 pN (14) (see Supplementary Materials for a detailed theoretical analysis that suggests that the previously measured 7-pN stall force likely resulted from an unintended electronic low-pass filtering during data acquisition). We analyzed the forces generated by the full-length yeast dynein construct, VY97-GFP [a GFP-tagged full-length motor (14)], at trap stiffnesses between 0.03 and 0.05 pN/nm (Fig. 4). Analysis of trap stiffnesses below 0.03 pN/nm was not feasible due to the limited reach of our optical trap (< 300 nm), as escape of the dynein-bound beads from the trap occurred at lower trap stiffnesses. In a buffer system containing 30 mM Hepes and 100 mM KAc (potassium acetate stock solution pH adjusted to 7.0; see Materials and Methods), we did not detect any significant effect of trap stiffness on force (Fig. 4B), and we obtained an average stall force of 4.5 ± 0.7 pN

(\pm SD; Fig. 4C). This is in agreement with our recent work (13) but is somewhat smaller than the previously published value (14) for the same construct (see Supplementary Materials for discussion). Therefore, even in 100 mM KAc, yeast dynein is sufficiently processive for its run length to have no impact on the observed forces. These trap stiffness-independent results also serve as internal control to show that our trapping instrument was accurately calibrated and functioning as expected.

Force generation of kinesin-1 becomes sensitive to trap stiffness at high ionic strength

We next analyzed the plus end-directed motor kinesin-1 using the well-characterized construct, K560-GFP, a 560-amino acid-long, tail-truncated construct of human conventional kinesin-1, with a C-terminal F64L/S65T variant of GFP (48–50). Because this construct has been previously shown to display significant salt-dependent reductions in run length (50), we considered it to be an ideal candidate to test the relationship between processivity and force generation for a kinesin motor.

To first confirm the functionality of the K560 construct, we conducted an MT-gliding assay, which yielded a velocity of 670 ± 10 nm/s (\pm SEM) at 1 mM ATP in Pipes-Hepes buffer (PHB). This is almost twice the velocity previously reported by Thorn *et al.* for the same construct in buffer containing 12 mM Pipes, 2 mM MgCl₂, and 1 mM EGTA (pH 6) (BRB12) (50), but is comparable to the value reported by Friedman *et al.* (51) (also in BRB12). For optical trapping experiments, the GFP moiety fused to K560 was used to specifically couple the motor to beads coated with anti-GFP antibodies. We then determined the single-molecule stall force of K560 in 80 mM Pipes, 2 mM MgCl₂, and 1 mM EGTA (pH 6.8) (BRB80), a buffer system typically used to study kinesin. This yielded a value of 5.7 ± 0.9 pN (\pm SD; stall plateaus ≥ 200 ms; Fig. 5, A to C), which is in agreement with the previously reported stall forces between 5 and 7 pN (39, 52–56). In addition, both the stall forces and the detachment forces were independent of the trap stiffness in BRB80 (Fig. 5B). The stall force was also found to be ~ 1.4 times greater than the detachment force, which is in good agreement with the ratio reported for kinesin-1 by Furuta *et al.* (6.8/5.2 pN ~ 1.3 , BRB12) (55).

When force measurements for K560 were obtained in PHB, we observed a slight dependence of the detachment force and distance on trap stiffness (Fig. 5D). However, the stall force F_s that resulted from the fit of Eq. 4 to the measured detachment forces (5.6 ± 0.1 pN, \pm SEM; Fig. 5D), agreed well with the stall forces (≥ 200 ms) measured for the highest trap stiffness in PHB (5.7 ± 0.2 pN, mean \pm SEM; 0.09 pN/nm) and with the stall force in BRB80 (Fig. 5C), indicating that the intrinsic stall force of K560 is not significantly reduced by the PHB system.

Last, to determine whether a systematic increase in buffer ionic strength results in a more pronounced effect of the trap stiffness, the PHB system was supplemented with increasing amounts of KAc. We observed a gradually heightened sensitivity to the trap stiffness with the addition of 20 to 40 mM KAc (Fig. 5D), whereas at high trap stiffnesses, the extrapolated force plateaus were similar to the stall force observed in BRB80 (Fig. 5, C and D). Only the buffer with the highest KAc concentration resulted in a significantly decreased value for F_s of 4.6 ± 0.4 pN (\pm SE from the fit with Eq. 4). Our data therefore suggest that kinesin's processivity becomes more and more limiting with increasing salt concentration, leading to trap stiffness-dependent forces. This hypothesis is further substantiated

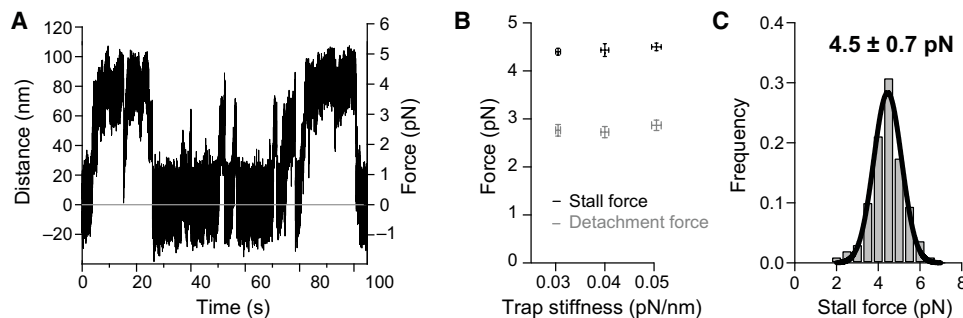


Fig. 4. Force generation of yeast dynein is insensitive to changes within a practical trap stiffness range. (A) Example trace showing stalling events and premature detachments of full-length yeast dynein ($k = 0.05$ pN/nm). Yeast dynein was specifically attached to beads coated with anti-GFP antibodies. (B) Forces versus trap stiffness. Values of stall forces (≥ 10 s, black) and of all events (gray) do not depend on the trap stiffness in the measured trap stiffness range (the error bars represent the 95% CIs of the means of the measured forces and trap stiffnesses). (C) Histogram of stall forces measured at various trap stiffnesses ($N = 486$; mean \pm SD).

by the finding that the y intercepts of the hyperbolic distance fits with Eq. 5 decay with increasing [KAC], suggesting a decreased run length (Fig. 5D). These observations—in combination with the data obtained for human dynein—thus indicate that the y intercept ($k = 0.00$ pN/nm) in a distance versus trap stiffness plot is a measure of a motor's run length at zero load.

DISCUSSION

Human dynein is an essential motor protein that is responsible for a multitude of transport and force-generating processes within the cell (1, 3). However, despite its central role in cellular biology, the biochemical and biophysical properties of dynein have not been fully deciphered. This is evidenced by the range of run lengths (17, 20, 22–25, 36) and forces (15–21) (see Introduction) that have been reported for this protein.

In this study, our analysis of human dynein in the absence of cofactors suggests that the forces generated by this protein in optical trapping assays are limited by the motor's processivity. This is indicated by the hyperbolic trap stiffness dependence that is observed for the measured forces and distances. The hyperbolic force plateau for all force-generating events at an infinite trap stiffness was found to coincide with human dynein's stall force (≥ 200 ms force plateau) at high trap stiffnesses (1.9 ± 0.1 pN, \pm SEM). High stall forces of up to 2.4 pN can be observed, even at low trap stiffness (0.01 pN/nm; see example traces in fig. S2B), but these are extremely rare. In addition, analogous to the detachment forces, our data imply that the run distance calculated for a trap stiffness of 0.00 pN/nm corresponds to dynein's run length in the absence of load (91 ± 5 nm, \pm SE).

Our hypothesis that trap stiffness-dependent forces act as an indicator for limiting motor processivity is further substantiated by trapping experiments with two other molecular motors. As expected, the highly processive yeast dynein motor (8, 11, 47) did not show trap stiffness-dependent behavior in our optical trap with a reach of ~ 300 nm, which allowed for trap stiffnesses equal to, or above, 0.03 pN/nm. Lower trap stiffnesses resulted in the motor frequently moving out of the trap due to its high processivity and yielded forces of ~ 4.5 pN (see Fig. 4), thereby preventing the determination of an accurate detachment force. The processivity of the kinesin-1 motor construct, K560, in contrast, could be sufficiently modulated by the addition of potassium acetate to the buffer. As predicted, increasing amounts of salt led to a heightened dependence on trap stiffness for both the detachment forces and run distances (see Fig. 5). Although

the stall forces, as determined by the force plateau of the hyperbolic plot, did not depend significantly on the salt concentration, the distances extrapolated to a trap stiffness of 0.00 pN/nm decreased with the addition of salt, suggesting a decreased run length at zero load (see Fig. 5). Thus, our observation of trap stiffness-dependent behavior for kinesin in elevated salt buffers, but not in the traditional low-salt kinesin buffer system, BRB80, reinforces the validity of our observations with human dynein. We hypothesize that such trap stiffness-dependent forces can be expected for other molecular motors with limiting processivity. However, whether such a behavior is measurable depends on a combination of parameters, including the reach of the optical trap and, thus, the range of usable trap stiffnesses, the presence of vertical force components (57), as well as the motor's processivity and its intrinsic stall force (see above, yeast dynein). In support of the broad relevance of this phenomenon, a similar trap stiffness-dependent behavior was reported for the molecular motor, kinesin-5 (Eg5), which generated a stall force of 1.5 pN under low-processivity conditions (58). Similar to human dynein in this study, Eg5 does not show clear stalling events before dissociation, indicative of low processivity (42).

The processivity of a motor is highly dependent on the experimental conditions under which it is measured, including the buffer system. Therefore, a range of observed forces would be expected for any given trap stiffness, depending on the buffer that is used. Moreover, different trap stiffnesses are expected to produce different observed forces in the same buffer system. Thus, the trap stiffness dependence revealed in this study could explain the range of in vitro forces, reported to be between 1 and 2 pN, for human dynein (15, 17, 18, 21). The higher forces reported by Walter *et al.* (16) and Toba *et al.* (19), however, are not consistent with our findings at the single-molecule level. Although the reason for this is unclear, possible explanations include the presence of motor aggregates, dynein cofactors, or posttranslational modifications on purified dynein in these publications. Alternatively, contamination with other molecular motors, such as the possible copurification of kinesin-1 with our StrepTrap-purified human dynein (see Supplementary Materials and fig. S6), is difficult to completely rule out, as trace amounts of protein that are undetectable by Western blot analysis could be sufficient to account for the observed single-molecule behavior.

A recent publication (18) has reported that forces measured in vivo for retrograde transport by dynein cluster in 2-pN increments, and the authors attribute this to the cooperation between two dynein dimers. However, our current findings would suggest that the unit

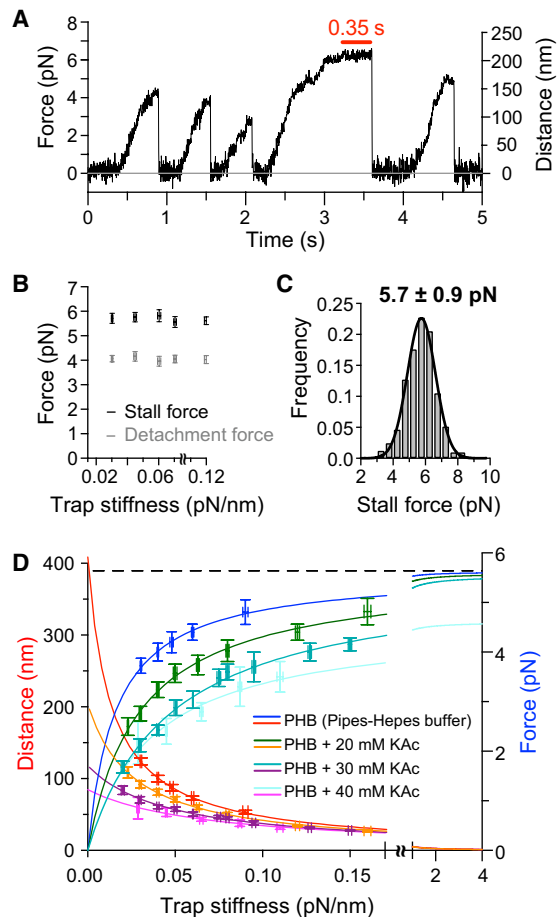


Fig. 5. Kinesin force generation becomes sensitive to changes in trap stiffness with increasing ionic strength. (A) Example trace of K560 in BRB80 trapping buffer showing a stalling event (red horizontal bar) and events without stalling ($k = 0.03$ pN/nm). (B) Stall forces (stalling for ≥ 200 ms; black crosses) and detachment forces (gray crosses) measured in BRB80 (the error bars are the 95% CIs of the mean forces and trap stiffnesses). (C) Stall force histogram measured in BRB80 ($k = 0.03$ to 0.12 pN/nm, $N = 409$, $F_{\text{stall}} = 5.7 \pm 0.9$ pN; mean \pm SD). (D) Trap stiffness dependence of all events in Pipes-Hepes trapping buffer (blue, red), Pipes-Hepes trapping buffer +20 mM KAc (green, orange), +30 mM KAc (dark cyan, purple), and +40 mM KAc (light blue, magenta). Fitting Eq. 4 to the detachment forces (right y axis, circles, diamonds, hexagons, and crosses) and Eq. 5 to the distances (left y axis, triangles, squares, and plus signs) reveals the same hyperbolic dependence (solid curves) on the trap stiffness as human dynein (see Fig. 3C) (the error bars are the 95% CIs of the means of the measured forces, distances, and the trap stiffnesses). The dashed line depicts the average stall force in BRB80 [5.7 ± 0.9 pN; see (C) for histogram].

force of a single dynein dimer is ~ 2 pN, in agreement with a recent study on recombinant full-length human dynein (12). Torisawa *et al.* (26) reported a force generation for human dynein of ~ 0.9 pN at a 0.01 pN/nm trap stiffness, a value that we also confirmed (Fig. 2A). However, this study further found that force generation does not change when the trap stiffness is increased to 0.028 pN/nm. Further studies will be required to resolve this apparent discrepancy.

CONCLUSION

In this study, we demonstrate that the peak force generation of mammalian cytoplasmic dynein measured in optical tweezers experiments is strongly influenced by its limited processivity and,

therefore, by the trap stiffness. At relatively weak trap stiffness, true stalling is exceedingly rare, because the weakly processive motor simply detaches from the MT before reaching a sufficient displacement to achieve its maximal force. As a result, the measured peak force generation increases as the trap stiffness is increased. We develop a theoretical model to describe the force generation as a function of processivity and trap stiffness and use this model to precisely and definitively determine human dynein's stall force and its zero-load processivity. Our findings resolve a longstanding uncertainty regarding the stall force and processivity of mammalian dynein while simultaneously providing a straightforward, self-consistent methodology for analyzing the single-molecule function of this and other weakly processive molecular motors. This framework provides a much more accurate and precise estimate than the analysis of rare stalling events at low trap stiffnesses and avoids the variability associated with arbitrarily chosen definitions of stalling (52).

This work provides key information on the intrinsic function of human dynein that is necessary to completely understand the effects of cofactors including dynactin and BicD. Using the results and experimental framework developed here will enable future experiments to unambiguously characterize the effects of these cofactors on both processivity and force generation independently. Moreover, although we have shown here that stall force and processivity are indeed distinct functional properties, the challenges encountered with measuring the stall force of a weakly processive motor illustrate the biological importance of processivity to generating force. For example, enhanced processivity (via cofactor association or multimotor ensembles) may be required to generate substantial forces when dynein associates with cargoes via highly compliant linkages such as cell membranes, regardless of the intrinsic maximal force generation capability. In other words, the strength of the motor can only be realized to the extent it can remain attached to its MT track. Last, the characterization of intrinsic dynein function will aid our understanding of biological scenarios in which dynein associates with organelles in the absence of dynactin (27, 28).

MATERIALS AND METHODS

Materials

Chemicals were obtained from Thermo Fisher Scientific or Sigma-Aldrich unless otherwise stated. All buffers were prepared using UltraPure distilled water from Life Technologies.

Human dynein-mfGFP-IC74 purification and characterization

HEK293 cells capable of doxycycline-induced mfGFP-IC74 (multi-functional GFP-tagged human cytoplasmic dynein intermediate chain) expression were obtained from T. Murayama [Department of Pharmacology, Juntendo University School of Medicine, Tokyo, Japan (37, 38)]. Cytoplasmic human dynein was purified from HEK cells essentially as described in (37, 38), utilizing the streptavidin tag contained in the mfGFP-IC in a one-step StrepTrap column purification (see Supplementary Materials). The purity and composition of the human dynein complex were analyzed using standard SDS-PAGE, native light blue PAGE, and Western blot analyses (see Supporting Material).

Kinesin-1 and yeast dynein purifications

Kinesin-1 [K560-GFP (48–50)] and full-length yeast dynein (VY97) were purified as described previously (8, 59). Active

motors were obtained in MT-binding release assays (protocol see below).

MT-binding release assay

To further purify the motor proteins used in this study (yeast dynein VY97, kinesin-1 K560, and mfGFP-IC74 human cytoplasmic dynein), the motors were subjected to an MTBR, in which the motors were bound to MTs in a strong-binding state [mimicked by the ATP analog AMPPNP (adenylyl-imidodiphosphate)], and functional motor proteins were subsequently released in the presence of salt and ATP (see Supporting Material).

Optical trapping assay

Coverslips for optical trapping assays were cleaned and coated as previously described (59, 60) using a series of cleaning steps (sonication in mucosal and plasma cleaning) followed by coating with APTES or AE-APTES [(3-aminopropyl)triethoxysilane and *N*-(2-aminoethyl)-(3-aminopropyl)triethoxysilane, respectively]. Fluorescently labeled MTs were covalently attached to the coverslips via glutaraldehyde.

Proteins were attached either nonspecifically to polystyrene beads (0.9 μm ; Bangs Laboratories Inc.) or specifically via anti-GFP antibodies (fig. S4). Antibody-bound beads were prepared as described previously (59). Motors were diluted in pure buffer for non-specific binding and in buffer plus β -casein (1 mg/ml) when using antibody-coated beads. β -Casein used in these buffers was purified as described previously (59). Motor proteins at a given dilution were incubated with the beads for 10 min on ice before adding the final trapping solution. All final trapping solutions contained an ATP regeneration system [2 mM phosphoenol pyruvate, pyruvate kinase (0.1 mg/ml)], an oxygen scavenger system [22.5 mM glucose, pyranose oxidase (3 U/ml), 90 U/ml catalase (90 U/ml)], and 10 μM Taxol. Depending on the assay, trapping buffers used were as follows: PHB [50 mM Pipes, 50 mM Hepes, 2 mM MgCl_2 , 1 mM EGTA (pH 7.0), 10 mM dithiothreitol (DTT), 1 mM guanosine 5'-triphosphate (GTP) or varying amounts of ATP, β -casein (1 mg/ml)]; Pipes-Hepes-KAc trapping buffer [50 mM Pipes, 50 mM Hepes, 20 or 30 or 40 mM KAc, 2 mM MgCl_2 , 1 mM EGTA (pH 7.0), 10 mM DTT, 1 mM ATP, β -casein (1 mg/ml)]; Hepes-KAc trapping buffer [30 mM Hepes, 100 mM KAc, 2 mM Mg acetate, 1 mM EGTA (pH 7.2), 10 mM DTT, 1 mM ATP, β -casein (1 mg/ml)]; and BRB80 trapping buffer [80 mM Pipes, 2 mM MgCl_2 , 1 mM EGTA (pH 6.8), 10 mM DTT, 1 mM GTP or 1 mM ATP, β -casein (1 mg/ml)]. Trapping assays were performed at 25°C.

The optical trapping microscope was calibrated as previously discussed (59). Position and trap stiffness calibrations were performed for each bead tested. The trap stiffness was calculated using a “hybrid” method of calibration (61), combining the power spectrum and viscous drag analyses, which permits trap stiffness calibration without the need to know the drag coefficient. The microscope body is a Nikon Eclipse Ti-U. A single, custom-designed polychroic mirror guides various lasers into the objective and has high transmission bands for emission of several fluorescent dyes including Cy3/TMR (tetramethylrhodamine) to monitor TRITC (tetramethyl rhodamine isothiocyanate)-labeled MTs using a 532-nm laser. To visualize trapped beads, a 470-nm light-emitting diode lamp was used for bright-field illumination using a charge-coupled device camera. Trapping was performed with a 10-W, 1064-nm laser (IPG Photonics) chosen for its output power stability. Detection of

bead position with nanometer precision is accomplished by back focal plane detection using an 830-nm laser. Data were acquired using a custom-written software in LabVIEW (National Instruments) and MATLAB (Mathworks) and analyzed using customized MATLAB software.

For the dilution curves (processivity analysis), each bead was tested for at least 4 min before judging it as “moving” or “nonmoving” bead. Error bars were calculated assuming a binomial distribution: error bar = $\pm \sqrt{((1-f) \times f/N)}$, with f being the fraction of moving beads and N the number of beads tested. Two models were fit to the data. The first equation is based on the assumption that one or more motors are required for the observed force generation (processive model, see Supporting Material for the derivation)

$$F = 1 - \exp(-\lambda c) \quad (6)$$

where F is the moving bead fraction, c is the relative motor concentration, and λ is a fitting parameter that describes the dependence on the fraction of active motors. The second equation (“nonprocessive” model) approximates those dilution curves better that result from force generation events driven by two or more motors (see Supporting Material for the derivation)

$$F = 1 - \exp(-\lambda c) - \lambda c \exp(-\lambda c) \quad (7)$$

The coefficients of determination (R^2) at a significance level $\alpha = 0.05$ and AIC test were used to judge which model approximates the data better. All figures were prepared using Prism software (GraphPad), and all fitting procedures, including the AIC test, were performed therein.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/15/eaaz4295/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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