Displacement-Weighted Velocity Analysis of Gliding Assays Reveals that Chlamydomonas Axonemal Dynein Preferentially Moves Conspecific Microtubules

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ABSTRACT In vitro gliding assays, in which microtubules are observed to glide over surfaces coated with motor proteins, are important tools for studying the biophysics of motility. Gliding assays with axonemal dyneins have the unusual feature that the microtubules exhibit large variations in gliding speed despite measures taken to eliminate unsteadiness. Because axonemal dynein gliding assays are usually done using heterologous proteins, i.e., dynein and tubulin from different organisms, we asked whether the source of tubulin could underlie the unsteadiness. By comparing gliding assays with microtubules polymerized from Chlamydomonas axonemal tubulin with those from porcine brain tubulin, we found that the unsteadiness is present despite matching the source of tubulin to the source of dynein. We developed a novel, to our knowledge, displacement-weighted velocity analysis to quantify both the velocity and the unsteadiness of gliding assays systematically and without introducing bias toward low motility. We found that the quantified unsteadiness is independent of tubulin source. In addition, we found that the short Chlamydomonas microtubules translocate significantly faster than their porcine counterparts. By modeling the effect of length on velocity, we propose that the observed effect may be due to a higher rate of binding of Chlamydomonas axonemal dynein to Chlamydomonas microtubules than to porcine microtubules.

INTRODUCTION

Motor proteins drive a wide variety of motile processes, including the transport of organelles by kinesins and cytoplasmic dyneins, the contraction of muscle by myosins, and the beating of cilia and flagella by axonemal dyneins. An important tool for studying motor proteins is the in vitro motility assay in which purified motor proteins are studied on their own, without the complex regulatory machinery found in cells. In one form of the assay, the stepping assay, cytoskeletal filaments are fixed to a surface and labeled motor proteins are observed moving along them (1–3). In the other form, gliding assays, the motor proteins are fixed to a surface and labeled filaments are observed gliding across the surface (4–6). In vitro motility assays have revealed many molecular mechanisms underlying the generation of force (7).

In gliding assays with most motor proteins, the movement is generally steady, with only small fluctuations in speed attributed to the stochastic stepping of the motors along their filaments (8,9). However, in the case of axonemal dyneins, the movement is unsteady, with large changes in speed varying irregularly on the timescale of seconds, from zero to several micrometers per second (10,11). On the one hand, the unsteadiness is surprising, given that the beating of the axoneme appears smooth. On the other hand, perhaps the unsteadiness is expected given that dyneins on radially opposite sides of the axoneme likely switch their activity on and off at the beat frequency. Furthermore, when axonemes are subject to partial proteolysis, the doublets slide apart with large variations in velocity (12,13). The latter two observations suggest that unsteadiness may reflect an inherent switchability of axonemal dynein. Thus, the unsteady motility of axonemal dynein is potentially interesting.

Consistent with unsteady speed being an intrinsic property of axonemal dyneins, unsteadiness appears to be independent of the in vitro assay conditions. For axonemal dyneins from Chlamydomonas reinhardtii, the best studied system, unsteadiness is observed for a large number of different inner (14) and outer-arm dyneins (10,11) and is seen over a wide range of assay conditions, including different ATP and ADP concentrations (11,15–17), different protocols for treating the surfaces (11,14,18), and different methods of attaching the motors to the surfaces (11). However, before concluding that unsteadiness is a fundamental property that distinguishes axonemal dyneins from other motors, it is necessary to examine all possible alternative explanations.

In this study, we asked whether the unsteady motility of microtubules gliding on axonemal dynein from Chlamydomonas reinhardtii could be due to the source of tubulin used for the in vitro assays. The source of tubulin may be important because unsteady axonemal dynein gliding assays, unlike steady gliding assays with other motors, have thus far been done with microtubules polymerized...
from mammalian brain tubulin and axonemal dynein purified from species genetically distant from mammals, i.e., *Chlamydomonas*. Mammalian brain tubulin differs from *Chlamydomonas* axonemal tubulin in several respects. For example, mammalian tubulin, which consists of a diverse mixture of isoforms, differs in sequence by ~15% from the single isoform found in *Chlamydomonas* (see Figs. S1 and S2 in the Supporting Material). In addition, the relative abundance of tubulin isoforms differs between brain and axonemes (19). Furthermore, the posttranslational modification of tubulin (20) and isotype mixture (21) differs between axonemes (19). Furthermore, the posttranslational modification of tubulin (20) and isotype mixture (21) differs between brain and cilial microtubules. Any or all of these differences could contribute to the unsteadiness of axonemal dynein gliding assays.

Testing whether the source of tubulin is important for axonemal dynein motility requires overcoming the problem of purifying tubulin from cilia. Mammalian brain is a rich source of tubulin; the high abundance of protein allows purification through cycles of polymerization and depolymerization (22). *Chlamydomonas* is a poor source of tubulin; cycling does not work. We therefore used a recently developed chromatographic technique (23) to overcome the scarcity of axonemal tubulin and purify it from *Chlamydomonas* axonemes in sufficient quantity to perform gliding assays.

We found that microtubules polymerized from *Chlamydomonas* tubulin also glide unsteadily over surfaces coated with axonemal dynein from *Chlamydomonas*. This finding rules out another possible cause of unsteadiness. During these experiments, however, we observed subtle differences in the motility speed between the two sources of tubulin. The unsteadiness of the motion made quantification of these differences difficult. To circumvent this problem, we developed an analysis technique that weighs the distribution of gliding velocities by the distance traveled rather than by the time traveled, as is usually done. Using this displacement-weighted velocity analysis, we found that, although long microtubules move at similar speeds irrespective of the tubulin source, shorter *Chlamydomonas* microtubules translocate significantly faster than their porcine counterparts. By applying a model for gliding assays, we show that this effect may be due to a higher rate of binding of *Chlamydomonas* axonemal dynein to *Chlamydomonas* microtubules than to porcine microtubules.

**MATERIALS AND METHODS**

**Strains and media**

The *Chlamydomonas reinhardtii* strain used was *oda2-t-lc2-bccp*. As described in Furuta et al. (11), it was obtained by crossing *oda2-t*, which lacks the motor domain of γ-HC, with *wt-lc2-bccp*, which was obtained by inserting a *Chlamydomonas* light-chain 2 biotin-carboxyl-carrier protein (LC2-BCCP) construct into the *oda12* strain. This strain was used to bind αδ-heavy chain outer-arm dynein complexes to a streptavidin-coated substrate in a site-specific manner in the gliding assays (11). The LC2 was biotinylated in vivo, and its location near the tail domain of dynein (24) helped to ensure that the microtubule binding domain on the stalk was free to bind to the microtubule (11).

The cells were grown in liquid Tris-acetate-phosphate (TAP) medium (20 mM Tris, 7 mM NH₄Cl, 0.40 mM MgSO₄, 0.34 mM CaCl₂, 2.5 mM PO₄³⁻, and 1000-fold diluted Hutners trace elements (25), titrated to pH 7.0 with glacial acetic acid) with continuous aeration and 24 h of light at room temperature. 60 L of cell culture were grown to a density of 5–10 × 10⁶ cells/mL.

**Dynin purification**

The *oda2-t-lc2-bccp* cells were harvested and the axonemes were isolated by standard methods (26). Briefly, cells were harvested by centrifugation at 800 × g for 7 min. They were deflagellated by 1.5 min of exposure to 4.2 mM dibucane-HCl. The flagella were separated from the cell bodies by centrifugation (1100 × g for 7 min and 1100 × g for 20 min on a 30% sucrose cushion). The flagella were concentrated by resuspending the pellet after centrifugation (28,000 × g) in 10 mL of HMDE (30 mM HEPES, 5 mM MgSO₄, 1 mM DTT, and 1 mM EGTA, titrated to pH 7.4 with KOH) with 0.4 μM Pefabloc (Sigma-Aldrich, St. Louis, MO). The flagella were demembranated by the addition of 0.2% IGEPAL CA-630 (Sigma-Aldrich) and then washed in HMDE. The dyneins were extracted from the axonemes by incubation in HMDE + 0.6 M KCl. The dynein extract was diluted fivefold in HMDE and clarified by centrifugation (125,000 × g for 10 min).

The dyneins were purified from the extract by standard methods (27). The dynein extract was applied to a MonoQ 10/100 GL (GE Healthcare, Piscataway, NJ) ion-exchange column. The dyneins were eluted with a linear gradient of 150–400 mM KCl in HMDE and collected in 2-mL fractions. The fractions of interest were pooled, desalted, and concentrated using 10-kDa molecular mass cut-off centrifugal filters (Ultra-15, PLHK Ultracel-PL Membrane, EMD Millipore, Billerica, MA). The protein concentration was determined by measuring the absorbance at 280 nm (NanoDrop, Wilmington, DE). Each pool was diluted to 100 μg/mL in 30% saturated sucrose and HMDE and stored at –80°C.

**Tubulin purification and labeling**

Porcine brain tubulin was purified by standard methods (22). Briefly, porcine brains were homogenized and clarified by centrifugation. Active tubulin and microtubule-associated proteins in the supernatant were purified with polymerization and depolymerization cycles. Tubulin was separated from the microtubule-associated proteins with a phosphocellulose column (Whatman P11, Piscataway, NJ) by incubating the dye with polymerized microtubules in a 10:1 dyetubulin molar ratio for 40 min at 37°C. To ensure that the dye did not impair microtubule polymerization, the active labeled tubulin was purified with polymerization and depolymerization cycles. The final protein concentration and labeling stoichiometry were determined using standard techniques (as in ThermoScientific Tech Tip #31; NanoDrop).

Axonemal tubulin from *Chlamydomonas* was purified using a recently developed chromatographic technique (23). Briefly, the axoneme pellet obtained after dynein extraction was resuspended in HMDE + 50 mM CaCl₂. It was sonicated for 10 × 1 min on/1 min off cycles in an ice-cold sonicating bath to induce the axonemal tubulin to depolymerize. It was centrifuged at 67,000 × g for 10 min. The supernatant was diluted to fivefold the volume in HMDE to reduce the CaCl₂ concentration, applied to a TOG12 domain column (23), and eluted with BRB80 (80 mM PIPES, 1 mM MgCl₂, and 1 mM EGTA titrated to pH 6.9 with KOH) + 0.5 M KCl. The tubulin fractions were pooled and concentrated using 10 kDa molecular weight cut-off centrifugal filters (Ultra-15, PLHK UltraCel-PL Membrane, Amicon). The concentration was determined by measuring the absorbance at 280 nm.
Microtubule preparation

Rhodamine-labeled porcine brain microtubules, hereafter called porcine microtubules, were polymerized from 1.7 μM 1.6:1 dye/protein tubulin and 33 μM unlabeled tubulin (35 μM porcine brain tubulin total) in the presence of 1 mM GTP, 4 mM MgCl2, 5% DMSO, and BRB80 for 45 min at 37°C. They were stabilized by diluting them in BRB80 + 20 μM taxol. Rhodamine-labeled _Chlamydomonas_ microtubules, hereafter called _Chlamydomonas_ microtubules, were polymerized from 8 μM unlabeled _Chlamydomonas_ axonemal tubulin and 0.4 μM 1.6:1 dye/protein porcine brain tubulin (8.4 μM tubulin in total contained 95% _Chlamydomonas_ tubulin and 5% porcine brain tubulin) in the presence of 1 mM GTP, 4 mM MgCl2, 5% DMSO, and BRB80 for 45 min at 37°C. They were stabilized by dilution in BRB80 + 20 μM taxol.

Microtubule gliding assays

Microtubule gliding assays (11) were performed in 5-μL flow channels made from an 18 × 18-mm coverslip (Corning B.V., Life Sciences, Amsterdam, The Netherlands) spaced ~100 μm from a 20 × 20-mm coverslip (Corning) by Parafilm M (Pechiney Plastic Packaging, Chicago, IL). The flow channels were filled sequentially with the following solutions: 1), 20 μL HMDE; 2), 10 μL 1 mg/mL biotinylated bovine serum albumin (BSA); 3), 20 μL HMDE; 4), 10 μL 1 mg/mL streptavidin; 5), 20 μL HMDE; 6), 10 μL 5 mg/mL BSA; 7), 20 μL HMDE; 8), 10 μL 100 μg/mL dynein; 9), 20 μL HMDE + 1 mM ADP; 10), 10 μL microtubule solution (~0.5 μM tubulin dimers, 40 μM taxol, and 1 mM ADP in HMDE); 11), 20 μL HMDE + 1 mM ADP + 40 μM taxol; and 12), 10 μL motility solution (1 mM ATP, 1 mM ADP, 40 μM taxol, 4 μg/mL catalase, 10 μg/mL glucose oxidase, 40 mM D-glucose, and 142 mM β-mercaptoethanol). All solutions containing protein were incubated in the flow channel for at least 5 min. Microtubules were imaged translocating using a Zeiss Axiovert 200 M microscope with a Zeiss 100×/1.46 α-Plan-Apochromat Oil Ph3 objective at 23°C. Images were acquired with a Metamorph (Molecular Devices, Sunnyvale, CA) or Andor iQ (Andor TechnoLogy, Belfast, United Kingdom) software-driven Andor DV887 Ixon camera. Fluorescence excitation was provided by a mercury arc lamp coupled through an optical fiber. TRITC filter sets (Chroma Technol-ogy, Bellows Falls, VT) were used to image rhodamine. The exposure time was 100 ms, and 2000-frame movies were recorded continuously. Movies were analyzed only if there was no decrease in speed due to ATP depletion.

Microtubule tracking

Each microtubule was tracked with 50-nm precision and the displacement along the microtubule’s path, along with microtubule length, were calculated on every frame using Fluorescence Image Evaluation Software for Tracking and Analysis (FIESTA) (28). FIESTA collected the microtubule’s position data from a sequence of frames into data sets called tracks. The tracks contained data on the microtubule’s position, its displacement along its translocation path, and its length. Tracks were terminated when microtubules collided, crossed, or intersected the edge of the field of view. Data points where the microtubule length changed greatly from the previous and subsequent frames were filtered out. Only tracks with total microtubule displacement >10 μm were used in the analysis. Occasionally, FIESTA was unable to track the microtubule in isolated frames, or the points were filtered out. The missing displacement along the path data points were estimated by linear interpolation. Then, the data were smoothed using a third-degree Savitzky-Golay filter with a span of nine data points (29) to reduce the experimental noise but preserve the details of the unsteadiness.

Time-weighted mean velocity calculation

The time-weighted velocity, \( p_i(v) \) (see Results), was calculated by binning the instantaneous velocities in \( N \) 200 ms/s bins, totaling the number of instantaneous velocity intervals in each bin, and normalizing by the total number of 0.4-s intervals over which velocity was measured. The mean velocity, \( \mu_i \), was calculated using

\[ \mu_i = \frac{\sum_{j=1}^{N} v_j p_j(v_j)}{N} \]

where \( v_j \) is the velocity and \( p_j(v_j) \) is the probability density of the \( j \)th bin. The standard deviation of the velocity, \( \sigma \), was calculated using

\[ \sigma^2 = \sum_{j=1}^{N} \left( v_j^2 p_j(v_j) \right) - \mu_j^2. \]

Displacement-weighted mean velocity calculation

The displacement-weighted velocity, \( p_i(v) \) (see Results), was calculated by binning the instantaneous velocities, summing the microtubule displacement during each interval in each bin, and normalizing by the total microtubule displacement. Note that

\[ p_i(v_j) = \frac{\sum_{j=1}^{n} d_j}{d} \]

where \( n \) is the number of 0.4-s velocity intervals and \( d_j \) is the microtubule displacement during the \( j \)th interval in the \( j \)th bin, and

\[ d = \sum_{j=1}^{N} \sum_{i=1}^{n} d_j \]

is the total microtubule displacement. The mean velocity, \( \mu \), was calculated using

\[ \mu = \sum_{j=1}^{N} \sum_{i=1}^{n} \left( v_j^2 p_j(v_j) \right) \]

The standard deviation, \( \sigma \), was calculated using

\[ \sigma^2 = \sum_{j=1}^{N} \left( v_j^2 p_j(v_j) \right) - \mu^2. \]

RESULTS

Conspecific tubulin does not eliminate unsteadiness

To determine whether matching the source of tubulin to the source of dynein reduced the unsteadiness of the microtubule gliding velocity (10, 11), we compared the kymographs of microtubules polymerized from _Chlamydomonas_ axonemal tubulin (Fig. S3) (23) with those purified from porcine brain (22) moving over axonemal outer-arm dynein purified from _Chlamydomonas_ (Fig. S4) (11). Both types of microtubules were visualized by fluorescence microscopy using the same small mole fraction (5%) of rhodamine-labeled porcine brain tubulin (see Methods). Unsteadiness was observed in the kymographs of both microtubule types (Fig. 1 a). Because the unsteadiness was not eliminated by using a conspecific system, it may be an important
To characterize the variability in velocity further, we calculated the probability density function of the instantaneous velocities, \( p_i(v) \). \( p_i(v) \) is the likelihood that the microtubule is gliding at velocity \( v \) at any time, \( t \). \( p_i(v) \) is normalized such that \( \int_{-\infty}^{\infty} p_i(v)\,dv = 1 \). The mean of \( p_i(v) \), \( \mu_i = \langle v \rangle_i = \int_{-\infty}^{\infty} vp_i(v)\,dv \), is the time-weighted mean velocity of the gliding microtubule. The variance of \( p_i(v) \) is \( \sigma_i^2 = \langle v^2 \rangle_i - \langle v \rangle_i^2 \), where \( \langle v^2 \rangle_i = \int_{-\infty}^{\infty} v^2 p_i(v)\,dv \). An example microtubule’s time-weighted velocity histogram is shown in Fig. 1 d. The example microtubule had a mean velocity of \( \mu_i = 2.68 \mu\text{m/s} \) and a standard deviation, which is a measure of the unsteadiness, of \( \sigma_i = 1.69 \mu\text{m/s} \). The technique was demonstrated here for a single microtubule; however, it can be used to account for many microtubules gliding in many movies by pooling the data into a single \( p_i(v) \) function.

**Displacement-weighted velocity analysis**

The characterization of unsteady microtubule gliding using \( \mu_i \) and \( \sigma_i \) is biased by periods of very slow or no gliding motility, because \( p_i(v) \) is weighted by the time microtubules spend at a velocity. Because we are interested in the biophysical properties of the motor proteins, and very slow periods (like that in the track of our example microtubule between 6 and 7.5 s in Fig. 1 b) and seen as the bar at \( v = 0 \) in the histogram of velocities in Fig. 1 d) contain little information about motility, this bias masks important motile properties of the underlying motors. To eliminate this bias, we characterized the gliding by weighting the data by the total microtubule gliding displacement using the displacement-weighted velocity probability distribution function, \( p_d(v) \). This approach is a systematic way to quantify unsteady motility without introducing arbitrary constraints or judgments.

The displacement-weighted velocity probability distribution, \( p_d(v) \), is the proportion of total displacement, \( x \), a gliding microtubule covers at a given velocity, \( v \). \( p_d(v) \) is normalized such that \( \int_{-\infty}^{\infty} p_d(v)\,dv = 1 \). The mean of \( p_d(v) \), \( \mu_d = \langle v \rangle_d = \int_{-\infty}^{\infty} vp_d(v)\,dv \), is the displacement-weighted mean velocity of the gliding microtubule. The variance of \( p_d(v) \) is \( \sigma_d^2 = \langle v^2 \rangle_d - \langle v \rangle_d^2 \), where \( \langle v^2 \rangle_d = \int_{-\infty}^{\infty} v^2 p_d(v)\,dv \). An example microtubule’s displacement-weighted velocity histogram is in Fig. 1 e. The example microtubule had a displacement-weighted mean velocity of \( \mu_d = 3.69 \mu\text{m/s} \) and unsteadiness of \( \sigma_d = 1.35 \mu\text{m/s} \). As with the calculation of \( p_i(v) \), the technique was demonstrated here for a single microtubule; however, it can be used to account for many microtubules gliding in many movies by pooling the data in a single \( p_d(v) \) function.

We applied the displacement-weighted velocity analysis to the question of whether the source of tubulin affects unsteadiness. We tracked hundreds of porcine and *Chlamydomonas* microtubules and calculated the instantaneous velocity for each of them along their tracks. By comparing \( p_i(v) \) and \( p_d(v) \) for all the microtubules, the advantage of displacement-weighted velocity analysis is demonstrated.
We found that the time-weighted velocity histograms were highly asymmetric and strongly biased to low velocities for both *Chlamydomonas* and porcine microtubules (Fig. 2, a and b, respectively), due to the high time-weighted probability that a microtubule was moving very slowly. In contrast, the displacement-weighted velocity histograms, \( p_x(v) \), were more nearly Gaussian for both *Chlamydomonas* and porcine microtubules (Fig. 2, c and d, respectively), making them easier to analyze and reducing the measurement bias toward low speeds. There are other ways of eliminating the low-velocity bias. For example, one could analyze only microtubule gliding events that have long-enough-sustained periods of high velocity. However, such a procedure is arbitrary, and highly dependent on the definition of long-enough-sustained periods of high velocity. This has significant effects on the quantification of both the average velocity and the unsteadiness. The displacement at a velocity analysis eliminates the low-velocity bias while still systematically accounting for all the data.

By comparing the displacement-weighted velocity distributions, we found both similarities and differences between the tubulin sources. We found that the displacement-weighted standard deviation, \( \sigma_x \), of *Chlamydomonas* microtubules was nearly identical to that of porcine microtubules: \( \sigma_x = 2.88 \, \mu m/s \) (Fig. 2 c) compared to \( \sigma_x = 2.89 \, \mu m/s \) (Fig. 2 d, respectively), confirming that the unsteadiness is not only present, but also equal, in the two sources of tubulin. We also found that the displacement-weighted mean gliding velocity, \( \mu_x \), was 1.05-fold higher for *Chlamydomonas* microtubules than for porcine microtubules: 5.76 \( \mu m/s \) compared to 5.48 \( \mu m/s \) (Fig. 2, c and d, respectively). This difference was small but statistically significant (Welch’s t-test, \( p = 0.03 \) for 1183 *Chlamydomonas* microtubules and 799 porcine microtubules).

### Short *Chlamydomonas* microtubules translocate faster than porcine microtubules on dynein coated substrates, but longer ones do not

The velocity of filaments in gliding assays with low-duty-ratio motors, such as myosin-2 (30) and axonemal dynein (10), increases with filament length. Low-duty-ratio motors spend only a small fraction of their time attached to their filaments and so many motors are needed for continuous motility. We suspected the length effect was obscuring more significant differences in axonemal dynein motility. Therefore, we characterized the gliding as a function of microtubule length by fitting the displacement-weighted mean velocity-length data (Fig. 3 a) to a Michaelis-Menten-like equation (10),

\[
v(L) = v_{max} \frac{L}{L_0 + L},
\]

where \( L \) is the microtubule length, \( L_0 \) is the length of the microtubule whose gliding velocity is half the saturating velocity, and \( v_{max} \) is the saturating displacement-weighted mean velocity reached in the limit of long microtubules. We found that the characteristic length, \( L_0 \), was shorter for *Chlamydomonas* than for porcine microtubules: 3.8 \( \pm \) 0.3 \( \mu m \) compared to 6.7 \( \pm \) 0.5 \( \mu m \) (least-squares fit parameter \pm SE of the fit, Fig. 3 a). This 1.8-fold difference is highly significant (\( p<1 \times 10^{-5} \)).

The displacement-weighted mean gliding velocity, \( \mu_x \), of short \((L<6 \, \mu m)\) *Chlamydomonas* microtubules was higher than that for porcine microtubules of the same length: 4.42 \( \pm \) 2.48 \( \mu m/s \) (\( \mu_x \pm \sigma_x \), Fig. 3 b) compared to 3.36 \( \pm \) 2.24 \( \mu m/s \) (\( \mu_x \pm \sigma_x \), Fig. 3 c). This 1.32-fold difference is highly significant (Welch’s t-test, \( p\)-value \( <1 \times 10^{-5} \), for 345 *Chlamydomonas* and 152 porcine microtubules). However, the displacement-weighted mean gliding velocity, \( \mu_x \), of long \((L>20 \, \mu m)\) *Chlamydomonas* microtubules was not significantly different (Welch’s t-test, \( p = 0.4 \)) from porcine microtubules of the same length: 7.61 \( \pm \) 2.86 \( \mu m/s \) (\( \mu_x \pm \sigma_x \), \( N = 110 \), Fig. 3 d) compared to 7.31 \( \pm \) 2.64 \( \mu m/s \) (\( \mu_x \pm \sigma_x \), \( N = 157 \), Fig. 3 e), respectively. Thus, whereas long microtubules move at the same speed, the shorter *Chlamydomonas* microtubules move faster than shorter porcine ones. In addition, note that the unsteadiness, \( \sigma_x \), is similar for both sources of tubulin and independent of microtubule length.

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**FIGURE 2** Displacement-weighted velocity characterizes unsteady gliding in an unbiased and systematic way. (a) Time-weighted velocity histogram \( p_v(v) \) of all of the analyzed *Chlamydomonas* microtubules. The mean velocity is \( \mu_v = 2.80 \, \mu m/s \) and the unsteadiness is \( \sigma_v = 2.96 \, \mu m/s \) for 1183 microtubules and 25,706 instantaneous velocities. (b) Time-weighted velocity histogram \( p_v(v) \) of all of the analyzed porcine microtubules. The mean velocity is \( \mu_v = 2.17 \, \mu m/s \) and the unsteadiness is \( \sigma_v = 2.79 \, \mu m/s \) for 799 microtubules and 26,588 instantaneous velocities. (c) Displacement-weighted velocity histogram \( p_x(v) \) of all of the analyzed *Chlamydomonas* microtubules. The mean velocity is \( \mu_x = 5.76 \, \mu m/s \) and the unsteadiness is \( \sigma_x = 2.88 \, \mu m/s \) for 1183 microtubules and 29.6 mm of total gliding displacement. (d) Displacement-weighted velocity histogram \( p_x(v) \) of all of the analyzed porcine microtubules. The mean velocity is \( \mu_x = 5.48 \, \mu m/s \) and the unsteadiness is \( \sigma_x = 2.89 \, \mu m/s \) for 799 microtubules and 24.2 mm of total gliding displacement.
assumes that microtubules are rigid and dynein is a two-state motor. When a microtubule glides over the dyneins, an individual dynein in the bound state translocates the microtubule at an instantaneous velocity equal to the instantaneous speed of an individual dynein undergoing its powerstroke,

\[ v_0 = \frac{\delta}{t_{\text{bound}}^*} = \frac{\delta}{1/k_{\text{off}}} \]  

where \( \delta \) is the microtubule displacement caused by the powerstroke, \( t_{\text{bound}}^* = 1/k_{\text{off}} \) is the time dynein is bound to the microtubule, and \( k_{\text{off}} \) is the dissociation rate of dynein from the microtubule. Having multiple motors in the bound state is redundant, and the microtubule moves at an instantaneous velocity of \( v_0 \), as if only one dynein were bound. An individual dynein in the unbound state does not contribute to the microtubule’s movement.

The duty ratio, \( r \), is the fraction of time that an individual motor spends in the bound state and moving the microtubule,

\[ r = \frac{t_{\text{bound}}}{t_{\text{bound}} + t_{\text{unbound}}} = \frac{1/k_{\text{off}}}{1/k_{\text{off}} + 1/k_{\text{on}}}. \]

where the unbound time is \( t_{\text{unbound}} = 1/k_{\text{on}} \) and \( k_{\text{on}} \) is the association rate of dynein to the microtubule. Assuming that the binding of a motor is random and independent of the state of the other motors, the probability of at least one dynein bound is \( p = 1 - (1 - r)^n \), where \( n \) is the total number of motors that are close enough to the microtubule to interact with it. \( n = \rho aL \), where \( \rho \) is the dynein density, \( a \) is the microtubule diameter, and \( L \) is the microtubule length.

According to this model, previously used for dynein (10) and myosin (30), the average velocity of a microtubule is the speed of the powerstroke, \( v_0 \), times the fraction of time that at least one motor is bound,

\[ v = v_0 [1 - (1 - r)^n]. \]  

According to Eq. 10, the maximum gliding velocity is the instantaneous speed of an individual motor undergoing its powerstroke, \( v_{\text{max}} = v_0 \). For low-duty-ratio motors \( r \ll 1 \), the maximum velocity is achieved at high dynein densities or for long microtubules, when there are a large number of motors driving the movement. In addition, for low-duty-ratio motors, the minimum velocity, \( v_{\text{min}} = 0 \), occurs at very low dynein densities or for very short microtubules, when there are only a small number of motors available to interact with the microtubule \( (n \rightarrow 0 \text{ in Eq. 10}) \). To satisfy both Eq. 10 and Eq. 7,

\[ L_0 = \frac{b}{r}, \]

where \( b = \ln(2)/(\alpha p) \) (see Supporting Material) under the assumption of low duty ratio \( r \ll 1 \).

Modeling of microtubule velocity versus length data reveals that the on rate is twofold larger for porcine than for Chlamydomonas microtubules

To relate gliding assay results to the properties of the underlying motors, we used a kinetic model for gliding assays that
To relate the mechanochemical parameters of axonemal dynein to the experimental results, we first note that according to Eq. 11, the 1.8-fold lower \( L_0 \) can be accounted for by a 1.8-fold larger \( r \) for Chlamydomonas microtubules than for porcine microtubules. We also note that, according to Eq. 8, \( k_{\text{cat}} \) is unaffected by the source of tubulin, assuming \( \delta \) is the same for Chlamydomonas and porcine microtubules, because the gliding velocity is the same for long microtubules. This predicts that the total ATP hydrolysis cycle time (\( t_{\text{total}} = t_{\text{unbound}} + t_{\text{bound}} \)) is 1.8-fold higher for porcine microtubules according to Eq. 9, and \( k_{\text{on}} \) is 1.8-fold higher for Chlamydomonas microtubules, assuming that \( r \ll 1 \). In other words, the difference in motility between Chlamydomonas and porcine microtubules is simply explained by a higher rate of attachment of Chlamydomonas axonemal dynein to the Chlamydomonas axonemal microtubules. An increased attachment rate increases the speed of short microtubules because the microtubules spend a larger fraction of time moving, but it does increase the speed of longer microtubules because they are already saturated with attached motors.

These results suggest that the ATP turnover rate, \( k_{\text{cat}} = 1/t_{\text{total}} \), should be nearly twice as high for Chlamydomonas microtubules as for porcine microtubules. Despite considerable effort, the high basal activity of axonemal dynein and the high concentration of microtubules needed to stimulate the ATPase (31) precluded direct testing of this prediction.

**DISCUSSION**

In this study, we developed a displacement-weighted velocity probability distribution function analysis technique and used it to show that conspecific and heterologous axonemal dynein-tubulin gliding assays exhibit the same unsteadiness. Using this analysis technique, we also showed that the source of tubulin affects the gliding motility of microtubules. Short Chlamydomonas microtubules are moved faster than short porcine brain ones. This difference is intrinsic to the source of tubulin, as the labeling ratio (5% rhodamine-labeled porcine brain tubulin) and the polymerization protocol (with 1 mM GTP and taxol stabilization) were closely matched. These findings highlight the importance of using conspecific motor-protein-tubulin systems for in vitro assays.

**Comparison to literature study**

In this study, we found that the mean velocity for long porcine microtubules, 7.3 ± 2.6 \( \mu m/s \) (Fig. 3 e), was similar to the previously reported value, 6.8 ± 1.3 \( \mu m/s \) (11). These slight differences could be due to the different set of outer-arm dynein complex proteins purified by the MonoQ column used in this study and the UnoQ column used by Furuta et al. (11) (see Supporting Material for more details on the purification). In addition, we note that the standard deviation was larger for our characterization because it includes the inherent unsteadiness of each microtubule in addition to the microtubule-to-microtubule variation captured by Furuta et al. (11).

**Displacement-weighted velocity characterizes unsteady gliding assays in a systematic and unbiased manner**

To better understand the differences between traditional gliding assay analysis techniques and our displacement-weighted velocity analysis, consider an analogy between a car traveling on a highway and a microtubule gliding on axonemal dynein. Assume we are interested in characterizing the properties of the car’s motor just as we are interested in characterizing the properties of the motor proteins translocating the microtubule.

One measure of the car’s performance is the total distance traveled divided by time. This measure is equivalent to the time-weighted mean velocity, \( \mu_\tau \), and it can be dominated by long periods of time when the car is moving very slowly. Slow moving periods may not be a function of the motor’s performance; they may be due to traffic, for example.

Another measure of the car’s performance is the maximum sustained velocity. This measure requires defining arbitrary limits for what constitutes maximum sustained velocity, and it excludes the data outside the defined limits from the analysis, essentially ignoring the slow periods. However, this excluded data may be an indicator of the motor’s performance; the car may slow from its maximum speed as it is climbing hills, for example.

We argue that a better judge of the car’s motor is how it performs over the greatest distances traveled: the displacement-weighted mean velocity, \( \mu_\ell \). This measure of motor performance is unbiased by long periods when the car is stopped or moving very slowly, and it is systematic in its treatment of the data without discarding any potentially important information about the unsteadiness of the motor due to its innate properties. Thus, by applying the displacement-weighted velocity analysis to unsteady gliding assays, the underlying properties of the motor proteins are revealed in an unbiased and systematic way.

The relationship between the displacement-weighted velocity analysis and the time-weighted velocity analysis can be understood through the relationship between the probability density functions, \( p_\ell(v) \) and \( p_\tau(v) \),

\[
p_\ell(v) = \frac{v p_\ell(v)}{\mu_\ell} \tag{12}
\]

where \( v p_\ell(v) \) is the displacement of microtubules while gliding at a velocity \( v \). By substituting \( v = \mu_\ell \) into Eq. 12, it follows that \( p_\ell(\mu_\ell) = p_\ell(\mu_\ell) \). Thus, the transformation from \( p_\ell(v) \) to \( p_\ell(v) \) preserves \( p_\ell(\mu_\ell) \). For \( v < \mu_\ell \), \( p_\ell(v) < p_\ell(v) \), and
for \( v > \mu, p_x(v) > p_t(v) \). Therefore, the transformation to \( p_x(v) \) diminishes the probability distribution at slow velocity and amplifies it at high velocity. By substituting Eq. 12 and the definitions of \( \mu_x \) and \( \sigma_x \) into the definitions of \( \mu_t \) and \( \sigma_t \), we find that \( \mu_x \) is related to \( \mu_t \) and \( \sigma_t \) by

\[
\mu_x = \mu_t + \frac{\sigma_t^2}{\mu_t}, \tag{13}
\]

and \( \sigma_x \) is given by

\[
\sigma_x^2 = \sigma_t^2 \left( 1 - \frac{\sigma_t^2}{\mu_t^2} \right) + \frac{\gamma^3}{\mu_t^2}, \tag{14}
\]

where \( \gamma^3 \) is the third central moment about the mean of \( p_t(v) \) (the normalized skewness of \( p_t(v) \)). Note that these types of relationships can also be derived for the skewness (\( \gamma_x \)) of \( p_x(v) \) (see Supporting Material). Therefore, the distance-weighted velocity analysis is related to traditional time-weighted velocity analysis techniques through the statistical moments of the distributions.

A closer examination into how transforming from \( p_t(v) \) to \( p_x(v) \) affects the data reveals that in contrast to the experimentally observed \( p_t(v) \), which tends to be exponential or the combination of an exponentially and a normally distributed subpopulation (Fig. 2, a and b), \( p_x(v) \) tends to be more normally distributed (Fig. 2, c and d). The tendency of \( p_t(v) \) analyses to comprise multiple underlying distributions makes them difficult to compare quantitatively. Therefore, the transformation to \( p_x(v) \) helps with statistical comparisons, because Gaussian distributions, characterized by \( \mu_x \) and \( \sigma_x \), can be compared using t-test hypothesis testing.

Not all \( p_x(v) \) data are well described by the Gaussian distribution. For example, the subpopulations of short and long microtubules are skew normal (Fig. 3, b–e). Short microtubules have positive skew (Fig. 3, b and c), suggesting that although most of the displacement traveled by short microtubules is at low speed, they can reach faster speeds. This is a natural consequence of having a lower bound on the velocity of 0 μm/s. Long microtubules have negative skew (Fig. 3, d and e), suggesting that although most of the displacement traveled by long microtubules is at high speed, they can reach slower speeds. This is a natural consequence of having an upper bound on the velocity of the instantaneous speed of an individual dynein undergoing its power stroke (Eq. 8). Thus, we argue that skewness indicates that the mean gliding velocity approaches a physical limit.

**Effect of tubulin source on mechanochemical properties of axonemal dynein**

The results show that the unsteadiness of axonemal gliding assays is a characteristic of tubulin-axonemal dynein systems independent of tubulin source (Fig. 1). Previous studies showed that controlling two possible sources of unsteadiness—ADP concentration (11,15–17) and motor-surface interactions (using an LC2-BCCP mutant (11))—also fails to eliminate unsteadiness. Although this study has eliminated another possible source, there are several untested sources of unsteadiness attributable to possible artifacts of the experiment. For example, a subpopulation of the axonemal dynein motors could be dead, there could be an uneven distribution of motors on the slide, or the presumably random orientation of the dynein with respect to the gliding microtubule could affect motility (52). If either of the first two were the source, our model for gliding assays would predict that the unsteadiness is a function of microtubule length, but it is not. If the third were the problem, sliding assays, in which a disintegrated axoneme slides apart, would be steady, but they are reported to be unsteady (32). Therefore, it is possible that the unsteadiness of axonemal gliding assays may be the result of the inherent properties of the motors.

Although we found that unsteadiness remains in conspecific gliding assays, we found that the motility of axonemal dynein on short conspecific tubulin is faster than on heterologous tubulin. This result raises questions about the properties of the different microtubules that could account for variations in behavior.

The tubulin orthologs share only 85–90% sequence identity (Figs. S1 and S2) between a consensus estimate of the six \( \alpha \) and eight \( \beta \) porcine tubulin isoatypes and the one \( \alpha \) and one \( \beta \) *Chlamydomonas* tubulin isoatype. The specific amino acid sequences of tubulins responsible for motility differences are unknown. Differences in the glutamic-acid-rich C-terminal E-hooks, which share only 78% and 62% sequence identity for \( \alpha \)- and \( \beta \)-tubulin, respectively, in the consensus porcine and *Chlamydomonas* tubulin sequences and contain the axonem-specific sequences (21,33), are possibly responsible, because the highly charged E-hook can regulate cytoplasmic dynein (34) and kinesin I (35) motility, likely due to the electrostatic interactions between motor proteins and tubulin. This hypothesis is particularly attractive because electrostatics often affect binding and unbinding kinetics, including \( k_{on} \), which our results suggest is higher in *Chlamydomonas* tubulin than in porcine tubulin.

A second possibility is that dynein recognizes differences in the posttranslational modifications of *Chlamydomonas* axonemal tubulin and porcine brain tubulin. Tubulin is highly modified after posttranslation, and the abundance of different isoforms varies with the organism, tissue, and organelle (19,20). It is not known how these differences in isoform influence axonemal dynein motility, but there is some evidence that it may indeed be important for axonemal motility. A mutation in a glutamic acid ligase involved in polyglutamylation caused reduced motility in *Chlamydomonas* (36,37) and *Tetrahymena* (38) cilia. In addition, it has been observed that the B-tubule of microtubule doublets, to which axonemal dynein binds in an ATP-dependent
manner, is more heavily detyrosinated (39) and polyglutamylated (36,38) than the A-tubule.

**Summary and outlook**

Displacement-weighted velocity analysis reveals that *Chlamydomonas* axonal dynein has greater motility on *Chlamydomonas* microtubules than on porcine microtubules. This highlights the importance of using conspecific assays for the in vitro analysis of motor proteins. Displacement-weighted velocity analysis may extend beyond the analysis of axonemal dynein to other unsteady motor systems. For instance, it could be used to analyze intracellular cargo transport by motor proteins acting in an antagonistic manner (40,41), motor stepping assays (42,43), RNA polymerase transcription (44,45), and whole-cell motility (46–48) and migration (49). Thus, future studies on a variety of systems could benefit from this approach.

**SUPPORTING MATERIAL**

Four figures, two tables, supporting methods, and references (50,51) are available at [http://www.biophysj.org/biophys/supplemental/S0006-3495(13)00380-9](http://www.biophysj.org/biophys/supplemental/S0006-3495(13)00380-9).

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