A fluid membrane enhances the velocity of cargo transport by small teams of kinesin-1

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Kinesin-1 (hereafter referred to as kinesin) is a major microtubule-based motor protein for plus-end-directed intracellular transport in live cells. While the single-molecule functions of kinesin are well characterized, the physiologically relevant transport of membranous cargos by small teams of kinesins remains poorly understood. A key experimental challenge remains in the quantitative control of the number of motors driving transport. Here we utilized “motile fraction” to overcome this challenge and experimentally accessed transport by a single kinesin through the physiologically relevant transport by a small team of kinesins. We used a fluid lipid bilayer to model the cellular membrane *in vitro* and employed optical trapping to quantify the transport of membrane-enclosed cargos versus traditional membrane-free cargos under identical conditions. We found that coupling motors via a fluid membrane significantly enhances the velocity of cargo transport by small teams of kinesins. Importantly, enclosing a cargo in a fluid lipid membrane did not impact single-kinesin transport, indicating that membrane-dependent velocity enhancement for team-based transport arises from altered interactions between kinesins. Our study demonstrates that membrane-based coupling between motors is a key determinant of kinesin-based transport. Enhanced velocity may be critical for fast delivery of cargos in live cells. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5006806>

I. INTRODUCTION

Kinesin-1 is a major molecular motor that actively transports cellular materials (“cargos”) along microtubule tracks in live cells.¹ This process is critical to biological function and health; dysfunctions in transport are linked to diseases including neurodegeneration. Single-molecule biophysical studies utilizing optical trapping^{2–6} have uncovered a great deal about the function of individual kinesins in minimal cell-free systems, empowering investigations into the roles of physiologically relevant factors in kinesin-based transport. Fundamental understanding of these factors is necessary for developing future strategies to combat transport malfunctions in cells and to improve human health.

In live cells, a cargo is often enclosed in a fluid lipid membrane and transported by a small team of approximately two kinesins,^{7–10} a scenario that is not captured in classical single-molecule investigations. To bridge this gap, several *in vitro* studies, including our own,^{11,12} have begun to adapt single-molecule approaches to examine transport by a team of kinesins.^{13–21} While these investigations have revealed important emergent behaviors of small teams of kinesins, the cargos utilized in these and most *in vitro* studies still lack a physiologically relevant membrane. The use of model membranes to investigate motor-based transport^{22–27} and the development of membrane-enclosed cargos that are directly compatible with optical trapping^{26–29} have undergone important advances in

recent years. Excitingly, a fluid lipid bilayer and a membrane-mimicking flexible linkage were recently demonstrated to impact cargo transport by two major molecular motors (the actin-based myosin Va²⁴ and the microtubule-based cytoplasmic dynein,³⁰ respectively); the transport speed of kinesins was also demonstrated to differ for membrane-bound versus glass-anchored motors in a recent study using microtubule-gliding experiments.²⁵ Whether or how a fluid membrane impacts cargo transport by small teams of kinesins, however, has remained unclear.

A key experimental challenge in these investigations is that it is difficult to quantify the number of motors driving transport. Precise determination of motor number is fundamentally limited, as equilibrium binding of motors to cargos dictates that the total number of motors on a cargo is not well defined, even when the number of binding sites on the cargo is defined.^{18,30} Due to the three-dimensional nature of the cargo, the interface between the microtubule and the cargo surface is also highly curved, in contrast to the flat geometry in microtubule-gliding experiments. As a result, when the cargo size exceeds the extension length of the motor (such as the $\sim 0.5\ \mu\text{m}$ diameter of the cargo optimized for optical trapping³¹ vs. the $\sim 0.08\ \mu\text{m}$ length of the native kinesin motor^{32,33}), only a fraction of the motors present on the cargo surface are within reach of the microtubule to contribute to transport. For membrane-free cargos, this fraction depends non-trivially on the Poisson statistics, cargo size, and motor length.^{2,12,34} The presence of a fluid membrane further complicates the problem, as motors can in principle exploit diffusion to cluster near the microtubule, thereby increasing the fraction and

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thus the number of motors on the cargo that can contribute to transport.

For membrane-free cargos, “motile fraction” has served as a quantitative guide for motor number in classical single-molecule studies utilizing optical trapping.² Motile fraction, which indicates the probability that a cargo undergoes directed motion along the microtubule, is directly sensitive to the number of motors driving cargo transport.² The greater the motor number, the larger the motile fraction. In contrast to motor number, motile fraction is readily tuned and measured in experiments, particularly when an optical trap is used to confine individual cargos to the vicinity of the microtubule for interaction.^{2,3,12} A motile fraction of ≤ 0.3 has served as a quantitative guide for reaching the single-molecule range in optical trapping-based investigations.⁵ We recently extended the range of this quantitative guide and experimentally determined the one-to-one correspondence between motile fraction and motor number for a range of higher motile fractions.¹² Our recent work indicates that motile fractions of 0.3–0.8 encompass transport by a single kinesin through physiologically relevant transport by a small team of approximately two kinesins.¹²

In the current study, we applied motile fraction to tune the number of motors driving membrane-enclosed cargos at the start of transport. We used a single-beam optical trap to determine the motile fraction of membrane-enclosed cargos, as we did for standard membrane-free cargos. We also matched the size of our membrane-enclosed cargos to that of our membrane-free cargos, in order to preserve the one-to-one correspondence between motile fraction and motor number determined in our recent study.¹² Note that although membrane-bound motors can exploit diffusion to cluster near the microtubule, this clustering requires the cargo to break its rotational symmetry with respect to the microtubule. Because the optical trap acts as a frictionless bearing and the cargo rotates freely within the trap,³⁵ clustering cannot take place before the cargo demonstrates directed motion along the microtubule, and thus clustering cannot impact motile fraction. We quantified the transport of membrane-enclosed versus membrane-free cargos across six motile fractions. We then determined the impact of the fluid membrane on cargo transport at each motile fraction. Our data demonstrate that a fluid cargo membrane significantly enhances the transport velocity of cargos carried by small teams of kinesin motors.

II. MATERIALS AND METHODS

A. Materials

Bovine brain tubulin was purified over a phosphocellulose column as previously described.³⁶ Recombinant kinesin protein (human K560, polyhistidine-tagged) was purified as previously described.³⁷ Lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA(Ni)), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (rhodamine PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Silicon beads (0.5 μm in diameter) were purchased from Bangs Laboratories,

Inc. (Fishers, IN, USA). Carboxylated polystyrene beads (0.5 μm in diameter) were purchased from Polysciences, Inc. (Warrington, PA, USA). Chemicals including 1,4-piperazinediethanesulfonic acid (PIPES), ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), Ethylenediaminetetraacetic acid (EDTA), adenosine triphosphate (ATP), and guanosine triphosphate (GTP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

B. Microtubule preparation

Purified tubulin was diluted to 40 μM in PM buffer (pH 6.9, 100 mM PIPES, 1 mM MgSO_4 , 2 mM EGTA) supplemented with 0.5 mM GTP and incubated at 37 °C for 20 min for assembly. The assembled microtubules were mixed with an equal volume of PM buffer supplemented with 40 μM taxol, followed by a second incubation at 37 °C for 20 min. After assembly, microtubules were kept at room temperature in a dark box and used within four days of preparation.

C. Preparation of membrane-enclosed cargos

Membrane-enclosed cargos were prepared as previously described^{26–29} with modifications. A lipid mixture of 94.95% DOPC, 5% DGS-NTA(Ni), and 0.05% rhodamine PE was dried under vacuum at room temperature overnight. The dried lipid film was resuspended to a stock concentration of 4 mM in HNa100 buffer (pH 7.0, 20 mM HEPES, 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol) via five freeze-thaw cycles, in which the lipid solution was flash frozen in a liquid nitrogen bath, followed by thawing in hand and vortexing briefly. The stock lipid solution was flash frozen in 100- μl aliquots and kept at –20 °C until use. Silica beads (0.5 μm in diameter, Bangs Laboratories) were bath-sonicated in methanol, resuspended in 1 N KOH, bath-sonicated again, washed seven times in nanopure water, and bath-sonicated prior to use. Aliquots of lipid stock were diluted in an equal volume of HNa100 to 2 mM and passed through a mini-lipid extruder (30-nm polycarbonate membrane, Avanti Polar Lipids) 21 times to create small unilamellar liposomes.²⁹ Immediately after extrusion, lipids were incubated with freshly washed silica beads for 30 min at room temperature in a dark box, with occasional gentle tapping to prevent bead sedimentation. The resulting membrane-enclosed cargos were washed four times in HNa100, resuspended in casein (5.55 mg/ml) in PMEE buffer (pH 7.2, 35 mM PIPES, 5 mM MgSO_4 , 1 mM EGTA, 0.5 mM EDTA) for 30 min at room temperature in a dark box, and kept at 4 °C for use within two days.

D. Preparation of motor/cargo complexes

Recombinant kinesin was incubated with membrane-enclosed cargos or carboxylated polystyrene beads (membrane-free cargos) in motility buffer (pH 6.9, 67 mM PIPES, 50 mM $\text{CH}_3\text{CO}_2\text{K}$, 3 mM MgSO_4 , 1 mM dithiothreitol, 0.84 mM EGTA, 10 μM taxol) for 10 min at room temperature. This solution was supplemented with an oxygen-scavenging solution (250 $\mu\text{g/ml}$ glucose oxidase, 30 $\mu\text{g/ml}$ catalase, 4.6 mg/ml glucose) and 1 mM ATP prior to motility measurements. The concentration of membrane-enclosed cargos and the concentration of membrane-free cargos were kept constant at

3.38×10^5 particles/ μl and 3.25×10^5 particles/ μl , respectively. These cargo concentrations were empirically determined to optimize the number of cargos in our field of view for optical trapping (data not shown). The concentration of kinesin was empirically varied to give rise to a range of motile fractions, as previously described.^{2,12}

E. Motile fraction and transport measurements

Motile fraction and cargo transport were measured in flow chambers using a single-beam optical trap, imaged via differential interference microscopy, and video-recorded at 30 Hz as previously described;¹² identical conditions were employed for membrane-enclosed cargos and membrane-free cargos. Briefly, we used a custom-constructed single-beam optical trap^{31,38} to locally confine individual kinesin/cargo complexes to the neighborhood of the microtubule and scored the fraction of cargos that demonstrated directed motility along the microtubule (“motile fraction”). To limit the interference from external load, we used a very low trap power (<20 mW at fiber output) such that the optical trap was sufficient to position individual cargos but could not stall cargos carried by a single kinesin.¹² Upon observation of directed motion along the microtubule, we turned off the optical trap to enable cargo transport in the absence of external load.

F. Data analysis

Cargo trajectories were particle-tracked to 10 nm resolution (1/3 pixel) using a template-matching algorithm as previously described.³⁹

The run length of a motile cargo was determined as the net displacement of the cargo along the microtubule between binding and dissociation. To account for the time that elapsed during manual shut-off of the optical trap, only trajectories with >300 nm of motion were analyzed. The cumulative probability distribution of the measured run lengths was fitted to the cumulative probability function of a single exponential distribution, $1 - Ae^{-x/d}$. The mean run length and standard error for each distribution were determined from the best-fit decay constant d and uncertainty, respectively.

The association time of individual kinesin/cargo complexes with the microtubule was determined as the time between landing and dissociation. To account for the human reaction time during manual shut-off of the optical trap, only trajectories >0.3 s were analyzed. The cumulative probability distribution of the association time was fitted to the cumulative probability function of a single exponential distribution, $1 - Ae^{-x/t}$. The mean association time and standard error for each distribution were determined from the best-fit decay constant t and uncertainty, respectively.

Pausing during continuous cargo motion was identified without operator bias using the Bayesian statistics-based automatic software⁴⁰ that parses each cargo trajectory into a series of segments of constant velocity. To evaluate cargo velocity under no load, only portions of each trajectory >300 nm were analyzed. A pausing event was identified when the velocity fell below 100 nm/s for at least 0.5 s. The frequency of pausing was determined as the number of pauses per second of cargo trajectory. The cumulative probability distribution of pause duration was fitted to the cumulative probability function of

a single exponential distribution, $1 - Ae^{-x/p}$. Unless specified as the arithmetic mean, mean pause duration and standard error were determined from the best-fit decay constant p and uncertainty, respectively.

The velocity of each trajectory was determined as the average velocity of its non-pausing segments, weighted by the corresponding segmental durations. The median velocity and standard error for each experimental condition (motile fraction and cargo type) were determined as the median and the associated standard error of the individual cargo velocities, respectively.

G. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine significant differences between multiple distributions of pause frequency or velocity measurements. The rank-sum test was used to detect significant differences between two distributions of measurements of pause duration or run length. Welch’s t -test was used to determine the significance of differences between two distributions of velocity measurements with unequal variances.

III. RESULTS AND DISCUSSION

We used optical-trapping-based measurements to determine the impact of a fluid cargo membrane on kinesin-based transport *in vitro* (Fig. 1). We employed traditional polystyrene beads as our membrane-free cargos.^{5,12,15} For membrane-enclosed cargos, we employed a DOPC-based lipid bilayer to model the fluid cellular membrane^{24–27} and used a silica core to control cargo size and to ensure compatibility with optical trapping^{2,28} (Fig. 1). Note that the silica core does not influence the fluidity of our model membrane⁴¹ but minimizes deformation of the supported membrane during kinesin-based transport.²⁸ Importantly, the presence of a lipid membrane does not significantly impact the refractive index or the physical size of the membrane-coated cargo^{28,42,43} and thus does not impact optical trapping or the one-to-one correspondence between motile fraction and motor number determined in our recent study.¹² For each cargo type, we empirically tuned the input ratio of kinesins to cargos to achieve six motile fractions and measured 97–231 trajectories per motile fraction. Using these measurements, we characterized three key transport characteristics (run length, pause, and velocity) for membrane-enclosed cargos and membrane-free cargos under identical conditions. We used cumulative probability distributions, as opposed to histograms, to better illustrate the potential difference or agreement between two sets of measurements [for example, Fig. 2(a)]. As detailed below, these key transport characteristics critically impact the distance and speed of cargo delivery *in vivo* and are likely sensitive to changes in inter-motor interactions associated with a fluid cargo membrane.

A. Cargo run length is similar between membrane-enclosed and membrane-free cargos across six motile fractions

We first examined the impact of a fluid membrane on cargo run length (Fig. 2). Extended run length is important for

FIG. 1. Experimental schematic (not to scale). (a) Optical trapping-based experiments were carried out in flow chambers, identically for membrane-enclosed cargos [panels (b) and (c)] and standard membrane-free cargos (not shown). The flow chamber was constructed by adhering a cover glass and a microscope slide together using two thin strips of double-sided Scotch tape. Microtubules were immobilized on the cover-glass surface; kinesin/cargo complexes were freely dispersed in solution in the flow chamber. (b) A single-beam optical trap was used to confine individual cargos to the vicinity of a microtubule to reduce the initial interaction distance between the microtubule and kinesins on the cargo. The trap was turned off upon observation of directed cargo motion along the microtubule, eliminating external load. For the membrane-enclosed cargos illustrated here, a silica bead supports a DOPC-based lipid bilayer, ensuring compatibility with the optical trap and matching the size of membrane-enclosed and membrane-free cargos for direct comparison. (c) Polyhistidine-tagged kinesins were specifically recruited to membrane-enclosed cargos via a nickel-functionalized lipid incorporated in the bilayer membrane.

achieving transport across large spatial distances in cells. Previous studies of membrane-free cargos revealed that the run length increases as the motor number increases.^{11,12,15–20} Here we used the motile fraction to match the number of motors driving each cargo type at the start of transport. For membrane-free cargos, this motor number remains unchanged during transport as the motors are uniformly and statically anchored on the cargo surface.² For membrane-enclosed cargos, however, the number of motors driving the cargo can in principle increase during transport as the motors exploit diffusion to cluster near the microtubule. We thus anticipated that at the same motile fraction, the run length of membrane-enclosed cargos would be significantly longer than that of membrane-free cargos.

For membrane-free cargos and consistent with previous studies, we found that the run length increased gradually but

significantly with increasing motile fraction (Fig. 2). In the single-molecule range (motile fraction ~ 0.3), the mean run length was $1.60\ \mu\text{m}$ [black line, Fig. 2(a–i)], in excellent agreement with previous reports of single-kinesin run length for a similar recombinant construct.^{13,18,44} At a motile fraction of 0.8, the cargo run length was significantly longer than that at a motile fraction of 0.3 ($p = 0.028$, rank-sum test) and agreed well with that expected for a small team of kinesins linked to membrane-free cargos.^{11,12,15–20}

Contrary to our expectation, at none of the six motile fractions was the run length significantly longer for membrane-enclosed cargos versus membrane-free cargos (Fig. 2; $p \geq 0.21$, rank-sum test). Note that at the highest motile fraction examined (0.8), although the average run length for membrane-enclosed cargos was somewhat higher than that for

FIG. 2. Run length does not differ between membrane-enclosed cargos (red) and membrane-free cargos (black). (a) Cumulative probability distributions of cargo run length at three motile fractions. Mean run length ($\pm$ standard error) and p -value (rank-sum test) are indicated ($n = 117$ –197). The two distributions measured at each motile fraction do not differ significantly from each other ($p \geq 0.27$, rank-sum test). (b) Mean run length ($\pm$ standard error) at six motile fractions ($n = 117$ –197). The two mean run lengths determined at each motile fraction do not differ significantly from each other ($p \geq 0.21$, rank-sum test).

membrane-free cargos [$2.48\ \mu\text{m}$ vs. $2.04\ \mu\text{m}$, Fig. 2(a-iii)], this difference was not statistically significant ($p = 0.28$, rank-sum test). Our data suggest that in the physiologically relevant few-kinesin range, a fluid membrane does not substantially impact the number of kinesins driving transport. We do not rule out the possibility of a subtle increase in motor number for membrane-enclosed cargos, but the effect is not sufficient to significantly increase the run length.

Our finding is surprising, as diffusion within the fluid membrane ought to significantly enhance the number of kinesins driving membrane-enclosed cargos. The time scale required for a kinesin to explore the cargo surface to come within reach of the microtubule ($\sim 0.56\ \text{s}$ using a diffusion constant of $1.4\ \mu\text{m}^2/\text{s}$, Ref. 25) is substantially shorter than the mean association time between the membrane-enclosed cargo and the microtubule ($2.67\text{--}4.78\ \text{s}$ depending on motile fraction, data not shown); the single kinesin also has a significant, >5 -fold faster associated rate than the dissociation rate for the microtubule.^{6,21,22} However, the area on the cargo directly accessible to the microtubule may not remain constant during transport. A cargo can “tumble” as individual motors stochastically bind to and unbind from the microtubule.^{17,24,34} With each tumble, a different portion of the cargo surface would be exposed to the microtubule, and the clustering process would start afresh. We thus speculate that although the fluid membrane can promote motor clustering, its effect on the run length is limited by stochastic tumbling of the cargo during transport. In principle, stochastic tumbling should occur less frequently for larger cargos. However, because the time scale of motor diffusion scales quadratically with cargo size, the effect of motor clustering is also more limited for larger cargos. Owing to the spherical symmetry of cargos used in most *in vitro* studies, direct observation of tumbling remains difficult. Future developments, such as anisotropic labeling of spherical particles,

will empower investigations into the extent of cargo tumbling and its impact on the clustering of membrane-bound motors during transport.

B. Membrane-enclosed cargos pause similarly to membrane-free cargos across six motile fractions

We next examined the impact of a fluid membrane on cargo pausing (interruptions during continuous motion). *In vivo*, pauses can delay the delivery of cargos to target destinations but can also contribute to cargo sorting at the pause site.⁴⁵ The mechanism underlying single-motor pausing has remained unclear. When functioning in a small team, the paused motor lags behind and pulls against moving motors via their common cargo. This force-based interaction is delayed for membrane-enclosed cargos because the attachment point of the motor to the cargo can “slide” within the membrane to alleviate force in the direction parallel to the cargo surface.^{24,25} A delayed interaction between motors can potentially increase the likelihood that the paused motor stochastically resumes motion before the cargo stops, thereby reducing the sensitivity of cargo transport to pausing of individual motors.

We did not detect a substantial difference in the pausing frequency between cargo types [Fig. 3(a)]. For both membrane-enclosed and membrane-free cargos, the frequency of pausing remained relatively low and did not change appreciably with increasing motile fraction ($p \geq 0.41$, ANOVA). Averaging across six motile fractions, we determined a mean pausing frequency of $0.040 \pm 0.006\ \text{s}^{-1}$ for membrane-enclosed cargos, in good agreement with a mean pausing frequency of $0.035 \pm 0.005\ \text{s}^{-1}$ for membrane-free cargos [Fig. 3(a)].

We also did not detect a significant difference in pausing duration between cargo types [Figs. 3(b) and 3(c)]. Due to

FIG. 3. Pausing does not differ between membrane-enclosed (red) and membrane-free (black) cargos. Arithmetic mean (a) frequency ($n = 4\text{--}5$ experiments) and (b) duration ($n = 11\text{--}48$ pauses) of pauses at six motile fractions. (c) Cumulative probability distribution of pause duration, pooled from measurements at all six motile fractions ($n \geq 175$ pauses). Mean pause duration ($\pm$ standard error) is indicated. These two distributions do not differ significantly from each other ($p = 0.23$, rank-sum test).

the low pausing frequency [Fig. 3(a)], the number of pauses that we measured was limited for each cargo type at each motile fraction ($n = 11$ –48 pauses). Despite this limitation, our data indicate that the associated arithmetic mean duration of pauses agreed well between cargo types at each motile fraction and remained approximately constant across motile fractions for both cargo types [Fig. 3(b)]. To increase the statistical power of our analysis, we combined measurements from all motile fractions for each cargo type [Fig. 3(c)]. These combined data again revealed similar pausing durations between cargo types ($p = 0.23$, rank-sum test), giving rise to a mean duration of 1.53 ± 0.01 s for membrane-enclosed cargos and 1.69 ± 0.01 s for membrane-free cargos [Fig. 3(c)]. These mean durations are in good agreement with the single-motor pausing duration previously reported for a similar kinesin construct.⁴⁴

Our data suggest that individual kinesins pause asynchronously when functioning in small teams. The low frequency of single-kinesin pausing [~ 0.040 s⁻¹ at a motile fraction ~ 0.3 , Fig. 3(a)] corresponds to an extended interval of ~ 25 s between successive pauses, which is >5 -fold longer than the average association time of our cargos with the microtubule (2.7–4.8 s depending on motile fraction, data not shown). When motors are not synchronized with each other, the probability that two or more motors pause at the same time is low. In this scenario, the frequency of cargo pausing is largely approximated by the frequency of single-motor pausing, which is in good agreement with our observations [Fig. 3(a)].

Further, our data indicate that the fluid membrane does not alter the sensitivity of a cargo to single-kinesin pausing. While sliding of motors within the membrane may delay the force-based interference between the paused motor and the moving motors, the time scale of this delay is likely substantially shorter than the typical duration of single-kinesin pausing [~ 1.6 s, Fig. 3(c)]. It is possible that the fluid membrane is effective in limiting the sensitivity of the cargo to significantly shorter single-motor pauses. Future identification of factors that significantly reduce single-kinesin pausing duration will help shed light on this intriguing possibility.

C. Membrane-enclosed cargos move faster than membrane-free cargos at higher motile fractions

We next examined the impact of a fluid membrane on cargo velocity. The velocity of a single motor is not typically uniform within a population of kinesins.^{46,47} During transport by a small team of kinesins, the faster motors lead the cargo and the slower motors lag behind; force-based interactions between motors tend to prompt the lagging kinesin to dissociate prematurely from the microtubule,⁶ causing the cargo to tumble forward to the position of the leading motor. Sliding of motors within the lipid membrane delays force-based interactions between motors and enables the leading kinesin to move forward further before the lagging motor dissociates. We thus predicted that when transported by small teams of kinesins, membrane-enclosed cargos would move faster than membrane-free cargos under identical conditions.

For membrane-free cargos, we detected a significant change in the distribution of cargo velocity with increasing motile fraction ($p = 3 \times 10^{-8}$, ANOVA) [Fig. 4(a), left]. In the single-motor range (motile fraction ~ 0.3), this distribution revealed a subpopulation of slow runs: 26% of cargos moved at <0.6 $\mu\text{m/s}$ [Fig. 4(b), top]. Heterogeneity in single-kinesin velocity has been previously observed for the same motor construct.^{46,47} Here, we found that the fraction of slow runs increased substantially as the density of motors on the cargo increased. For example, at a motile fraction of 0.8, 56% of cargos moved slower than 0.6 $\mu\text{m/s}$ [Fig. 4(b), top]. The median velocity of these slow runs remained largely constant at different motile fractions [Fig. 4(c), top] as did the median velocity of cargos moving at ≥ 0.6 $\mu\text{m/s}$ [Fig. 4(d), top]. Together, our data demonstrate a significant, 19% reduction in median velocity at a motile fraction of 0.8 versus a motile fraction of 0.3 ($p = 9 \times 10^{-8}$, Welch's t -test). This observation is consistent with the previous reports of reduced cargo velocities at higher motor densities.^{15,17}

By contrast, the velocity distribution of membrane-enclosed cargos did not change significantly with motile fraction ($p = 0.80$, ANOVA). Irrespective of motor density, most cargos ($\geq 70\%$) moved faster than 0.6 $\mu\text{m/s}$ [Fig. 4(a), right] and the median velocity of these fast runs remained approximately constant [Fig. 4(d), bottom]. Interestingly, the median velocity of slow runs was somewhat faster at higher motile fractions, increasing by 1.5-fold from 0.34 ± 0.03 $\mu\text{m/s}$ at a motile fraction of 0.3 to 0.51 ± 0.03 $\mu\text{m/s}$ at a motile fraction of 0.8 [Fig. 4(c), bottom]. Because the fraction of slow runs was limited ($<30\%$), velocity changes in slow runs did not significantly impact the overall median velocity of membrane-enclosed cargos (including both slow and fast runs), which remained approximately constant across motile fractions, with $<5\%$ variation [Fig. 4(a), right].

At a motile fraction of 0.3 (approximately single motor range), the distribution of the velocities of membrane-enclosed cargos did not differ significantly from that of the membrane-free case [$p = 0.68$, Welch's t -test, Fig. 5(a–i)]. Both cargo types moved at a similar median velocity of ~ 0.7 $\mu\text{m/s}$ [Fig. 5(a–i)], in good agreement with the previously reported single-motor velocity of similar kinesin constructs.^{13,18,44} This observation is expected because a fluid membrane should not impact the intrinsic function of the motor at the single-molecule level.

At higher motile fractions, the distribution of the velocities of membrane-enclosed cargos differed significantly from that of membrane-free cargos [for example, $p \leq 0.004$, Welch's t -test, Figs. 5(a–ii) and 5(a–iii)]; the median velocity of membrane-enclosed cargos was substantially faster than the membrane-free case [as indicated by positive values in Fig. 5(b)]. We did not detect any indication of membrane-free cargos moving faster than membrane-enclosed cargos at any motile fraction, as the velocity difference between the two cargo types did not fall substantially below zero [Fig. 5(b)]. At a motile fraction of 0.8, the mean velocity of membrane-enclosed cargos demonstrated a significant, 0.15 ± 0.03 $\mu\text{m/s}$ increase over the membrane-free velocity [$p = 3.7 \times 10^{-9}$, Welch's t -test, Figs. 5(a–iii) and 5(b)].

FIG. 4. Changes in velocity distributions at distinct motile fractions differ between cargo types. (a) Dot plot of velocity measurements for each experimental condition ($n = 97$ –208). Horizontal bars—30th percentile, median, and 70th percentile. ** $p < 0.005$ versus a motile fraction of 0.3 (Welch's t -test). [(b) and (c)] Fraction and median velocity of cargos moving at $<0.6 \mu\text{m/s}$. (d) Median velocity of cargos moving at $\geq 0.6 \mu\text{m/s}$. Error bars in [(b)–(d)]—standard error. Blue lines are guides to the eye. At higher motile fractions, we detected a substantially smaller fraction of slow runs (b) and a somewhat faster median velocity of these slow runs (c) for membrane-enclosed cargos than membrane-free cargos.

Our data indicate that for cargo transport by a small team of kinesin-1 motors, coupling motors via a fluid membrane reduces the fraction of slow runs [Fig. 4(b)] and increases the median velocity of these slow runs [Fig. 4(c)]. The observed

reduction in the subpopulation of slow runs is consistent with a model in which the membrane delays force-based interactions between kinesins: upon dissociation of the lagging motor, a membrane-enclosed cargo tumbles forward a larger distance

FIG. 5. Membrane-enclosed cargos (red) move faster than membrane-free cargos (black) at higher motile fractions. (a) Cumulative probability distributions of velocity at three motile fractions. Median velocity ($\pm$ standard error) and p -value (Welch's t -test) are indicated ($n = 97$ –139). (b) The difference in the median velocity of two cargo types at each motile fraction. A positive value indicates that the membrane-enclosed cargos move faster than membrane-free cargos. The pink line is a guide to the eye.

than does a membrane-free cargo. A motor may lag due to intrinsic heterogeneity in single-kinesin velocity [Fig. 4(a) and Refs. 46 and 47], stochastic pausing of the motor (Fig. 3), and/or the potential presence of inactive motors within protein purification.⁴⁸ In this model, the effect of the membrane on velocity is smaller for cargos with less-fluid membranes. We are working to develop new cargos with altered membrane fluidity to test this possibility. Because the mobility of the motor's attachment point on the cargo surface is limited by the length of the motor, it will also be interesting to explore how the effect of the membrane can be modulated by the length of the motor's tail domain. We note that the exact mechanism underlying the slowing velocity of membrane-free cargos is not fully understood. In addition to the force-based interactions between motors discussed here, non-mechanical interactions (for example, the physical spacing of motors on the microtubule) can also influence velocity for membrane-free cargos.^{18,20,49} It is possible that a delay in force-based interactions may also impact non-mechanical interactions as well as force-based interactions between motors, further contributing to the observed velocity increase of membrane-enclosed cargos versus membrane-free cargos.

Our finding is perhaps surprising as a recent study using microtubule-gliding experiments revealed a slower transport velocity for kinesins anchored atop a fluid lipid membrane versus a traditional glass surface.²⁵ However, a key difference between studies is that the interface between the cargo and the microtubule is highly curved in optical-trapping experiments (Fig. 1) but flat in microtubule-gliding experiments.²⁵ Sliding of the motor's attachment point within the cargo membrane alleviates the component of force parallel to the cargo surface, but not the component of force perpendicular to the cargo membrane. *In vivo*, the curvature of the cargo/microtubule interface can differ between cargo types. Our study raises the exciting possibility that the impact of a fluid membrane combines nontrivially with cargo geometry to control kinesin-based transport.

IV. SUMMARY

Here we applied classical single-molecule methodologies to dissect the impact of a fluid membrane on cargo transport by a small team of kinesin-1 motors. We used motile fraction as a quantitative guide to tune the number of kinesins at the start of cargo transport. We applied optical trapping to determine the motile fraction and to quantify the transport of membrane-enclosed cargos versus membrane-free cargos under identical conditions. In the single-motor range (motile fraction ~ 0.3), we detected the same run length, pause frequency/duration, and velocity for both cargo types; our measurements are in good agreement with previous reports of single-kinesin values. For transport by a small team of kinesins (for example, motile fraction ~ 0.8), we found that coupling motors via a fluid membrane significantly increased cargo velocity, while leaving the run length and pausing unchanged. The lack of effect of a membrane on the run length may reflect stochastic tumbling of cargos during transport. The increased velocity of membrane-enclosed cargos supports a model in which a lipid membrane delays force-based interactions between kinesins,

allowing the cargo to maintain the single-motor velocity rather than slowing as in the membrane-free case. Our study highlights the fluid cargo membrane as an important determinant of the fundamental biological process of transport by kinesins. An enhanced velocity is likely important for fast delivery of cargos in cells.

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