Myosin Va molecular motors manoeuvre liposome cargo through suspended actin filament intersections in vitro

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Intracellular cargo transport relies on myosin Va molecular motor ensembles to travel along the cell’s three-dimensional (3D) highway of actin filaments. At actin filament intersections, the intersecting filament is a structural barrier to and an alternate track for directed cargo transport. Here we use 3D super-resolution fluorescence imaging to determine the directional outcome (that is, continues straight, turns or terminates) for an ~10 motor ensemble transporting a 350 nm lipid-bound cargo that encounters a suspended 3D actin filament intersection in vitro. Motor-cargo complexes that interact with the intersecting filament go straight through the intersection 62% of the time, nearly twice that for turning. To explain this, we develop an in silico model, supported by optical trapping data, suggesting that the motors’ diffusive movements on the vesicle surface and the extent of their engagement with the two intersecting actin tracks biases the motor-cargo complex on average to go straight through the intersection.

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The final step in the delivery of secretory vesicles such as insulin granules to the cell membrane relies on myosin Va (myoVa) molecular motors to manoeuvre their cargo through the cell’s cortical actin meshwork (Fig. 1a). The actin cortex is a dense, three-dimensional (3D) cytoskeletal highway in which the plus-ends of individual actin filaments are biased towards the cell membrane, which is the direction in which myoVa travels. However, this seemingly random highway, with its numerous actin filament intersections, makes efficient straight-line cargo delivery from point A to B directionally challenging (Fig. 1a). In addition, the actin cortex can act as a structural barrier to transport when cargo diameters approach the mesh size of the dense actin network. Therefore, the need to define how myoVa motor ensembles deliver their cargo in a directed manner is emphasized by myoVa genetic mutations leading to mislocalized cargo such as melanosomes and endoplasmic reticulum in melanocytes and Purkinje neurons, respectively, which in turn cause albinism and neurological defects in humans and the dilute mouse.

Due to the complexities of studying myoVa cargo transport in cells, investigators have characterized how individual myoVa or ensembles of these motors transport non-physiological cargo in vitro (for example, silica beads, DNA scaffolds) along single actin filaments or simple actin cytoskeletal models. As the next step to understanding how myoVa motor ensembles meet the mechanical and directional challenges of the cell’s complex 3D actin cytoskeleton, we create an in vitro network of suspended actin filaments with numerous intersections (Fig. 1c). This network is designed to directionally challenge constitutively active myoVa motor ensembles transporting more physiologically relevant, lipid-bound vesicle cargos. Motor–cargo complexes travelling along an actin filament that encounter and physically interact with a suspended intersecting filament continue straight through the intersection on the original filament it is travelling on 62% of the time. This is surprising given that the intersecting filament is both a structural barrier and an alternate track to travel on. To explain this observation, we develop an in silico, mechanico model that describes the diffusive movement of motors on the vesicle surface, their engagement with the two intersecting actin tracks (Fig. 1b) and the ensuing ‘tug-of-war’ between the two myoVa ensembles that eventually dictates the directional outcome at an intersection.

Results

3D actin intersection and 3D myoVa transport characterization. 3D actin filament intersections were created by suspending actin filaments between 3 μm silica beads, DNA scaffolds that adhered electrostatically to the glass surface of a microfluidic chamber (Methods section). By flowing fluorescent Alexa647-phalloidin–labelled actin filaments into the chamber through orthogonal ports, a sparse actin network was created with actin filaments running parallel to the glass surface at different heights. The height for any filament relative to the microscope’s focal plane was determined by 3D super-resolution STORM imaging with 5 nm precision (Methods section). Imaged filaments (Fig. 1c–e) demonstrate uniform colours along their length, reflecting a constant height for the actin filament along its entire length. By this approach, nearly perpendicular actin filament intersections (when viewed from above in a 2D projection, Fig. 1d,e) were formed with up to 250 nm centre-to-centre filament separation at the intersection (parameter d in Fig. 2a). Fluorescent Dil–labelled liposomes (~350 nm dia.), comparable in size to physiological cargos, with ~10 surface-bound myoVa motors (Supplementary Fig. 1) were then introduced into the chamber. The ability for motors to diffuse on the liposome’s fluid-like surface (0.92 μm² s⁻¹ (ref. 17)) resulted in ensembles of motors assembling at sites of actin filament engagement, which distinguishes this study from most previous studies in which the motors’ positions on the cargo were fixed and thus far from physiological. The liposome transport trajectories were tracked in 3D with high spatial precision (17 nm X, 18 nm Y, 30 nm Z) and temporal (100 ms) resolution, using an intentionally induced optical astigmatism so that the shape of the liposome’s fluorescent image defined its Z-position relative to the actin filament on which it travelled (Supplementary Fig. 2).

Before encountering an intersection, motor–cargo complexes travelled at velocities of 423 ± 24 nm s⁻¹ (mean ± s.e.m., n = 67) with run lengths limited by the length of the actin filaments suspended between the beads (3.7 ± 0.2 μm, mean ± s.e.m., n = 135), since nearly all runs terminated at a bead (Fig. 1c). When compared to single-motor run lengths (~1 μm) (ref. 17), multiple-myov motors on the cargo surface must be simultaneously engaged with the actin filament (see below) to account for the nearly four-fold increase in run lengths. Motor–cargo complexes followed a spiralling path (average left-handed pitch: 2.160 ± 40 nm, n = 14; Supplementary Fig. 3A,B) around the suspended actin filament, allowing the motor–cargo complex to approach the intersection at any angle (parameter z in Fig. 2a) relative to the intersecting actin filament. Whether or not the liposome interacts with the intersecting actin filament at any point during its transit through the intersection is a simple geometric consideration, depending on the liposome diameter, its approach angle (z, Fig. 2a), and the filament separation (d, Fig. 2a). The geometries leading to whether an interaction occurs or not are graphically represented as a function of the actin-filament parameter d in Fig. 2a).
of $\alpha$ and $d$ on a polar plot (Fig. 2b). Given the liposome’s 350 nm diameter, the motor–cargo complex will interact with the intersecting filament for all $d$ between 0–225 nm as long as the motor–cargo complex approaches the intersection with $\alpha < 90^\circ$ on either side of the original filament it is travelling on (Supplementary Fig. 4). At approach angles greater than $\pm 90^\circ$, there are certain combinations of $\alpha$ and $d$ where the motor–cargo complex can’t physically interact with the intersecting filament. Knowing the importance of these spatial relationships, we characterized the directional outcomes at an intersection (that is, continued straight, turned or terminated) for those motor–cargo complexes (94 out of 103) that were geometrically predicted to interact with the intersecting filament (Fig. 2b), because those predicted not to interact with the intersecting filament (9 out of 103) went straight, as expected. For those that were spatially positioned to interact, 62% continued straight along the originally bound actin filament (Supplementary Movie 1), 33% turned (Supplementary Movie 2), while 5% either remained at the intersection or detached and terminated their runs (Fig. 2c). Interestingly, these directional outcomes are strikingly different compared with 2D intersections that are formed by adhering actin filaments directly to a glass surface.

MyoVa transport at 2D actin intersections. To create 2D intersections where one filament lying over the top of the other could be distinguished, two separate populations of actin filaments were fluorescently labelled with either TRITC- or FITC-phalloidin and then introduced into the microfluidic chamber in sequential order. In this assay, motor–cargo complexes that approached the intersection on the bottom filament preferred turning (51%, total $n = 96$; Fig. 2c, Supplementary Fig. 5D, Supplementary Movie 3) onto the upper filament at the intersection rather than continuing straight through (33%) (Fig. 2c, Supplementary Fig. 5C, Supplementary Movie 4). To go straight would have required the motor–cargo complex to first transfer onto the intersecting filament (a step up of 7 nm) and then immediately transfer back down to the original filament. In contrast, when approaching the intersection on the upper filament, the motor–cargo complex continues straight...
Mechanistic model of 3D transport and laser trapping. To explain the 3D intersection data, we developed a mechanistic model that simulates the emergence and dynamic nature of motor ensembles on the liposome surface as they engage the suspended actin filaments and then mechanically interact to determine the directional outcome. In brief, the model assumes that the 350 nm vesicle and actin filaments are rigid and that 10 myoVa motors diffuse individually across the liposome's ideally fluid membrane surface (Supplementary Fig. 8; Supplementary Methods). Each motor has elastic properties both in extension (1 pN nm$^{-1}$) and torsion (0.25 pN nm rad$^{-1}$) via their linkage to the liposome. Once bound to an actin filament, a motor takes 36 nm forward steps that are occasionally short (31 nm) or backwards (36 nm) with all step lifetimes being dependent on the resistive load that a motor experiences$^{18-22}$. Resistive loads originate from partner motors within an ensemble attempting to transport the liposome simultaneously and from motors that form within a second ensemble and engage the intersecting actin filament (Fig. 1b). The fact that a myoVa motor can take a short step under resistive load$^{20,23}$ is the underlying basis for the predicted spiral trajectory (Supplementary Fig. 3B, inset). This trajectory is similar in pitch (left-handed: 2.120 ± 200 nm, (10 simulations, mean ± s.d.)) to both the experimental observations reported here (Supplementary Figs 3B and 9) and that of a single myoVa on an actin filament tightrope or multiple motors bound to a glass surface$^{23,24}$. As a motor occasionally steps short on the actin helix, this motor within the ensemble places a torque on the liposome that is relieved once one of the other motors in the ensemble detaches, at which point the cargo re-centres itself over the remaining attached motors, biasing the liposome to follow a spiral trajectory. This cargo re-centring is one factor in the model that contributes to motor–cargo complexes travelling straight past an intersecting actin filament even if it presents a structural barrier (see below).

The model predicts that even though ten motors rapidly diffuse on the liposome’s surface, most often three engaged motors form an ensemble at their site of engagement to the actin filament (Supplementary Fig. 10). This limited number is due to the geometric constraints dictated by the surface area of the liposome from which motors can reach the actin filament and the mechanical properties of the motors themselves. In fact, once one motor binds to actin, the spatial freedom of the liposome is restricted so that another motor joining the ensemble or engaging another filament is less probable (Fig. 4d, Supplementary Fig. 10). This three-motor ensemble prediction was experimentally confirmed by laser trapping a motor–cargo complex travelling along a single actin filament (Fig. 4a; Methods section). For ease of trapping, 500 nm silica beads were lipid coated using the identical liposome preparation so that the lipid coating has the same membrane fluidity and motor surface density. Although 43% larger in diameter, this cargo’s surface area from which motors can engage the actin filament is only 23% larger than that of the 350 nm liposomes. Once engaged, the motor–cargo complex moves until stalling due to the opposing force of the trap. Three distinct populations best described the stall force distribution with peaks at 1.9 ± 0.7, 4.1 ± 0.6 and 5.9 ± 0.4 pN (n = 400). The lowest stall force population is presumably that of a single motor, given that 1.7 ± 0.6 pN (n = 28) stall forces were observed for experiments at limiting motor density (Fig. 4c), which agrees with the 1.8 pN forces we reported previously$^{19}$. Therefore, as predicted by the model for the liposome size and motor density of our experiments, no more than three motors can engage an actin filament (Fig. 4c, d).

3D intersection geometric analysis and modelling. Finally, according to the model, the directional outcome at an intersection is the resolution of a simple ‘tug-of-war’ between the motor ensembles engaged with each of the intersecting actin filaments, where the ensemble with the greater number of motors wins. In our experiments, evidence for a ‘tug-of-war’ is most apparent under conditions where both the liposome diameter (> 350 nm) and motor number (> 25) are higher than in the present system, resulting in deformation of the liposome at a 2D intersection as the liposome is being tugged in two different directions (Supplementary Fig. 6).

The model was used to simulate trajectories that encompassed a range of approach angles ($\alpha$: 0°–360°) and filament separations ($d$: 50–250 nm). When we matched these to our experimental observations, the model predicts (six simulations at each actin intersection, mean ± s.d.) average directional outcomes of 61 ± 5% going straight, 33 ± 5% that turn and 6 ± 4% that terminate, in good agreement with the experimental results for
those motor–cargo complexes that geometrically are predicted to interact with the intersecting filament (Figs 2c and 3, Supplementary Fig. 12B). Interestingly, these overall directional outcomes are independent of the plus-end orientation of the intersecting actin filament, that is, whether it faces left or right (Fig. 2c, inset). Therefore, the turning direction is determined only by the polarity of the intersecting filament and not the side of the original filament the motor–cargo complex is travelling on (that is, \( \alpha \) of \( 0^\circ \text{–}180^\circ \) versus \( 180^\circ \text{–}360^\circ \)). This might explain the equal probability of turning left or right experimentally (Fig. 2c) since there should be no bias in the actin polarity when the filaments are suspended from the beads.

To gain additional insight into how the directional outcome may be influenced by approach angle and filament separation, we plotted the straight-to-turn ratio for the model and experimental data onto a polar plot, as in Fig. 2b. With the side of the original filament on which the motor–cargo complex approaches the intersection not affecting the directional outcome (see above, Fig. 2c, Supplementary Fig. 12A), we simplified the plot by mirroring all motor–cargo complex approach angles onto a \( 0^\circ \text{–}180^\circ \) range (Fig. 3). Given the large parameter space in \( \alpha \) and \( d \) for this plot, we binned the straight-to-turn ratio into five spatial regimes. By visual inspection, in large part there is good agreement between the model predictions and experimental results with the straight-to-turn ratio being \( >1.0 \) over the entire range of \( \alpha \) and \( d \) (Fig. 3). Therefore, regardless of the approach angle and filament separation, as long as the motor–cargo complex interacts with the intersecting filament, the probability for the complex to go straight is greater than the probability to turn. The model provides insight to this result. Prior to the intersection, the model predicts that two to three motors comprise the ensemble that is transporting the liposome. This ensemble restricts the liposome's spatial freedom as described above (Fig. 4d, Supplementary Fig. 10), which in turn limits the number of accessible actin-binding sites on the intersecting filament that a second ensemble of motors from the remaining pool of diffusing motors on the liposome surface can bind (Fig. 4d). Therefore, the likelihood that this second ensemble can develop sufficient force to win the tug-of-war and result in a turning event is low. At this point, the original ensemble will attempt to go straight but will be prevented by the intersecting filament acting as a structural barrier (Fig. 4e, Supplementary Movie 5). Thus, the motor–cargo complex hesitates at the intersection and during that period the number of motors within the original ensemble can fluctuate due to a motor's stochastic rates of attachment (\( \sim 2.4 \text{ s}^{-1} \)) and detachment (\( \sim 0.35 \text{ s}^{-1} \)). With each motor detachment and new motor attachment, the liposome spatially re-centres over the engaged motors and by doing so allows the liposome to change its approach angle (Fig. 4f,g) to the point where the intersecting filament is no longer a barrier and the motor–cargo complex is free to continue straight along the original filament (Fig. 4h). However, due to these stochastic motor number fluctuations in each ensemble, scenarios will arise in which the ensemble on the intersecting filament wins.
the tug-of-war, resulting in a turning event (Figs 1d,2c and 3), which is far less frequent (Supplementary Movie 6).

**Discussion**

Although the simple 3D actin network and the motor–cargo complex described here are first steps to bridging in vitro model systems to intracellular cargo transport, additional complexities such as denser actin networks where the motor–cargo complex could interact with multiple actin filaments simultaneously will bring both technical and modelling challenges. The addition of different motor types (for example, kinesin) to the cargo and their respective microtubule tracks to the 3D filament network are exciting future directions. Based on the present in vitro and in silico efforts, the force generating capacity of motor ensembles, fluid membranes on intracellular cargos and the geometric organization of 3D actin networks are all linked and may contribute to insuring directed myoVa intracellular cargo transport. Changes to any of these properties could then provide the cell regulatory capabilities that might allow transport to be tuned to accomplish specific cellular functions.

**Methods**

**Liposome preparation and myoVa conjugation.** Phospholipid liposomes of 350 nm diameter, composed of (motor ratio) 84 parts DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), 5 parts PEG-ylated phospholipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]-2000), 5 parts cholesterol, 5 parts 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramidine] (MBP:PE) and 1 part carboxyamine dye DiD or DiO Cell-labelling Solution (Thermo Fisher Scientific) were created through extrusion using filter membranes. In brief, the lipid mixture was mixed then dried under a nitrogen stream followed by 1 h under vacuum (Rotavap; Eppendorf). The mixture was then rehydrated to 5 mg ml⁻¹ using PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, 2 mM Na KHPO₄) at pH 7.2. Using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) and a pore size specific to the desired final liposome diameter, which varied between 100 and 650 nm (Supplementary Fig. 1). A double headed, heavy meromyosin myosin Va (myoVa) construct was tagged at the C-terminus with an 88-aa biotin ligase recognition sequence. The myoVa was coexpressed with calcium-insensitive catalase, 100 mM DTT, with 3.5 mg ml⁻¹ glucose, 40 mg ml⁻¹ glucose oxidase, 100 mM NaATP to a final concentration of 1 mM NaATP to a final concentration of 100 mM, were added to the flow chamber and imaged. Run length and velocities were determined from independent filaments which contained no actin–actin interactions. Run lengths were determined using a Fourier–Matter survival estimator44 since a large fraction of DiO labelled actin was chased off the end of the actin before terminating. Velocities for these experiments were calculated by first measuring the distance travelled using the ImageJ v1.47 plug in MTrack and dividing by the travel time. 2D experiments as presented in Fig. 2c were performed three times with different preparations on separate days to ensure repeatability.

**Determination of liposome size and density of bound motors.** Liposome size was determined by dynamic light scattering. Liposome diameter was measured using a Wyatt Technology Dynapro model MXS-TX on suspended liposomes in phosphate buffered saline at pH 7.4. The measured liposome diameter was analysed using Dynamics V6 software and determined by comparing the measured diameter to a standard curve created using polystyrene beads of known size (Supplementary Fig. 1D,E). To estimate the number of myoVa motors conjugated to each liposome we used an alternative myoVa construct that in addition to its C-terminal biotin was the presence of an N-terminal Yellow Fluorescent Protein (YPF) on each motor domain (YPF-myoVa) (Supplementary Fig. 1A), and employed a fluorescence photobleaching approach described extensively in Nayak and Rutenberg. To apply this method to our system, the YFP-myoVa was conjugated to the DOPC liposomes as described above. The liposomes were then imaged on a bare glass surface, using total internal reflection fluorescence (TIRF) conditions. As the liposomes landed on the glass surface, the integrated intensity of each liposome was measured along with the intensity decay over time as the YFP fluorophores on the YFP-myoVa photobleached (Supplementary Fig. 1B). The intensity per fluorophore (v) was then calculated using equation (6) from Nayak and Rutenberg.

\[
\nu = \frac{1}{\tau} \frac{\partial \phi}{\partial t_i}.
\]

**3D actin tight rope and intersection assay.** Silica beads of 3 μm diameter were used to suspend single actin filaments off the glass surface. To adhere the actin to the beads, the beads were incubated under mild agitation in a solution of 400 μg ml⁻¹ poly-L-lysine for a minimum of 12 h at room temperature. The beads were then washed extensively in 1 M TRIS pH 8 buffer and diluted to a final concentration of 1% solids. The beads were then passed into a custom flow chamber with orthogonal inflow ports and an internal volume of ~30 μl and then allowed to settle to the glass surface and attach electrostatically. This was followed by a 3 × volume wash with 1 M TRIS pH 8 and then a 2 min incubation of AB–BSA buffer. Alexa 647 phalloidin-labelled actin (100 nM) was then flowed into the chamber and allowed to incubate for 2 min before being washed out. The flow helped to string actin filaments between beads. To create actin intersections, actin was introduced through the orthogonal inflow ports followed by a single AB wash. 3D STORM imaging of the actin filaments was performed in AB buffer with 1 mM NaATP and 77 μg ml⁻¹ of beta-mercaptoethanol. MyoVa coated DiI-labelled liposomes were diluted 400 x to a final concentration of 50 μM and then very gently introduced into the flow chamber as not to disturb the suspended filaments.

**Microscopy.** Fluorescent liposomes were imaged in both 2D and 3D experiments at an exposure time of 100 ms using epifluorescence. Under intense excitation the FITC–phalloloid actin quickly photobleached, which allowed for the later imaging of either labelled DOPC liposomes that share a similar excitation and emission spectra to FITC. At interactions, one can distinguish between the top and bottom filament knowing the sequential order in which the fluorescent actin filament actins were introduced into the flow cell. Specifically, TRITC–phalloloid labelled actin were introduced first followed by the FITC–phalloloid labelled actin. After imaging the actin intersections, myoVa-bound 350 nm DiO-labelled liposomes with an average of 10 myoVa motors diluted 200 x into AB with 1 mM NaCl and 1 mM NaATP to a final concentration of 100 pM, were added to the flow chamber and imaged. Run length and velocities were determined from independent filaments which contained no actin–actin interactions. Run lengths were determined using a Fourier–Matter survival estimator since a large fraction of DiO labelled actin was chased off the end of the actin before terminating. Velocities for these experiments were calculated by first measuring the distance travelled using the ImageJ v1.47 plug in MTrack and dividing by the travel time. 2D experiments as presented in Fig. 2c were performed three times with different preparations on separate days to ensure repeatability.

**2D actin filament intersections motility assay.** The creation of 2D actin filament intersections was achieved in a flow chamber by the following steps as described previously. (i) The glass surface of 20 μl flow cells were coated with N-ethyl maleimide-modified skeletal myosin in myosin buffer (0.3 M KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl₂, 10 mM DTT, pH 7.4). (ii) 5 x volume actin buffer (AB) wash (25 mM imidazole, 4 mM MgCl₂, 1 mM EGTA, 25 mM KCl, DOPC liposomes, with 3.5 mg ml⁻¹ glucose, 40 mg ml⁻¹ glucose oxidase, 100 mM NaATP to a final concentration of 1 mM NaATP) were then added and incubated for 2 min before being washed out. The flow helped to string actin filaments between beads. (iii) 20 μl AB buffer with 1 mg ml⁻¹ BSA. (iv) 5 x volume AB wash. (v) 20 μl 100 mM TRITC–phalloloid-labelled actin filaments in AB. (vi) 3 x AB wash. (vii) 20 μl 100 mM FITC–phalloloid-labelled actin filaments in AB. (viii) 3 x AB wash with 1 mM NaATP. Chicken skeletal actin was used for all experiments and prepared as described previously.

The actin filament intersections bound to the glass surface were imaged at an exposure time of 100 ms using epifluorescence. Under intense excitation the FITC–phalloloid actin quickly photobleached, which allowed for the later imaging of either labelled DOPC liposomes that share a similar excitation and emission spectra to FITC. At interactions, one can distinguish between the top and bottom filament knowing the sequential order in which the fluorescent actin filament actins were introduced into the flow cell. Specifically, TRITC–phalloloid labelled actin were introduced first followed by the FITC–phalloloid labelled actin. After imaging the actin intersections, myoVa-bound 350 nm DiO-labelled liposomes with an average of 10 myoVa motors diluted 200 x into AB with 1 mM NaCl and 1 mM NaATP to a final concentration of 100 pM, were added to the flow chamber and imaged. Run length and velocities were determined from independent filaments which contained no actin–actin interactions. Run lengths were determined using a Fourier–Matter survival estimator since a large fraction of DiO labelled actin was chased off the end of the actin before terminating. Velocities for these experiments were calculated by first measuring the distance travelled using the ImageJ v1.47 plug in MTrack and dividing by the travel time. 2D experiments as presented in Fig. 2c were performed three times with different preparations on separate days to ensure repeatability.

\[I = \frac{I_0}{1 + \frac{C_1}{C_2}} \exp\left(-\frac{t}{\tau}\right)\]

\[t = \frac{1}{\nu} \frac{\partial \phi}{\partial t_i}.
\]

\[v = \frac{1}{\tau} \frac{\partial \phi}{\partial t_i}.
\]
excitation of Alexa 647-phalloidin-labelled actin and 3D cylindrical lens added to the light path.

3D image acquisition and calibration. Approximately 20,000 images were collected to generate the actin super-resolution 3D reconstruction. Minimum and maximum intensity thresholds were determined on a slide-to-slide basis, however, fluorophores with an axial ratio greater than 1.3 were eliminated from the Nikon software reconstruction. DiO-labelled liposomes navigating the actin–actin intersections were excited using a 532 nm laser. The built in NIS Nikon drift correction software and pinhole focus settings were applied to suppress alignment of the liposomes and actin within the same imaging plane over the duration of any individual imaging session. Images of actin intersections and liposomes were performed by overlaying the liposome movie images to the super-resolution reconstruction using ImageJ. The 3 μm silica beads were visible in both the actin and liposome imaging channels and were used as fiducial marks to safeguard against image drift in addition to the Nikon drift correction system. The Z-position of actin filaments and their relationship to each other in 3D intersection experiments were determined by selecting and averaging the Z-positions stored in the Nikon 3D reconstruction particle table for specific regions of individual actin filaments. The difference in separation between the two actin filaments was calculated using particle analysis functions in ImageJ. A propagation of error calculation was performed on the angle approach using the uncertainties on the actin filament position, liposome position and radius of the liposome. The error was calculated to be a function across the possible values of approach angle from 0 to 180°. The maximum error was calculated to be ±11.6° at approach angles of 0 and 180° and the minimum to be ±3.5° at an approach angle of 90°. The average error across all angles was ±7.9°. Filament separation (d) represented the centre-to-centre perpendicular distance between the two intersecting actin filaments at the point of the intersection (Fig. 2a). The mean Z-position of each filament comprising the intersection was determined from the STORM 3D fluorophore localizations within region of interest analysis of the images on both sides of the intersection (see Supplementary Fig. 14 and legend for additional details). Once obtained, the filament separation at the intersection was simply the difference between the two filaments’ Z-positions. The reported directional outcomes data are only for intersections that had a filament separation <250 nm, as larger separations could lead to spatial geometries beyond our calibrated Z-position range.

Liposome approach and intersecting filament separation. For 3D intersection experiments, the approach angle was determined by using the Pythagorean relationship between the measured 3D positions of the originally bound actin filament and the liposome for the 10 frames immediately before reaching the actin intersection. The approach angle (θ) were calculated relative to the actin filament intersection. A 0° approach angle represented a fully vertical liposome travelling on the same side of the actin filament as the intersecting actin filament, while 180° represented a liposome travelling on the opposite side of the actin filament away from the intersecting actin filament (Fig. 2a). A propagation of error calculation was performed on the angle approach using the uncertainties on the actin filament position, liposome position and radius of the liposome. The error was calculated to be a function across the possible values of approach angle from 0 to 180°. The maximum error was calculated to be ±11.6° at approach angles of 0 and 180° and the minimum to be ±3.5° at an approach angle of 90°. The average error across all angles was ±7.9°. Filament separation (d) represented the centre-to-centre perpendicular distance between the two intersecting actin filaments at the point of the intersection (Fig. 2a). The mean Z-position of each filament comprising the intersection was determined from the STORM 3D fluorophore localizations within region of interest analysis of the images on both sides of the intersection (see Supplementary Fig. 14 and legend for additional details). Once obtained, the filament separation at the intersection was simply the difference between the two filaments’ Z-positions. The reported directional outcomes data are only for intersections that had a filament separation <250 nm, as larger separations could lead to spatial geometries beyond our calibrated Z-position range.

Directional outcomes at actin filament intersections. In both 2D- and 3D-intersection experiments, intersection directional outcomes were determined by overlaying the movies of myoVa-bound liposomes over the actin tracks. A trajectory terminated if the myoVa disengaged from the actin filaments while the liposome was positioned over the intersection’s spatial centre in the 2D experiments or in the 3D experiments when the 3D spatial position of the liposome was such that it would be physically interacting with the intersecting filament. 'Straight' events were defined by the liposome exiting the interaction on the originally bound filament and a 'turn' event when exiting on the intersecting filament. Trajectories were determined to be left or right defined by the direction of projection from the perspective of a viewer looking top down so that the interaction formed the cardinal directions of a compass. In this analogy, all trajectories were assigned so vesicles would start at 'South' and move 'North' towards the intersection. Turning right at the intersection would be a turn toward 'East', turning left would be a turn toward 'West'. 3D experiments as presented in Fig. 2c were performed 23 times with different preparations on separate days to ensure repeatability.

Liposome approach angle and intersecting filament separation. For 3D intersection experiments, the approach angle was determined by using the Pythagorean relationship between the measured 3D positions of the originally bound actin filament and the liposome for the 10 frames immediately before reaching the actin intersection. The approach angle (θ) were calculated relative to the actin filament intersection. A 0° approach angle represented a fully vertical liposome travelling on the same side of the actin filament as the intersecting actin filament, while 180° represented a liposome travelling on the opposite side of the actin filament away from the intersecting actin filament (Fig. 2a). A propagation of error calculation was performed on the angle approach using the uncertainties on the actin filament position, liposome position and radius of the liposome. The error was calculated to be a function across the possible values of approach angle from 0 to 180°. The maximum error was calculated to be ±11.6° at approach angles of 0 and 180° and the minimum to be ±3.5° at an approach angle of 90°. The average error across all angles was ±7.9°. Filament separation (d) represented the centre-to-centre perpendicular distance between the two intersecting actin filaments at the point of the intersection (Fig. 2a). The mean Z-position of each filament comprising the intersection was determined from the STORM 3D fluorophore localizations within region of interest analysis of the images on both sides of the intersection (see Supplementary Fig. 14 and legend for additional details). Once obtained, the filament separation at the intersection was simply the difference between the two filaments’ Z-positions. The reported directional outcomes data are only for intersections that had a filament separation <250 nm, as larger separations could lead to spatial geometries beyond our calibrated Z-position range.

Non-interaction and interaction geometries. Supplementary Fig. 4 describes the spatial geometries that predict a physical interaction between the motor–cargo complex and the intersecting filament in relation to filament separation (d) and approach angle (θ). As the liposome’s approach angle increases from 0° (travelling on the same side of the actin filament as the intersecting filament) to an approach angle of 180° (travelling on the opposite side), the centre of the liposome follows an arc as the cargo rotates around the originally bound filament. A sinusoidal function is needed to predict the maximum filament separation at each approach angle, which would allow the motor–cargo complex to still interact with the intersecting filament (Supplementary Fig. 4).
protected from temperature increases by suspension in an ice bath. The liposomes were then centrifuged at 5,000 g for 10 min to remove any metal shards from the probe tip sometimes used. An aliquot of 50 μl of 250 nm diaclyr silica beads (at a concentration of 2% solids (Duke Standard) was rinsed with (1 ml) methanol, then vacuum dried and then re-suspended to 200 μl of 10 mM HEPES, 150 mM NaCl buffer. The liposomes were incubated at 60 °C for 20 min then mixed with the silica beads. The bead–liposome mixture was vortexed then shaken for 1 h to ensure adsorption of the liposomes onto the glass surface. Lipid-coated silica beads were separated from free liposomes by centrifugation at 5,000 for 3 min, removing the supernatant and then re-suspending the pellet in 200 μl of PBS pH 7.4. This was repeated for a total of three washes before final suspension of lipid-coated silica beads in 280 μl of PBS (pH 7.4). Liposomes were conjugated with myoVα in the same manner and concentration as described above, with the exception of the limiting motor condition used to define unitary stall force of a single motor.



**Code availability.** The code that supports the findings of this study are available from the corresponding author upon request.

**Data availability.** The data that support the findings of this study are available within the article and from the corresponding author upon request.

**References**

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Author contributions
A.T.L. and D.M.W. conceived project and designed experiments. A.T.L., S.R.N. and M.Y.A. performed experiments and analysed data. G.G.K. built the microscopes and optical trap used in this study and provided technical support for these systems. K.M.T. provided the myoVa protein. S.W. created and analysed the mechanistic model. D.M.W. and A.T.L. wrote the manuscript. All authors contributed to theoretical development and manuscript editing.

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