Cargo navigation across 3D microtubule intersections

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The eukaryotic cell’s microtubule cytoskeleton is a complex 3D filament network. Microtubules cross at a wide variety of separation distances and angles. Prior studies in vivo and in vitro suggest that cargo transport is affected by intersection geometry. However, geometric complexity is not yet widely appreciated as a regulatory factor in its own right, and mechanisms that underlie this mode of regulation are not well understood. We have used our recently reported 3D microtubule manipulation system to build filament crossings de novo in a purified in vitro environment and used them to assay kinesin-1-driven model cargo navigation. We found that 3D microtubule network geometry indeed significantly influences cargo routing, and in particular that it is possible to bias a cargo to pass or switch just by changing either filament spacing or angle. Furthermore, we captured our experimental results in a model which accounts for full 3D geometry, stochastic motion of the cargo and associated motors, as well as motor force production and force-dependent behavior. We used a combination of experimental and theoretical analysis to establish the detailed mechanisms underlying cargo navigation at microtubule crossings.

The microtubule (MT) network in eukaryotic cells is typically a dense, highly variable, 3D mesh. MT network topologies are known to vary widely between cells (1) and even between cells of the same type and lineage (2). Within a network, MTs converge to form crossings at a variety of filament separations and angles of intersection (3). Often, the crossings feature interfilament separations that are comparable to the scale of cargos found within the cell. At these “intersections,” a cargo, with multiple motors on its surface, can interact with several MTs simultaneously. This scenario belongs to a class of tug-of-war (ToW) processes (4, 5), although ToW events between dissimilar motors have received by far the most attention to date. As the result of a ToW, the cargo can pass along the original MT, switch to the crossing filament, pause, or detach. The probabilities of these outcomes are known to be sensitive to the 3D layout of the filaments (6–8), but the mechanisms of this phenomenon are unclear. Given that the architecture/topology of the MT cytoskeleton serves as a backdrop for cargo transport (9), its role in cargo routing warrants investigation.

The importance of MT cytoskeletal architecture is underscored by the fact that MT network remodeling occurs in various diseases and during normal cellular processes. For example, neutritic dearchitecture or restructuring is encountered in neurodegenerative diseases (10, 11). MT remodeling is also observed in neoplasias (12) and is associated with pathways disturbed in cancers (13). There is also evidence that the geometry of the MT network acts as a regulator to tune insulin granule secretion in mouse pancreatic β-cells (14, 15).

The cell can use multiple mechanisms to modulate its MT architecture. MTs can be locked in parallel (16) or antiparallel (17) via cross-linking. Axonal branching shows a preference for normal angles (18); low branch angles can arise from MT nucleating factors (19). The cell can set overall MT spacing by controlling the amount of polymerized tubulin, either via regulation of expression levels (20) or filament stability (21). Filament spacing can also be controlled via MT-associated proteins that cross-link MTs (22). MT and actin networks are often coupled, and remodeling of one can drive structural changes in the other (23, 24). External factors which affect cell shape can also regulate MT network topology (25). Finally, MT crossings are known to be special loci for network regulation (26). Currently, the implications of these topology variations for cargo logistics and overall biomechanics are not quantitatively understood.

Decoupling the influence of the MT network’s 3D topology from regulatory factors is challenging. The same pathways that drive network remodeling can also couple to motor regulation. As a result, the impact of geometric changes in MT networks on intracellular cargo transport has been difficult to isolate and quantify. It is common in biology to think in terms of chemical regulation. However, to understand how intracellular cargo transport functions, it is critical to gain a baseline understanding of how 3D MT geometry impacts cargo distribution, starting at the most fundamental level of the MT network: individual MT–MT intersections.

Studying cargo navigational behavior as a function of 3D network geometry poses considerable experimental challenges. In vivo investigations cannot easily decouple chemical and topological regulation, as discussed above. Moreover, although theoretical work
The 3D MT crossings and cargo navigational outcomes. (A) Illustration of in vitro 3D MT crossing with a cargo undergoing ToW and its possible outcomes. Bead handles (large spheres) are permanently bound to MTs (blue and red). They are held in 3D via holographically defined optical traps (light pink cones). MT plus ends are indicated (+). For clarity, we only depict two motors on the cargo (small bead), engaged on either MT. Navigational outcomes consist of the cargo moving on either MT toward the plus end (both cases shown), and the switch/pass classification of each outcome is indicated with labeled arrows. (B) Switching probabilities as a function of 3D MT arrangement. Higher switch rates are highlighted by a darker background. Significant differences (P < 0.05, Barnard's test) are indicated by links.

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Final Navigational Outcomes Depend on 3D Geometry. For each of the nine MT network arrangements, we quantified final cargo routing outcomes strictly in terms of “switching” or “passing” because detachment at intersections was negligible (~2% of events). We do not report pause events, because we characterized the entire navigational event, even in cases where the cargo's navigational choice took several seconds.

Our results suggest that 3D MT network topology can be an effective regulator of cargo routing (Fig. 1B) independent of other regulatory influences (chemical and biophysical). Geometries in the upper left corner of the table promoted switching, while those in the lower right corner promoted passing. The fact that certain geometries could promote switching was particularly unexpected, since it was not reported by Lombardo et al. (28) for the actin–myosin liposome system. One difference may be that the liposome system allowed motors to diffuse along the surface of the cargo, whereas our bead system did not. Our results for normal intersections and zero separations was in approximate agreement with the work of Ross et al. (7), where switching probability for underpass geometries on a glass slide was ~70%.

Routing outcomes are determined by multiple geometric factors interacting in nontrivial ways. Disentangling these factors by intuition alone is challenging; therefore, we relied on in silico modeling. Many fine mechanistic details were resolved by our experimental approach (see below), which constrained the in silico model.

Characterization of ToW Events. ToW is necessary for switching; therefore, we first characterized ToW occurrence and duration. The ToW was identified by looking at several parameters: motility track, motion of intersecting MTs, and force on bead handles. Any one parameter of the three was allowed to be ambiguous. Our spatiotemporal resolution was sufficient to establish whether a ToW took place and to precisely determine ToW durations.
Mechanisms of Cargo Routing. The ability to sensitively detect ToW events allowed us to quantify their probability. Our data indicated that ToW probability is sensitive to 3D geometry. It was higher for narrower filament separations and for acute/obtuse angles (Table 1). For 0-μm separations, ToW probability was so high that significant differences as a function of angle may not be practical to measure. A different pattern of navigational outcomes emerged when no-ToW events were omitted. Therefore, we also examined the probability for the cargo to pass or switch, conditional on ToW occurrence (Table 2 vs. Fig. 1C). Four of nine geometries showed switch probabilities close to 50%. Switch probabilities were significantly higher than 50% for the following geometries: 0 μm normal, 0 μm acute, and 0.5 μm acute (P < 0.05, Barnard’s test). We concluded that geometric constraints can promote or inhibit switching outcomes for ToW events.

**A Mathematical Model of Cargo Transport Reproduces Observed Switch Probabilities.** It is difficult to disentangle the mechanism through which each factor acts (in concert) to determine ToW and switching probabilities. Therefore, we constructed an in silico model of cargo transport. This model allowed us to examine experimentally unobservable details, such as how motor locations, bound states, and force states changed with time.

We modeled the cargo as a 3D solid sphere which underwent translational and rotational diffusion. Attached to the cargo were a number of motors, which may bind to MTs within reach and step and unbind from the MT with rates dependent on the force experienced by spring-like motor stalks (Fig. 3A). We derived the equations of motion of the cargo from force balance and simulated stochastic trajectories. Simulated MTs did not move, twist, or bend. The 500 cargo trajectories were simulated independently for each assay geometry, and probabilities of ToW and switching were determined analogously to the experimental data (Movies S3–S10). For full simulation details and parameter fitting procedure, see SI Appendix, sections 1 and 2. Briefly, two parameters could not be established from current experiments or prior literature: the number of motors on the cargo and motor on-rate. These two parameters were constrained by matching three experimental observations: ToW probability as a function of geometry (SI Appendix, Fig. S11), fraction of bead handle escape events, and cargo run lengths (SI Appendix, Fig. S12). Thus, the model was fully constrained and therefore predictive.

We found good agreement between experimentally observed and theoretically predicted probabilities of ToW and switching (Fig. 3B–D). We therefore used this simulation to investigate the mechanisms of cargo navigation at MT intersections.

**The Influence of Intersection Geometry on ToW Probability.** The longer a cargo spends within reach of the primary and crossing MTs (henceforth, the ToW zone), the more chances unbound motors; more motors were likely engaged on the MT, but not all were positioned to exert force during the ToW.

We identified ToW start and end by observing cargo displacements away from MT axes and associated deflections (Fig. 2A). Bead tracks for representative pass (Fig. 2B) and switch (Fig. 2C) events (Movies S1 and S2) are shown, along with their displacements projected along the axes of the intersecting MTs. A ToW start was identified with the beginning of a sustained displacement on the crossing MT’s axis. The ToW end was identified when the cargo snapped back upon dissociation from either the primary or crossing MT. Bead handle displacements can also be used as an indicator of a ToW (Figs. 1B and 2A, Middle, and D). A slowdown in cargo movement along the primary MT (Fig. 2B and C) was also often observed.

Bead handle displacements were also an indicator of how many motors were exerting force on the bead. We set the trap stiffness at ~1 pN per 100 nm, so that a single kinesin motor could not pull the bead handle out of the trap (escape event), but two or more motors working together could (SI Appendix, Fig. S1). Quantifying collective activity of multiple motors via trap escape forces is a well-established approach both in vivo (30, 31) and in vitro (32) and has been validated in silico (32). We controlled the surface density of motors on cargos by discarding assays in which bead handle escape fraction was >25% of total events. This provided refined control on top of the more coarse approach of controlling motor concentration at incubation time. It also provided confidence that ToWs in our assays were dominated by forces from one to two kinesin motors; more motors were likely engaged on the MT, but not all were positioned to exert force during the ToW.

| Table 1. ToW probabilities as a function of 3D MT geometry |
|-------------|-------------|-------------|-------------|
| Separation | Acute | Normal | Obtuse |
| 0 μm   | 0.79 (n = 38) | 0.93 (n = 59) | 0.89 (n = 27) |
| 0.5 μm | 0.83 (n = 46) | 0.70 (n = 60) | 0.80 (n = 40) |
| 1 μm   | 0.58 (n = 36) | 0.46 (n = 95) | 0.64 (n = 44) |

The distance between MTs (rows) and intersection angle (columns) provide full geometric specification for a MT intersection. The angles and distances are as visually illustrated in Fig. 1B. In each case, the number of measurements is given in parentheses. See Fig. 3B for comparison with modeling results.
mathematical model of navigational outcomes. (www.pnas.org/cgi/doi/10.1073/pnas.1707936115)

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Motors on the cargo have to engage the crossing MT. This phenomenon can be understood from a simple, heuristic model. If we assume the cargo has a single rate of binding to the crossing MT, given by $k_{on}^{\text{macro}}$, the probability the cargo binds to the crossing MT before leaving the ToW zone is

$$P_{\text{ToW}} = \frac{k_{on}^{\text{macro}}}{k_{on}^{\text{macro}} + \nu/d_{\text{ToW}}}$$

where $\nu$ is the cargo velocity and $d_{\text{ToW}}$ is the length of the ToW zone. This simple model reproduces experimental ToW probabilities for both the 0.5- and 1-μm separation distance geometries (SI Appendix, Fig. S2), but not for the 0-μm separation geometries, suggesting that there were mechanisms at play for 0-μm separations which were not present at other distances (see below).

On the other hand, our full model closely recapitulated all experimental ToW probabilities, including 0-μm separation data. The model captured several a priori intuitive features of the system. For 0-μm geometries, there was guaranteed to be a pool of motors able to bind the crossing MT (namely, the already engaged motors). Thus, we expected the ToW probability for all angles to be close to 1, which our model reproduced. For 0.5- and 1-μm geometries, we expected higher ToW probability for longer ToW zones (acute and obtuse angles). Indeed, simulated ToW probabilities were smallest for 90° intersections (Fig. 3B). Also, as expected, they were symmetric about the normal angle (since kinesin binding is not affected by MT polarity). Simulated ToW probabilities also increased when MT separation decreased from 1 to 0.5 μm, as expected. When the crossing MT encountered the cargo’s midsection, at 0.5-μm separations, it sampled more of the bead’s surface area as it attempted to pass. Hence, at 0.5-μm separation, more motors encountered the crossing MT, increasing ToW probability.

The Influence of Intersection Geometry on Navigational Outcomes, Given ToW. Experimentally, we observed a large range of geometries (most 0.5- and 1-μm geometries) where the conditional probability to switch was ~50%. At first glance, this appears intuitive: If ToW lasts ~1 s or more (our experimental ToW durations averaged ~3 s; SI Appendix, Fig. S3), then the motor team engaged on the crossing MT should have been able to reach steady state (33) and become equal in number to the primary motor team. With two equivalent ways to proceed, the

| Table 2. Probabilities of a switching outcome conditional on a ToW taking place, as a function of 3D MT network geometry |
|---------------------------------|-----------------|-----------------|
| Separation | Acute | Normal | Obtuse |
| 0 μm | 0.80* (n = 30) | 0.71* (n = 55) | 0.63 (n = 24) |
| 0.5 μm | 0.74* (n = 38) | 0.50 (n = 42) | 0.47 (n = 32) |
| 1 μm | 0.48 (n = 21) | 0.44 (n = 44) | 0.36 (n = 28) |

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**Fig. 3.** Mathematical model of navigational outcomes. (A) Snapshots of simulated cargo activity at an intersection (color coding as in Fig. 1B). Examples of Pre-ToW (A, Left), ToW (A, Center), and post-ToW (A, Right) states are shown. Motors bound to the primary MT are shown in cyan. Motors bound to the crossing MT are shown in magenta. Unbound motors are shown as green spherical protrusions from the cargo; sphere radius represents motor length. Geometries are shifted slightly horizontally to aid the eye. (B) Simulated probabilities to engage in ToW. Probability of undergoing ToW for each geometry is shown as thin symbols (error bars: SEM). Experimental data (Table 1) are shown as large symbols (error bars: 95% C.I.). Experimental data points for 0.5 and 1 μm, as expected. When the crossing MT encountered the cargo’s midsection, at 0.5-μm separations, it sampled more of the bead’s surface area as it attempted to pass. Hence, at 0.5-μm separation, more motors encountered the crossing MT, increasing ToW probability.

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0 μm Separation Intersection

0 μm Normal Geometry

Force From Crossing MT

0.5 μm Normal Geometry

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probability of either would have been \( \sim 50\% \). However, such a consideration is too simplistic, as we discuss below.

As in experiment, we identified ToW and probability of switching conditional on ToW in simulation (Fig. 3A–D). We assessed whether the number of motors in the two ensembles during ToW was in fact equal in our simulations. We found that, contrary to expectation, the number of engaged motors on the crossing MT was slightly but significantly lower than that on the primary MT (ratio of \( \sim 0.7 \); SI Appendix, Fig. S4). The reason is that the motors already engaged on the primary MT constrained the bead from a full range of linear and rotational diffusion. Once a ToW starts, the bead diffusion is even more constrained; this curtailed the number of motors that could reach the crossing MT. Thus, the primary MT team generally has an advantage. However, the two paths to proceed are not equivalent either. The crossing MT itself can exert a force which hinders the cargo from passing (Fig. 3G). The smooth decrease from passing to switching prevalence across our experimental geometries (Table 2) therefore reflected the balance between net motor activity (which is biased in favor of moving along the primary MT) and steric hindrance from the crossing MT.

The Limits of Geometric Regulation. Our results establish that a single MT intersection can significantly bias cargo routing toward switching or passing. The former was not reported for the 3D liposome motility system (28). Notably, in our system, the motors were anchored at fixed locations on the bead surface, whereas in the liposome system, they were free to diffuse. Protein anchoring in cells can range from fixed to readily movable, which raises the possibility that factors like membrane fluidity can directly affect cargo routing in cells. These effects are of great interest for future work.

Can a single MT intersection produce a near-100\% bias for switching or passing? What are the mechanisms which affect these limits? Approximately 100\% passing naturally occurs for intersections with filament separation much greater than cargo diameter. Is 100\% switching attainable? To address this, we considered two special cases which lead to elevated probability to switch and their broader implications for geometric regulation. For completeness, we also discuss each experimental geometry individually in SI Appendix, section 3.

Low Filament Separations. A closer look at the geometric setup (Fig. 3E) makes it clear that the 0-\( \mu \)m case is qualitatively distinct. At 0-\( \mu \)m separation, the crossing MT sterically hindered the motors engaged on the primary MT. To pass, the cargo could “hurtle” over the crossing MT due to Brownian motion, but this was improbable for our large silica beads (density \( \sim 2\times \) water). It would also be unlikely in the cytosolic environment, where cargo diffusion is often highly constrained (even for smaller cargos). The only other way for the cargo to pass is by first diffusing underneath the crossing MT, then having unengaged motors on its surface bind to the distal side of the primary MT (Fig. 4A and Movie S8). All other motors must then disengage before the distal ones.

This mechanism suggests passing at 0-\( \mu \)m separation should depend on the diffusion properties of the medium. Our simulations demonstrated that, for high viscosities, where cargo diffusion is suppressed, the preference to switch approaches 100\% (Fig. 3F and Movie S9).

Acute Angles/Intermediate Separations. Why is switching more prevalent for 0.5-\( \mu \)m acute geometry? Again, this is likely due to the crossing MT acting as a hindrance, but the mechanism cannot be the same as for low filament separations. Here, the cargo could not pass under the crossing MT, but it could hurdle over it. In this case, for switching probability to be elevated, this latter mechanism must be suppressed. The vertical forces associated with the ToW for 0.5-\( \mu \)m acute geometry pulled the cargo between MTs. This effectively wedged the cargo between them and prevented it from moving above the crossing MT (Fig. 3G and Movie S10). In our simulations, MTs were not allowed to bend. In experiment, the two motor teams in this scenario would have been expected to pull the two MTs closer together, making the crossing MT an even stronger obstacle. This is likely the reason experimentally observed switching probability for 0.5-\( \mu \)m acute geometry was somewhat higher than theoretical prediction.

Discussion

Cargos driven by multiple motors can exhibit incredibly complex behavior. Even when one considers this for a single cargo moving along a single MT, the complexity is sufficient to lead to highly nontrivial emergent behaviors (34, 35). The number of motors in biologically relevant systems is typically small, which necessitates detailed experiments and modeling to account for not only averaged behavior, but also the effect of fluctuations. The addition of just one more filament adds such complexity that the emergent behaviors can dramatically diverge to give rise to discrete outcomes: passing and switching. It is therefore a fascinating model system to study emergent behaviors in biology.

We have modeled this problem in a highly controlled experimental environment in which we imposed specific restrictions on cargo size and other variables. For example, cargos were larger than motor size, and motors could not diffuse along the cargo surface. We then performed modeling of our system in silico to generalize our experimental results. We were thus able to infer the key processes which underlie our observations. This enabled us to extrapolate how cargo routing might function in cells (and other environments).

Our analysis shows that the team of motors driving the cargo along the primary MT is generally at an advantage, even when the ToW is prolonged. This implies that there is an inherent bias to pass. However, our data show that, for many geometric conditions, passing and switching is balanced, and in some cases, switching dominates. The factor which shifts the balance between passing and switching is the extent to which the crossing MT acts to sterically hinder progression along the primary MT.

We show that the crossing MT can be an effective obstacle, especially when it intersects at an acute angle with intermediate or near-zero separation. Both geometries significantly inhibit passing but via different mechanisms: Intermediate separations with acute angles lead to cargos being wedged between the two MTs, while in near-zero filament separations, passing is only accessible via a multistep, and hence unlikely, process. These
results imply that the cell could selectively route cargos of different sizes by controlling MT intersection angle and spacing. Our simulations closely followed the experimental results, but two important deviations were seen: The conditional probability to switch given that ToW already started was higher in experiments than in simulations for some geometries. Also, the time a cargo spent at the intersection was higher in the experiment than in simulation (SI Appendix, Fig. S3). In both cases, we believe this discrepancy stemmed from modeling MTs as infinitely rigid rods. We analyze MT bending in SI Appendix, section 4 and highlight how model results could be changed by MT bending.

To date, MT rigidity has been mostly studied separately from MT-based transport. Our work suggests that MT bending, and, more generally, biomechanics of the MT cytoskeleton, must be taken into account in future studies of intracellular motility, as they are a nonnegligible contributor to cargo routing.

A faithful and detailed in silico model of cargo motility and the ToW process enabled us to speculate about cargo navigation patterns beyond our specific conditions. We show that as viscosity increases, switching is more strongly favored for near-zero filament separations. These simulations predict that local micro rheology can be a regulator of cargo routing.

Our work opens up a prospect of a comprehensive quantitative understanding of intracellular cargo fluxes from a single-molecule mechanistic perspective.

Materials and Methods
Our holographic optical trapping and bead assays were performed as described (29) with minor changes; see SI Appendix for more details of protocols and calibration. Much of our data are in the form of contingency tables, so Barnard’s exact tests were used to assess significance of differences between outcomes. Super resolution images were recorded with a Vutara microscope (Bruker) based on single-molecule localization biplane FPALM technology (36, 37); see SI Appendix for more experimental details. Numerical simulations were based on a hybrid Euler–Maruyama–Gillespie scheme, written in C; see SI Appendix for a detailed discussion.

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