Correlative live-cell and superresolution microscopy reveals cargo transport dynamics at microtubule intersections

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Intracellular transport plays an essential role in maintaining the organization of polarized cells. Motor proteins tether and move cargos along microtubules during long-range transport to deliver them to their proper location of function. To reach their destination, cargo-bound motors must overcome barriers to their forward motion such as intersection points between microtubules. The ability to visualize how motors navigate these barriers can give important information about the mechanisms that lead to efficient transport. Here, we first develop an all-optical correlative imaging method based on single-particle tracking and superresolution microscopy to map the transport trajectories of cargos to individual microtubules with high spatiotemporal resolution. We then use this method to study the behavior of lysosomes at microtubule–microtubule intersections. Our results show that the intersection poses a significant hindrance that leads to long pauses in transport only when the separation distance of the intersecting microtubules is smaller than ~ 100 nm. However, the obstructions are typically overcome by the motors with high fidelity by either switching to the intersecting microtubule or eventually passing through the intersection. Interestingly, there is a large tendency to maintain the polarity of motion (anterograde or retrograde) after the intersection, suggesting a high degree of regulation of motor activity to maintain transport in a given direction. These results give insights into the effect of the cytoskeletal geometry on cargo transport and have important implications for the mechanisms that cargo-bound motors use to maneuver through the obstructions set up by the complex cytoskeletal network.

STORM | dynein | kinesin | trafficking

Cells rely on a two-way transport system to deliver important proteins and organelles to their location of function. Kinesin and dynein motors are responsible for long-range transport along microtubules (1). Although dynein walks toward the (–) end of the microtubule (retrograde), carrying cargo toward the cell nucleus, most kinesins walk toward the (+) end (anterograde), carrying cargo toward the cell periphery (1). Microtubules organize into a complex, 3D network inside cells, and the intersections between microtubule filaments or between microtubules and other cytoskeletal filaments (actin, intermediate filaments) likely have important consequences on the efficiency and accuracy of cargo transport (2). For example, microtubule–microtubule intersections can serve as switching points or barriers that disrupt continuous transport in a given direction.

The effect of microtubule–microtubule intersections on the movement of individual motors and motor-decorated beads has been studied using *in vitro* reconstituted microtubules deposited on top of each other (3, 4). These studies showed that although single motors can have varied behavior (passing, dissociation, switching), at high motor densities dynein-decorated beads stop and tether at the intersection (3). On the basis of these results, it was suggested that microtubule intersections can act as a tethering point for cargo when it reaches the right destination and that the tethering can be tuned in the cell by regulating motor

stoichiometry (3). These studies constitute a starting point to understand how filament intersections can affect cargo transport; however, the implications for intracellular transport are not clear. The microtubule network has a more complex architecture inside the cell than what has been achieved in these *in vitro* experiments. In addition, it is likely that both polarity motors (dynein and kinesin) are present on the cargo simultaneously (5, 6), and their activity can be regulated through mechanisms that are not present *in vitro* (7).

Relating cargo dynamics to the underlying microtubule network inside cells is technically challenging. The high density and the complex organization of microtubules and the inherently dynamic nature of transport necessitate imaging tools with high spatiotemporal resolution. Microtubule track switching has been studied in neuronal cells by using high-resolution single-particle tracking of vesicles with time-lapse microscopy (8). In these studies, a displacement perpendicular to the direction of motion was taken to indicate a switch from one microtubule to another. However, as the microtubules were not imaged, their geometry and the effect of this geometry on transport were unclear. Conventional dual-color time-lapse microscopy has also been used to study the interaction of peroxisomes with microtubules (9, 10), but dissecting the 3D geometry of the microtubule network was not possible in these studies because of the diffraction-limited resolution of conventional fluorescence microscopy. In fact, microtubule intersections could only be discerned in select regions close to the cell periphery where the microtubule network was less dense.

Recent development of superresolution microscopy techniques such as stochastic optical reconstruction microscopy (STORM) or photoactivation localization microscopy (PALM) has made it possible to surpass the diffraction limit in fluorescence microscopy (11–13). These techniques rely on precisely determining the position of sparsely activated photoswitchable probes. A superresolution image can be reconstructed from repeated cycles of activation, localization, and deactivation. The microtubule network has been imaged using these methods in 3D with ~ 20 -nm lateral and ~ 55 -nm axial resolution inside fixed cells (14) and in 2D with ~ 70 -nm lateral resolution inside living cells (15). However, the imaging speed of live-cell superresolution microscopy has been limited because of the frame rates of modern cameras and the performance of photoswitchable fluorescent probes (15–18). Therefore, spatial and temporal resolution must often be balanced

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against each other in live-cell superresolution microscopy, making it challenging to observe fast cargo dynamics on the microtubule network with the needed spatiotemporal resolution.

We developed an all-optical correlative imaging method that combines single-particle tracking with superresolution microscopy such that fast intracellular dynamics can be interpreted in the context of subcellular ultrastructure. Using this method, we could map the transport trajectories of lysosomes onto individual microtubules with high precision and analyze cargo behavior at microtubule intersections inside cells. Our results show that the axial separation of intersecting microtubules plays an important role in whether the lysosome can pass through the intersection. However, even at small separations, microtubule intersections do not constitute a stopping point for lysosome transport, and motors can maneuver around these barriers by either switching to the intersecting microtubule or eventually squeezing through the intersection. There is a large tendency to preserve the polarity of transport direction after the intersection, which points toward regulatory mechanisms of motor activity.

Results

Correlative Live-Cell and Superresolution Imaging: Workflow and Requirements. Fig. 1 shows the workflow for all-optical correlative live-cell and superresolution microscopy. First, the cargo of interest (e.g., lysosomes) is labeled with a fluorescent marker (e.g., LysoTracker; Invitrogen) and a time-lapse movie is recorded. The cells are subsequently fixed in situ on the microscope stage and immunostained with primary and secondary antibodies against a target of interest (e.g., tubulin antibodies). After the sample preparation is complete, a superresolution (STORM) image of the immunostained target structure (e.g., microtubules) can be recorded. The transport trajectories of the cargos can be obtained from the time-lapse movie using a single-particle tracking routine that determines and links together the centroid position of their images. Fiduciary markers that are visible both in the time-lapse movie and the STORM image allow precise alignment of the two channels (Fig. S1 and ref. 19). The trajectories can then be mapped onto the STORM image of microtubules for further analysis.

We found that the in situ fixation was very rapid (faster than a single camera frame, which was 100–500 ms in our experiments), and after fixation the structures were preserved as they appeared in the final frame of the movie (Fig. S2). The image in

the final frame of the movie also matched well with the STORM image (Fig. S2). To map the transport trajectories of lysosomes to their corresponding microtubules, the microtubule network must be stable, such that the end-point STORM image corresponds with the position of the microtubules during the time-lapse movie. We could reduce the microtubule network dynamics from milliseconds to minutes by treating the cells with low concentrations of paclitaxel and nocodazole (*SI Methods*) and carrying out the imaging at room temperature (24°C). *Movies S1* and *S2* show the dynamics of the microtubule network before and after drug treatment, respectively. As is evident in *Movie S1*, the microtubules showed rapid growth, shrinkage, and buckling behavior before drug treatment. However, after treatment, these dynamic changes were substantially slower. We recorded simultaneous time-lapse movies of the microtubules and lysosomes and confirmed the stability of the microtubules from these time-lapse movies before further analysis.

Microtubule Stabilization Does Not Significantly Alter Lysosome Transport.

There was more cell-to-cell variability in terms of lysosome mobility in treated cells compared with physiological conditions. Although some cells had very few mobile lysosomes, likely because of a compromised cytoskeleton, others showed high lysosome mobility and a stable microtubule network. For our correlative studies we only imaged the latter cells containing a large fraction of highly mobile lysosomes, similar to physiological conditions. We tested the effect of microtubule stabilization on lysosome transport by measuring several transport parameters under physiological conditions (37°C) and at room temperature (24°C) with and without drug treatment (Table 1 and Fig. S3). Lysosomes were labeled with LysoTracker (Invitrogen) and their trajectories reconstructed from time-lapse movies in living cells using conventional fluorescence microscopy and single-particle tracking. To distinguish the transport mode, we determined the dependence of the mean-square displacement ($\langle \Delta r^2 \rangle$) on time (Δt) by plotting $\langle \Delta r^2 \rangle$ versus Δt on a logarithmic scale and fitting to a line (ref. 5 and *SI Methods*). We defined the mean-square displacement α -coefficient as the slope of this linear fit (5). In all cases we found two populations of lysosomes: processive ones with α greater than or equal to 1.5 and nonprocessive ones with α lesser than or equal to 1.0 (5). The percentage of processive lysosomes was slightly smaller in treated cells (60% in treated cells vs. 80% in nontreated cells; Fig. S3A). Among the processive lysosomes, the retrograde and anterograde speeds spanned a similar range in treated and nontreated cells (Fig. S3B). The average speeds were slightly slower in treated cells (Table 1), likely because of decoupling of the microtubule dynamics from the lysosome movement (10). The run lengths, defined as the total displacement during processive runs, showed a similar distribution (Fig. S3C), and the average retrograde and anterograde run lengths were similar in treated and nontreated cells (Table 1). The processive periods of motion were interrupted by periods of pausing, and the pausing times and pausing frequencies were similar in treated and nontreated cells (Fig. S3D and E and Table 1). Lysosome transport was bidirectional, and on average lysosomes reversed their transport direction with similar frequency in treated and nontreated cells (Fig. S3F and Table 1). Given these results, we conclude that the microtubule stabilization did not substantially affect lysosome transport. Finally, we note that GFP-tubulin transfection alone did not have an effect on lysosome mobility (Table S1).

We also tested the effect of drug treatment on microtubule posttranslational modifications. We focused on acetylation and deetyrosination because these modifications have been associated with stable microtubules (20). Immunofluorescence and Western blot analysis showed that deetyrosinated tubulin levels were unchanged after drug treatment (Fig. S4). In terms of acetylation, there was no discernible difference between drug-treated and

Fig. 1. Workflow of the all-optical correlative live-cell and superresolution imaging. A live-cell time-lapse movie is recorded at high temporal resolution. The sample is then fixed in situ and stained with antibodies conjugated with photoswitchable fluorophores for immunofluorescence and superresolution imaging (STORM). A STORM image of the microtubule network is then recorded. Single-particle tracking is used to obtain trajectories from the live-cell movie, and these trajectories are precisely aligned with the STORM image of the microtubules using fiduciary markers. IF, immunofluorescence.

Table 1. Average values of lysosome motility parameters measured under different conditions

Parameters (mean $\pm$ SD)	37°C		24°C		24°C + treatment	
	RG	AG	RG	AG	RG	AG
Average speed, $\mu\text{m/s}$	0.45 ± 0.19 (50)	0.41 ± 0.18 (50)	0.45 ± 0.18 (50, 0.93)	0.43 ± 0.22 (50, 0.62)	0.39 ± 0.24 (50, 0.20)	0.33 ± 0.17 (50, 0.01*)
Run length, μm	2.6 ± 1.9 (50)	2.3 ± 1.9 (50)	2.6 ± 2.0 (50, 0.99)	2.1 ± 1.5 (50, 0.55)	2.3 ± 1.4 (50, 0.36)	2.1 ± 1.5 (50, 0.54)
Processivity, s	6.8 ± 4.4 (50)	6.8 ± 4.8 (50)	6.0 ± 3.5 (50, 0.29)	5.3 ± 3.3 (50, 0.08)	7.3 ± 3.7 (50, 0.59)	7.4 ± 3.7 (50, 0.44)
Pausing time, s	6.0 ± 5.0 (112)		7.5 ± 6.7 (106, 0.07)		7.2 ± 5.8 (184, 0.07)	
Pausing frequency, events/min	5.1 ± 2.0 (50)		4.5 ± 1.6 (50, 0.13)		4.5 ± 1.8 (50, 0.11)	
Frequency of reversal, events/min	1.9 ± 2.5 (50)		1.3 ± 1.6 (50, 0.18)		1.2 ± 1.2 (50, 0.12)	
Mean-square displacement α -coefficient	1.7 ± 0.2 (30)		1.7 ± 0.2 (30, 0.43)		1.6 ± 0.2 (30, 0.15)	

The first three parameters have been split into retrograde (RG) and anterograde (AG) directions. The two numbers in the parentheses are the event number (n) and the P value for a two-tailed two-sample t test, respectively. $P < .05$ was taken to indicate statistical significance (*).

nontreated cells in immunofluorescence images (Fig. S4). Western blot analysis showed only a small increase (~ 1.5 -fold) in the levels of acetylated tubulin in treated cells (Fig. S4).

Correlation of Lysosome Trajectories with the Underlying Microtubule Cytoskeleton. We next aimed to correlate lysosome trajectories with the 2D superresolution images of the microtubule cytoskeleton. Fig. 2 shows multiple frames from an example time-lapse movie and the corresponding trajectory of a lysosome overlaid with the conventional and the STORM image of microtubules (Movie S3). Individual microtubules and their organization could not be resolved in the conventional image because of the diffraction limit. However, the microtubule network was clearly resolved in the STORM image, and the lysosome trajectory could be precisely mapped onto the individual microtubules. When the trajectory was aligned with the STORM image of microtubules, it became evident that the lysosome crossed several microtubule–microtubule intersections and switched microtubules multiple times. In the overlay with the conventional microtubule image, only the sharp changes in transport direction (e.g., at $t = 12$ s) could be interpreted as a potential change of microtubule track, whereas the lysosome behavior at the rest of the intersections was missed. This

example clearly demonstrates the power of correlative single-particle tracking and superresolution imaging in studying the interaction of cargos with their microtubules during motor protein-mediated long-range transport.

Because the trajectories were determined by tracking the centroid of the lysosome image, they only showed perfect overlap with the microtubules when the lysosome center was transported directly above or below the microtubule. However, lysosomes can bind to and translocate on the microtubule such that their centers are laterally displaced from the microtubule image. Given the size of an average lysosome (~ 600 nm), we assumed that the displacement between the lysosome and its associated microtubule can be as large as 300 nm. Thus, we took the microtubule that was closest to the lysosome trajectory to be the one with which the lysosome was associated. In a few cases, when the lysosome was moving between two parallel microtubules in very close proximity, we could not assign it to one specific microtubule and discarded these lysosomes from our analysis. In addition, in certain regions the microtubule network was too dense even for STORM to clearly resolve the individual microtubules and it was not possible to map the trajectories to individual microtubules in these regions. In some cases, lysosomes

Fig. 2. Correlative live-cell and superresolution imaging allows interpreting cargo dynamics in the context of the cytoskeleton. (Upper) Multiple frames from a conventional dual-color movie of lysosomes (red dotted circle shows the position of a single lysosome as determined from the lysosome image) and microtubules (green). The trajectory (red line) of the lysosome is overlaid with the image of the microtubules at multiple times. (Lower) The same region as Upper but with the conventional microtubule image replaced by the end-point STORM image of microtubules. The lysosome trajectory can be mapped to the individual microtubules in the STORM image with high fidelity.

seemed to fall off the microtubules during processive motion. We associated this effect to either a not-well-preserved microtubule structure after fixation or a low stability of microtubules in that particular region of the image. In regions in which the microtubule network structure was clearly visible, 65% of lysosome trajectories could be associated with the microtubules for their entire length; in 27% of cases the trajectory could be partially associated with the microtubules. There were very few cases (8%) in which the trajectory could not at all be matched with the microtubules.

Lysosome Behavior at Microtubule Intersections. We first used 2D STORM images of microtubules and identified points in which the images of two or more microtubules crossed as microtubule–microtubule intersections. We then analyzed the behavior of lysosomes when they approached these intersections by overlaying their trajectories with the STORM images of the microtubules. Of the intersections we analyzed this way, 50% were between two microtubules and 50% involved three or more microtubules. We observed four distinct behaviors of lysosomes at microtubule intersections (Fig. 3*A* and *Movies S4, S5, S6, and S7*). The majority of lysosomes (48.6%; $n = 108$) slowed down and paused when they arrived at an intersection point between multiple microtubules (Fig. 3*B*). We defined pausing as no net displacement of the centroid position for 1 s or longer. The second most common behavior (31.5%; $n = 70$) was given by lysosomes passing through the intersection unhindered and continuing to move on the same microtubule (Fig. 3*B*). A small percentage of lysosomes (14.5%; $n = 32$) switched to the intersecting microtubule when they encountered an intersection (Fig. 3*B*). Reversing transport direction and moving backward on the same microtubule was rare (5.4%; $n = 12$) (Fig. 3*B*).

To distinguish the behavior of anterograde and retrogradely moving lysosomes, we used the GFP–tubulin image to determine the position of the microtubule organizing center near the cell nucleus. The direction of transport was then classified as anterograde if the net displacement was away from the microtubule organizing center and as retrograde if the net displacement was

toward it. Trajectories for which the direction could not be determined on the basis of these criteria were discarded from the analysis. Retrograde lysosomes (dynein-mediated) were more likely to pass through intersections than to switch track or reverse direction, but the pausing happened with equal probability in both directions (Fig. 3*C*). When the full trajectory of the lysosomes was taken into account, the majority of pauses (71%) and direction reversals (69%) happened at microtubule intersections.

We further characterized the behavior of those lysosomes that initially paused near intersections. Unlike in vitro studies (3, 4), the pausing was not an end point for transport but, rather, a period spent overcoming the barrier presented by the intersection. In the majority of the cases (83%), lysosomes exhibited what we refer to as a secondary behavior after pausing (Fig. S5). The secondary behavior was equally split between switching to the intersecting microtubule and eventually passing through the intersection (Fig. 3*D*). As in the case of primary behavior, only a small percentage of lysosomes reversed direction after pausing (18%). The lysosomes that crossed the intersection after pausing appeared to slowly “crawl” through the intersection and increased their speed substantially once they were able to pass the intersection. Interestingly, when an anterograde-moving or retrograde-moving organelle switched to the intersecting microtubule either as a primary behavior or after pausing, the transport direction was maintained as anterograde (28/34 cases) or as retrograde (23/33 cases), respectively.

To ensure that drug treatment did not influence lysosome behavior at microtubule intersections, we also matched lysosome trajectories to STORM images of microtubules in untreated cells imaged at room temperature. Because of the dynamic rearrangements in the microtubule network, we could only match a small percentage of lysosome trajectories to their microtubules during a short period toward the end of the live-cell movie. Similar to cells with a stabilized microtubule network, pausing and passing were the majority primary behavior, and microtubule track switching or direction reversals were less common.

Effect of 3D Microtubule Network Geometry on Lysosome Transport. We next examined the correlation between the axial separation

Fig. 3. Lysosome behavior at microtubule intersections. (A) Examples showing the four different types of lysosome behavior at microtubule intersections. The lysosome trajectories have been color coded to show time according to the color scale bars. (Far Left) An example of pausing: The lysosome rapidly approaches the intersection (red arrow), indicated by the magenta-blue part of the trajectory, but spends extended time at the intersection, indicated by the blue-red part of the trajectory. (Middle Left) An example of passing: The lysosome moves with linear directed motion through the intersection (red arrow), indicated by the mostly uniform change of color of the trajectory. (Middle Right) Microtubule track switching at the intersection (red arrow). (Far Right) Direction reversing. (B) Histogram showing the percentage of lysosomes that pause ($n = 108$), pass ($n = 70$), switch track ($n = 32$), or reverse direction ($n = 12$) at microtubule intersections. (C) Histogram showing the lysosome behavior at microtubule intersections split into anterograde (red bars) and retrograde (blue bars) directions (anterograde: $n = 44$ pause, $n = 21$ pass, $n = 16$ switch, and $n = 6$ reverse; retrograde: $n = 52$ pause, $n = 44$ pass, $n = 15$ switch, and $n = 4$ reverse). (D) Histogram showing the secondary behavior of lysosomes after pausing. Passing ($n = 36$) and switching ($n = 38$) were equally likely; reversing ($n = 16$) was less common.

Fig. 5. Mechanistic model. When cargo arrives at a microtubule–microtubule intersection in which the axial separation of the intersecting microtubules is larger than 100 nm, there is minimal hindrance to the forward motion and transport continues on the same microtubule without disruption (A). When the axial separation of the intersecting microtubules is less than 100 nm, the intersection presents a major obstacle, stalling the motors and momentarily stopping forward motion until the obstacle is overcome (B). Cargos that are not positioned between the intersecting microtubules continue moving forward on the same microtubule without feeling the obstruction from the intersection (C).

pausing, which might correspond to the rearrangement of motors on the microtubule surface (22). Future studies that combine high-precision 3D tracking with superresolution microscopy can potentially reveal whether motors rearrange on the microtubule surface at intersections as opposed to other possibilities (cargo squeezing, microtubule deformation, etc.).

A subset of lysosomes could switch to the intersecting microtubule within less than 1 s even when the two microtubules were separated by large distances (up to 500 nm). Both dynein and kinesin motors take small steps (8–32 nm) relative to the distances between the intersecting microtubules that we observed (23). Thus, it seems unlikely that switching is mediated by an individual motor but, rather, by other excess motors present on the same cargo.

Interestingly, lysosomes had a strong tendency to maintain the polarity in their transport direction even after switching onto the intersecting microtubule. Similar “memory” has recently also been shown for lipid droplets in optical manipulation experiments (24). These studies showed that after pulling the lipid droplet off the microtubule by optical tweezers and allowing it to reattach, the majority of lipid droplets continued to move in the same direction. Membranous vesicles such as lysosomes have previously been shown to have both polarity motors (kinesin and dynein) present simultaneously during transport (5). Taken

together with these former studies, our results point toward a high degree of cellular regulation to maintain only a single motor type active at a time to ensure transport continues in a given direction to deliver cargo at the right location.

Finally, it is important to emphasize that our all-optical correlative method is not limited to studying cargo transport and that we expect that it will have a wide range of applications in biology, where putting dynamics into the context of nanoscale ultrastructural or molecular information is important.

Methods

For a detailed description of the methods, please refer to *SI Methods*. Briefly, African green monkey kidney cells (BS-C-1, American Type Culture Collection, ATCC) were treated with 120 nM paclitaxel and 120 nM nocodazole for 10 min at 37°C and maintained in serum and phenol-red free media containing these drugs throughout the imaging. For live-cell imaging, cells were transfected with a GFP–tubulin plasmid and lysosomes were labeled with 50 nM of LysoTracker orange (Invitrogen) for 10 min at 37°C. Live-cell and STORM imaging were carried out by a custom microscope fitted with a 100× 1.4 numerical aperture (NA) oil immersion objective, 488-nm laser for exciting GFP, 560-nm laser for exciting LysoTracker orange (Invitrogen), and 647-nm laser for exciting Alexa647 (Invitrogen). The fluorescence emissions from these markers were collected and filtered by three emission filters (ET525/50, ET605/52, and ET705/72m, respectively; Chroma Technology) and imaged onto an electron-multiplying charge-coupled device camera (Andor Technology) at a frame rate of 100–500 ms per frame for live-cell imaging and 20 ms per frame for STORM imaging. Temperature was maintained using a stage incubator system from Live Cell Instruments, and focus was maintained by a custom-built focus lock system as previously described (14, 21). After live-cell imaging, cells were fixed with 3% (vol/vol) paraformaldehyde and 0.1% glutaraldehyde in PBS and immunostained with anti- α -tubulin primary antibody and a secondary antibody labeled with an activator/reporter dye pair (Alexa405/Alexa647; Invitrogen), as previously described (25). Lysosomes in live-cell images were tracked with an automated single-particle tracking software (Kalaimoscope; Transinsight). STORM images were analyzed and rendered with custom-written software (STORMProcessor, a kind gift of Mark Bates, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany; Insight3, a kind gift of Bo Huang, University of California, San Francisco, CA).

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