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A Biophysical Analysis of Mitochondrial Movement: Differences Between Transport in Neuronal Cell Bodies Versus Processes

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Abstract

There is an increasing interest in factors that can impede cargo transport by molecular motors inside the cell. Although potentially relevant (Yi JY, Ori-McKenney KM, McKenney RJ, Vershinin M, Gross SP, Vallee RB. High-resolution imaging reveals indirect coordination of opposite motors and a role for LIS1 in high-load axonal transport. *J Cell Biol* 2011;195:193–201), the importance of cargo size and subcellular location has received relatively little attention. Here we address these questions taking advantage of the fact that mitochondria – a common cargo – in *Drosophila* neurons exhibit a wide distribution of sizes. In addition, the mitochondria can be genetically marked with green fluorescent protein (GFP) making it possible to visualize and compare their movement in the cell bodies and in the processes of living cells. Using total internal reflection microscopy coupled with particle tracking and analysis, we quantified the transport properties of GFP-positive mitochondria as a function of their size and location. In neuronal cell bodies, we find little evidence for significant opposition to motion, consistent with a previous study on lipid droplets (Shubeita GT, Tran SL, Xu J,

Vershinin M, Cermelli S, Cotton SL, Welte MA, Gross SP. Consequences of motor copy number on the intracellular transport of kinesin-1-driven lipid droplets. *Cell* 2008;135:1098–1107). However, in the processes, we observe an inverse relationship between the mitochondrial size and velocity and the run distances. This can be ameliorated via hypotonic treatment to increase process size, suggesting that motor-mediated movement is impeded in this more-confined environment. Interestingly, we also observe local mitochondrial accumulations in processes but not in cell bodies. Such accumulations do not completely block the transport but do increase the probability of mitochondria–mitochondria interactions. They are thus particularly interesting in relation to mitochondrial exchange of elements.

Keywords axonal transport, mitochondrial motion

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Correct transport of proteins and other vesicles involve molecular motors (1,2) and in axonal and dendritic compartments is important for proper functioning of the nervous system. Many neurodegenerative conditions including amyotrophic lateral sclerosis and Parkinson's disease (3–5) are linked to failures in axonal transport. Furthermore, molecular motor-based transport plays a critical role in healing of axonal injuries (6,7), which would thus be impaired by poor transport. Overall regulation of cargo

motion in neurons is still poorly understood, though for mitochondria, part of the regulation involves the Miro and Milton proteins (8,9).

Mitochondrial positioning in processes is crucial, as they may locally buffer calcium transients (10), and also produce ATP needed for multiple cellular activities, including formation of *de novo* synapses during development, and continuous maintenance/remodeling of synapses in

the adult nervous system. The architecture of axonal and dendritic processes in single neurons can be quite complex, and some processes are only slightly wider than the larger cargos (such as mitochondria) that move through them. As they move through such compartments, cargos are frequently very close to the membranes bounding the process, which we hypothesize is likely to increase resistance to the cargos' motion. Furthermore, the presence of these bounding membranes may make it difficult to push other objects out of the way because they have nowhere to move. Thus, theoretical studies (11) suggest that cargos likely encounter varying levels of opposition, at least some of which depend on cargo size, with larger cargos subject to increased resistance to motion. Because Lis1 helps groups of dynein motors add forces productively (12), a study where Lis1 alteration particularly affected neuronal transport of large lysosomes (1) is consistent with such a size-dependent opposition hypothesis. How important are such effects? Do they contribute significantly to the overall properties of cargo transport? Our data shows that in neurons cultured from the *Drosophila* brain, there is no correlation between mitochondrial size and motion in the cell body, but in the restricted confines of the processes, movement of these organelles is size dependent.

Results

Motion in the Cell Body Is Unaffected by Mitochondria Size

To evaluate the transport of mitochondria in neurons from the *Drosophila* CNS, primary cultures were prepared from the brains of late-stage pupae. All pupae were heterozygous for UAS-mitoGFP and one of three GAL4 driver lines targeting mitoGFP expression in different populations of neurons. With the *elav*-GAL4 driver, >70% of the neurons had GFP+ mitochondria, consistent with this being a pan-neuronal driver line. When using the GH298-GAL4 and GH146-GAL4 drivers, that are expressed in smaller subpopulations of neurons in the adult brain (13), GFP+ mitochondria were expressed in ~10% and <10 neurons, respectively, in each culture. Imaging was carried out at 2–4 days *in vitro*, using objective-based total internal reflection microscopy (TIRFM). Moving mitochondria were tracked with ~10 nm resolution (14). The run distances and velocities of mitochondria were determined

from video tracks (Figure 1A) of time-lapse TIRF images (5 fps) using the LVCORR software (14). There was no difference in any of the parameters measured between the three driver lines, so the data were pooled for the analysis presented. Neurons that contained abundant fluorescent mitochondria were chosen for analysis. The soma size ranged from 7 to 17 μm , with a mean of 10.2 ± 0.56 ($\pm\text{SEM}$, $n = 21$). In the cell body, $37.1 \pm 3.03\%$ of the visible mitochondria were moving. Overall, the above data reflect measurements from 19 cells in six different cultures, sampled over 6-min windows.

The mitochondria in the cell body and processes showed a wide distribution of sizes that ranged from 0.4 to 5.0 μm in length. To determine the effect of size on the transport properties of mitochondria, the population was divided into two groups: small (length ≤ 1500 nm with a mean of 0.72 ± 0.02 μm , cell bodies, and 0.91 ± 0.03 μm , processes); and large (length > 1500 nm with a mean of 3.56 ± 0.25 μm , cell bodies, and 2.4 ± 0.07 μm , processes). For a discussion of the relationship between apparent fluorescent size and actual size, see Supporting Information. The velocity and run distances (total displacement of mitochondria between two consecutive pauses) were calculated for each group. The averages of velocities (Figure 1B) and run lengths (Figure 1C) of the small and large mitochondria in the cell body were independent of size. Importantly, the average velocity of ~800 nm/second is typical of the unloaded velocity for both kinesin and dynein (12,15). Thus, in the cell body, the effect of drag appears negligible consistent with a previous lipid droplet study in *drosophila* embryos (2). Similar cargo-size-independent transport properties in cell bodies have recently been reported while studying the effect of lysosome diameters on their transport in mammalian (BS-C-1) cells (16).

Motion in Processes Depends on Mitochondria Size

Processes in neurons are frequently quite narrow, and in such environments cargo transport might face varying levels of impedance. The neuritic processes selected for analysis here contained easily detectable fluorescent mitochondria, and the image window was focused on the distal-most 30 μm segment of the process. In our hands, there was no significant difference in velocity, run lengths or pause frequency of mitochondria moving in proximal (within 10 μm of soma) versus distal locations in the processes (>25 μm

Figure 1: Transport parameters of mitochondria in neurons. A) Typical tracks of the mitochondria obtained from video tracking using LVCORR software. Relationship between the mitochondrial size and movement is different in cell bodies and in processes. The differences in the velocity (B) and run lengths (C) of small (S) and large (L) mitochondria in the soma and in the processes observed in wild-type neuronal cells are reduced when cultures are treated with hypotonic solution (osmolarity reduced from 250 to 200 mOsm) and Latrunculin A (1 and 5 μM). **t*-test, $p < 0.05$. ND, not significantly different.

from soma) (Figure S1, Supporting Information, $n = 49$ near and $n = 39$ far). The average diameter of the neurites in which we tracked mitochondrial movement, determined from all of those for which we had DIC images, was $1.16 \pm 0.1 \mu\text{m}$ (mean $\pm$ SEM, $N = 19$). Well above the diffraction limit of the point spread function ($>400 \text{ nm}$), the apparent and real sizes of the objects in differential interference contrast, and fluorescent images are approximately equal with slight overestimation of the real sizes ($\sim 10\%$, Figure S2A,B). Recent theoretical calculations predict that when cargos are similar in size to the process diameter, the wall effects and macromolecular crowding play a large role in regulating transport (11). Thus, we experimentally characterized the effect of size on mitochondrial transport ($N = 46$).

In this stage of culture, and without additional genetic markers, it was not possible to accurately identify axons versus dendrites, so we refer to these as 'processes'. We identified mitochondria as moving 'anterograde' or 'retrograde' when the location of the cell body could be definitively identified, but acknowledge that each group may include plus-end-directed as well as minus-end-directed transport. Ultimately, we found that motion was not statistically different in the two directions (see Supporting Information), so in subsequent analysis anterograde and retrograde data were pooled. We find that mitochondrial transport in the processes is predominantly unidirectional, with occasional reversals. Overall, $44.2 \pm 4.6\%$ of the visible mitochondria in the processes were mobile during the 10–20 min of continuous sampling. As in the cell body, there were more small mitochondria than large ones. Intriguingly, the average velocity and run lengths of all mitochondria in the process were roughly half of those of the averages in the cell body (Figure 1B,C). In addition, in contrast to the motion in the cell body, there is a significant inverse correlation between mitochondrion size and both velocity and run distance (Figure 1B,C). Because motors under load (opposition to motion) slow down, and are more likely to detach from microtubules (17,18), both observations suggest that mitochondria moving in the processes face significantly more opposition to motion than do those moving in the cell bodies. We observed occasional fission and fusion events but did not analyze those mitochondria that underwent fission or fusion during the recording time.

We briefly examined whether the distance from the cell body mattered for this effect (i.e. whether the effect was the same for motion in a portion of the process close to the cell body versus motion far away from the cell body), and found that the effect was approximately the same, regardless of the distance from the cell body (see Supporting Information).

We next analyzed motion as a function of mitochondrial size in more detail (Figure 2), and found that for mitochondrial sizes below 1.5 μm , velocity and run length were inversely correlated, but for mitochondria of 2 μm or larger, run length and velocity were approximately constant. Interestingly, while the mean velocities of the smallest moving mitochondria are ~ 600 nm/second, and approximately twice the means of the larger mitochondria, they have still not attained the presumably unloaded velocity of ~ 800 nm/second measured in the cell body, suggesting that in the processes even the small mitochondria are under significant load.

As our analysis suggests that larger mitochondria experience more opposition to motion in the processes (Figure 2A,B), we asked whether overall mitochondrial size might be chosen to decrease such opposition. Because our data suggest that the size cutoff for decreased opposition appears to be ~ 1.5 μm , we quantified the percentage of mitochondria, 1.5 μm or less, to be 71.2% in the processes and 64.6% in the cell body. The overall high percentage of these smaller mitochondria is consistent with the hypothesis that their size might have been tuned to minimize opposition to motion. While further investigation will be useful, we note that in non-neuronal cells, mitochondria are typically larger (longer) (e.g. In Cos1 cells, we observed average mitochondrial lengths of 5–6 μm). It will be particularly interesting to investigate whether cells with larger caliber processes (where boundary effects will be less important) tend to have more mitochondria above this cutoff size.

Origin of Opposition to Motion: The Role of the Bounding Membrane

This study was motivated by the hypothesis that the presence of the process membrane would increase opposition to motion on cargos moving close to it, and that such opposition to motion would depend on cargo size. We tested this by treating the cultured neurons with a hypotonic solution (to induce swelling, and, thus on average, to move the

Figure 2: Mitochondrial velocities and run distances in the processes. A) Histograms of average mitochondrial velocity distributions as a function of their length in control (Ctrl), hypotonic and Latrunculin A-treated cells. The velocities and run distances (B) are significantly longer for smaller mitochondria in Ctrl cells. The differences are minimized with hypotonic or Latrunculin A treatment.

process membrane away from the mitochondria). Analysis of mitochondrial velocities and run lengths under these conditions was consistent with our hypothesis: during exposure to hypotonic solution, the mean velocity and run lengths of mitochondria was increased in the processes (Figures 1B,C and 2), but not in the cell bodies (Figure 1B,C).

The Role of Actin

Actin might also play a role in mitochondrial motion, either promoting or opposing it. To test this, we used

Latrunculin A (Santa Cruz Biotech) to depolymerize actin, and again quantified velocities and travel distances in the cell body and in the processes. Depolymerizing actin had little effect on velocity or run lengths in the cell body, but did increase in the processes (Movies S1 and S2; see *Discussion*).

Increased Pause Time at Mitochondria Aggregates

Movement of each individual mitochondrion is not continuous, but is instead punctuated by frequent pauses, with

an average pause duration of 7.5 seconds. In addition to moving mitochondria, the majority of the processes had at least one aggregate of stationary mitochondria in the 30- μm segment in which mitochondria movement was assessed (see, for example, two red stars, Figure 3A,B first panel). Some aggregates were small (composed of 3–6 distinguishable mitochondria), whereas some were large and the mitochondria were tightly enough packed that by fluorescence microscopy individual mitochondria could no longer be distinguished. Consistent with a past report

Figure 3: Longer pause times associated with mitochondrial aggregates in neurites. A and B) Typical TIRF time-lapse images with an individual mitochondrion indicated by arrow; it moves through a large mitochondrial aggregate in the process. Scale bar = $1\ \mu\text{m}$. C) Average pause time of mitochondria when they are isolated (separated by $>2\ \mu\text{m}$ from any other mitochondria) is significantly shorter than when they encounter aggregates (7.36 ± 1.63 seconds, $n = 41$ for isolated, 24.67 ± 3.45 , $n = 51$ for aggregates). D) A similar percentage of small and large mitochondria are moving in the cell bodies. In the processes, the percentage moving is significantly higher for the small versus the large mitochondria.

characterizing transport in mutant axons in the fly (19), individual mitochondria appear to pass through both kinds of aggregates. However, the average pause time of mitochondria when they encounter aggregates is approximately four times longer than the pause time of mitochondria moving in a region with no other mitochondria (within 2 μm), when they paused (Figure 3C).

Were the mitochondria that exited the aggregates the same that entered? In general, we believe so. We could usually follow individual mitochondria as they passed through the smaller aggregates; such events comprised 73% of the 55 events involving the aggregates. In the remaining 27%, the aggregates were larger or organized in such a way that it was not possible to distinguish the mitochondrion once it entered the aggregate. In such cases, two criteria suggested that the mitochondrion that exited the aggregate after a delay was the same one that had entered earlier. First, the size of the mitochondrion entering and exiting were typically the same, to within experimental error. Second, the probability that a mitochondrion would exit an aggregate was low unless one was observed entering (in a time window of 60 seconds, if none entered, the probability of one exiting was about 6%, i.e. 1 out of 15 events).

A Larger Percentage of Small Versus Large Mitochondria Are Mobile in Neurites

Finally, we asked whether there was a correlation between mitochondrial size and the probability of moving. In the cell body, a similar percentage of the large and the small mitochondria were moving (Figure 3D). In contrast, in the processes, the percentage of small mitochondria that were moving was significantly higher than the percentage of large mitochondria. We hypothesize that this difference reflects one of the challenges of moving in processes: because the large mitochondria experience more opposition to motion in the processes, they are more likely to get stuck.

Discussion

This study was motivated by a theoretical analysis (11) suggesting that in neurons, motor-mediated transport of cargos in small-diameter neuronal processes would be more difficult than in the larger-diameter cell body. The

availability of *Drosophila* with genetically tagged fluorescent mitochondria makes this an attractive model to evaluate motor-mediated transport. However, it is difficult to evaluate mitochondrial movement in neurons, particularly their axons, in the intact *Drosophila* brain using the available imaging techniques. Therefore, this study focused on mitochondrial movement in neurons dissociated from the brains of late-stage pupae. When grown in cell culture for 2–3 days, the neurons regenerate processes (20–22) and we find that the movement of green fluorescent protein (GFP)-tagged mitochondria can be easily tracked in both the large-diameter cell bodies and in the small-diameter neurites. Although mitochondrial transport parameters have not been previously reported in the intact CNS, the rate of movement is likely to reflect that occurring *in vivo* based on an average velocity, 0.4–0.8 $\mu\text{m}/\text{second}$, that is similar to the velocity reported in motor axons in the segmental nerves of semi-intact third instar *Drosophila* larvae (19). However, when our analysis considered mitochondrial size, there was an inverse correlation between the size and velocity in the processes for smaller mitochondria. The average run length in the cultured neurons (0.7–1.7 μm) is somewhat shorter than that in the larval motor neurons (1.8–3 μm). Further studies will be necessary to determine whether this is due to differences in cell type, developmental stage and/or culture versus *in vivo*.

Motors slow down (23) when experiencing load that is a significant portion of their stalling force, so we suggest that opposition to movement in the small processes can likely reach at least 2–3 pN (see Supporting Information). In the processes of these *Drosophila* neurons – but not in the cell bodies of the same cells – mitochondrial size does indeed matter, with smaller mitochondria moving faster and further. Importantly, even the small mitochondria appear ‘loaded down’, and their velocities and run lengths are much lower than what is observed for all mitochondria moving in the cell body.

It is formally possible that these differences result from very tight differential regulation of the mitochondrial transport apparatus as a function of mitochondrial size and spatial location. Nonetheless, because two sets of perturbations – hypotonic treatment and actin depolymerization – dramatically decrease the difference between motion parameters in the cell body and in the processes, we

favor a simpler interpretation: in each case, the transport machinery is the same, independent of mitochondrial size and location, and motion differences result from increased opposition to motion in the process.

What is the source of the opposition to motion in the process? Well-established physics suggests that it is difficult to move objects close to boundaries, both because of non-slip boundary conditions (and hence increased local effective viscosity) and also because in a confined space it is difficult to move extended objects out of the path of motion as they have nowhere to go. We favor this interpretation here, supported by the experimental observation that the hypotonic treatment to increase the process' caliber (and move the process membrane away from the mitochondria) did decrease apparent opposition to motion in the process but not in the cell body, as judged by increased travel velocities and run lengths (Figures 1 and 2) only in the processes.

How then should the alterations in motion due to actin depolymerization be interpreted? Loss of actin filaments improved transport in the processes consistent with a previous report studying role of actin filaments in axonal transport but had little effect in the cell bodies, suggesting that actin contributes to opposition to motion predominantly in the processes. Our favorite model is that in the process, the actin contributes to forming a cortex that the membrane attaches to; in the absence of such actin, the membrane is then freer to expand locally, and thus motion is less opposed.

Alternatively, myosin motors on the mitochondria might also grab on to actin filaments in the process, opposing microtubule-based transport and providing drag. One interesting possibility is that much of the actin is not cross-linked to other actin filaments and is thus dragged along by the moving mitochondria. In such a case, the drag provided by these filaments would be increased due to the presence of the bounding membrane. Because the magnitude of the hypotonic effect is approximately the same as that due to the actin depolymerization, we find this idea intriguing, and worthy of additional study.

Regardless of the causes, the overall differences in mitochondrial motion as a function of size are interesting because they suggest that at least in small-caliber processes,

mitochondrial fragmentation can be used to significantly alter the overall mitochondrial motion.

In addition to containing many moving mitochondria, the processes in the cultured neurons also contained both small and large mitochondrial aggregates. While some large aggregates of non-moving mitochondria had been described in diseased neurons (24,25), and they had occasionally been observed as present in wild-type processes as well (6,7), they were more common than we expected, with typically at least one in any 30- μ m segment chosen. Many such 'aggregates' were relatively small, consisting of 3–6 closely spaced unmoving mitochondria. Nonetheless, the presence of these aggregates altered the transport of other mitochondria moving in either the anterograde or retrograde direction. As previously reported in *Drosophila* motor neurons, the aggregates do not completely block the movement of mitochondria (19), but we observe that in the cultured brain neurons, they significantly increase the pause time. Thus, in principle, the frequency of such aggregates could be used to tune the overall rate of mitochondrial transport. More likely, these long pauses of moving mitochondria when they encounter aggregates suggest increased opportunities for mitochondria–mitochondria interactions: there are reports of transient and permanent fusions of mitochondria (26) which are believed to be a way of regenerating aged mitochondria, and these exchanges and fusions are presumably enhanced by repeated slow contacts between mitochondria. Thus, these swellings where sustained mitochondria–mitochondria interactions occur could be important for promoting the exchange of mitochondrial components, and ultimately mitochondrial health in the distal axons. It remains for future work to determine, if indeed, these swellings are useful, instead of harmful as has previously been assumed.

Materials and Methods

Fly lines

Pupae were obtained from the mating of mito-GFP lines (Bloomington Stock Center No. 8442 or 8443) that express GFP with a mitochondrial import signal to one of the three enhancer trap lines to label mitochondria in different neuronal subpopulations: *elav^{C155}*-GAL4 (Bloomington Stock Center, No. 458), GAL4 broadly expressed in neurons throughout adult CNS; GH298-GAL4 (Bloomington Stock Center, No. 37294), GAL4

expressed in adult neurons including local neurons (LNs) in the antennal lobe; and GH146-GAL4 (obtained from Luquin Luo Lab), GAL4 expression restricted primarily to projection neurons (PNs) in the adult antennal lobe.

Cultures

Primary neuronal cultures were prepared from the brains of late-stage pupae as previously described (27,28). Cultures were maintained in a humidified 5% CO₂ incubator at 22–24°C, and imaging experiments were performed between 2 and 4 DIV.

Imaging

Movies of fluorescent mitochondria were recorded using the objective-based TIRFM. TIRFM is built on Nikon TE200 inverted Differential Interference Contrast (DIC) microscope by using a high numerical aperture objective (Nikon 1.49 NA, 100× objective, 1.4 NA condenser). The cultured cells grown on special dishes have either genetically encoded GFP or stained with 10 nM Tetramethylrhodamine (TMRM) dye. The medium was exchanged with HBSS (Hepes-buffered salt solution, in mM, 120 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 15 glucose, 20 HEPES, 10 NaOH and 0.001% phenol red, pH 7.2–7.4) before imaging. Very low level of excitation light from 488 nm Ti-Sapphire laser (Coherent, Inc.) is used for imaging, and the emitted light is collected using suitable notch filter to filter out the excitation laser from reaching the camera. An EMCCD camera from Photometrics (QuantEM 512SC) is used to grab and save the images on to the disc using Photometrics Micromanager Software. The images have a spatial resolution of 60 nm/pixel (After 2.5× lens attachment in front of the camera) and were captured at five frames a second. To reduce the photo-toxicity and bleaching, the circularly polarized excitation laser was synchronized with the automated opening of shutter and its intensity kept minimum. While imaging with the optimized intensity level, there was no considerable bleaching for up to 10 min of acquisition. Before acquiring the fluorescence images, the DIC images were also acquired after reducing the exposure time and EM gain of the camera to avoid the pixel intensity saturation. The diameter of the processes was estimated from the DIC images through further image analysis.

Actin depolymerization

The neuronal cells were treated with 1, 5 and 10 μM Latrunculin A (Santa Cruz Biotechnology, sc-202691) in HBSS solution for 15 min at 25°C prior to imaging. The analysis was carried out on 1 and 5 μM only as the motion stopped in cells treated with 10 μM after about 15 min. In our hands, 1 μM Latrunculin A solution was sufficient to alter the morphology of Cos1 cells attached to coverslips from well spread out to round shapes, thus confirming its effectiveness in depolymerizing actin networks.

Hypotonic conditions

Hypotonic solution was prepared from HBSS. The osmolarity of HBSS is 250 mOsm. Hypotonic HBSS was prepared by adding distilled water to achieve an osmolarity of 200 mOsm. The treatment method for hypotonic solutions was similar to Latrunculin A treatment. The solution with

200 mOsm osmolality was exchanged with culturing medium 15 min prior to imaging. The mitochondrial morphology was affected in solutions with <200 mOsm osmolality.

Video tracking

Template matching method is used to generate the video tracks. Custom-written labview-based LVCORR software is used to track the positions of the mitochondria in the processes. The template generation for shorter mitochondria is carried out by selecting the whole organelle. For larger ones, a portion of the leading end is considered as the template and LVCORR program did well in generating the tracks of isolated mitochondria with this approach. The tracks were broken whenever a mitochondria passed through a docked mitochondria. In such cases, the position of the mitochondria was scored by generating the intensity line profile of the passing mitochondria to join the broken tracks. During the linear motion/run, if the average displacement in four subsequent frames is <100 nm, that mitochondria was considered to be in pause state.

Length of the mitochondria was estimated by generating the intensity line profile of the straight mitochondria in five successive gray-scale images/frames and by calculating the average length using a custom-written algorithm in labview. A threshold of 10% of maximum intensity was selected to find the edges of the mitochondria. The images for estimating the length were chosen in such a way that the mitochondria remained straight and there was no deformation or defocusing of mitochondria in the images. The raw data used for the analysis are presented in Figure S4.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Proximal and distal transport parameters are not significantly different. Average speeds (A), run lengths (B) and pause frequencies (C) for the small mitochondria moving in the processes within 10 μm from the soma (proximal) and >25 μm from the soma (distal) are not significantly different.

Figure S2: Use of microscope images to determine the cargo size. A) Fluorescence recording of 200 and 540 nm red fluorescent beads acquired using the TIRF imaging reveals their distinct sizes. Scale bar = 3 μm. B) Calibration procedure was cross-examined in differential interference

video contrast microscopy using the standard size silica beads showing a close correlation between the real and apparent sizes.

Figure S3: Estimation of penetration depth using IMAGEJ plugin (Calc TIRF) shows that the penetration depth of evanescent field when using 1.49 NA objective lens is close to 900 nm at the critical angle of incidence.

Figure S4: Estimation of average run lengths and velocities based on raw data. A, C, E and G) Velocity (B, D, F and H: run length) distributions of mitochondria in wild-type, 200 mOsm hypotonic solution, 1 and 5 μM Latrunculin A solutions.

Movie S1: Mitochondrial motion in the processes of wild-type *Drosophila* primary neurons. Scale bar = 1 μm (frame rate is 12 $\times$ real time).

Movie S2: Mitochondrial motion in the processes of *Drosophila* neurons treated with 5 μM Latrunculin A to depolymerize actin filaments. Notice the higher speeds due to lowered opposition to motion when compared with motion in wild-type cells (Movie S1). Scale bar = 1 μm (frame rate is 12 $\times$ real time).

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