Author

Hyuna Lee, working under Professor Jeon, used a unique microfluidic platform to perform her biological research. Using this platform, she was able to culture neurons to isolate the axons from the soma, so that she could observe GFP-tagged mitochondrial movement in the axons. Through her research experience, Hyuna developed an appreciation for reading papers describing how other researchers conducted their studies. She found the constant friendship and support of the people in her lab to be particularly valuable. Hyuna intends to attend graduate school to further develop the passion she has developed for innocent lives shortened by incurable diseases.

Key Terms

- Axonal Transport
- Microfluidic Platform
- Mitochondria
- Mitochondrial Trafficking
- Mitochondrial Transport
- Neurons

Developing a Control Model of Mitochondrial Trafficking in Neurons Using a Novel Microfluidic Culture Platform

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Biological Sciences

Abstract

Neurodegenerative diseases, such as Alzheimer's disease, lead to permanent disabilities in muscular movements, memory and dementia. Mitochondrial transport is closely linked to neuronal function. Mitochondrial trafficking along microtubules delivers energy in the form of adenosine triphosphate (ATP), which is necessary for signal transmission and sustaining viability. Controversy remains regarding the direction and mechanisms by which mitochondria move in aging and diseased neurons. It is unclear whether axonal transport impairment is due to mutations in mitochondrial DNA or other cytoplasmic factors. We used a microfluidic culture platform to analyze mitochondrial trafficking patterns in axons by transfecting neurons with Mito-GFP and observing them under time-lapse microscopy to analyze differences in mitochondrial morphology, movement and interaction. Elongated mitochondria in soma and dendrites were less motile but moved steadily. Mitochondria in axons generally had short ovular shapes with rapid bidirectional movements and varying speeds. Many clusters of mitochondria were found in locations that required large amounts of ATP. We also observed mitofusion or mitofission, which may be indicators of neuron survival or death, respectively. This research is a first step in developing a model to closely reflect neuronal mitochondrial trafficking patterns.

Faculty Mentor

Understanding the relationship between mitochondrial axonal transport and neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's is important in the search for successful treatments for these diseases. In tracking mitochondrial movement, it is important to distinguish the morphology of the dendrites and axons. The microfluidic culture platform Hyuna Lee used in her research establishes this distinction, providing a clear look at trafficking patterns throughout the neuron. The results of this study offer a better understanding of mitochondrial transport, a potential first step toward complete elucidation of the pathology of neurodegenerative diseases. Hyuna's work demonstrates the success that can be achieved by undergraduate students who passionately devote themselves to their research.

Noo Li Jeon
The Henry Samueli School of Engineering

Introduction

Research has shown that damage in axons induced by the accumulation of impaired proteins and abnormal tangles of microtubule filaments are linked to a number of progressive neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's diseases. While these disease proteins and microtubule tangles have been intensely investigated over the last five years, far less attention has been focused on the role of the axonal transport in the aggregation of proteins and in mechanisms underlying the neurodegenerative process. Therefore, recent neurodegenerative disease research has been focusing on the mechanisms of axonal transport. Components including human motor proteins, gene mutations, and amyloid precursor protein (APP) plaques and tau are involved in neuronal death (Holzbaur and Chevalier-Larsen, 2006).

Elucidating the mechanisms of mitochondrial axonal transport and its relationship with neurodegenerative diseases may lead to treatments. However, the mechanism of mitochondrial axonal transport is not fully understood. For example, axonal transport in mouse models was found to be disrupted long before there was any identification of disease proteins or disruption of organelle transport. Therefore, it is still unclear whether axonal transport impairment causes or is a consequence of neurodegenerative diseases. It is also still unclear whether the genetic mutations of axonal transport inhibit transport functions or create negative domino effects (Goldstein and Duncan, 2006; Holzbaur and Chevalier-Larsen, 2006).

One approach to investigating these questions would be to observe the movement and location of mitochondria in healthy neuronal axonal transport. Mitochondrial transport in axons can serve as a general model for how organelles move because mitochondria move and change direction along the microtubules in response to physiological signals (Saxton and Hollenbeck, 2005). Recent research suggests that mitochondrial movements closely reflect the functional condition of the cell (Malaiyandi et al., 2005; Reynolds and Santos, 2005). Experimental data reveals that many mitochondrial trafficking dysfunctions in the axonal transport are identified in the majority of neurodegenerative diseases (Chang et al., 2006; Ebneth et al., 1998; Malaiyandi et al., 2005; Piccionin et al., 2002; Szeto, 2006). Mitochondria are of interest because their transport is associated with their function, which in turn determines the neuronal cellular viability. Understanding how mitochondrial trafficking sustains neuronal health will therefore reveal how irregular trafficking induces pathogenic conditions and cellular dysfunctions.

To track mitochondrial movement in the axon, morphology of the dendrites and axons must be distinguished correctly. In conventional mitochondrial trafficking research, neurons are cultured on coverslips or cell culture chambers, and axons and dendrites are distinguished based on well-established morphological characteristics (Hollenbeck et al., 1995; Ligon and Steward 2000; Sheng 2005). However, experimental analysis requires single, intact neurons to be selected. For example, the neuron has to be spatially isolated for unambiguous identification of the processes (Hollenbeck et al., 1995). Also, only neurons that do not have regions of overlaps and crossings, have axons twice the length of other processes and do not move relative to the substratum, and sections of axons with thin and uniform caliber are chosen (Sheng et al., 2005). Although some researchers confirm the morphology with immunocytochemical labeling (Ligon and Steward, 2000), distinction depending on the morphologic criteria may not be completely accurate and the extra step for confirmation may become tedious and time consuming. In this study, we developed a method to understand the conflicting elements of axonal transport observed in neurodegenerative diseases by analyzing the innate characteristics of mitochondria in embryonic rat neurons.

Materials and Methods

The obstacles of using the conventional method to correctly identify intact neuronal processes can be greatly reduced by using a 450 μm neuron microfluidic device. This neuronal platform is an oxygen-permeable "molded elastomeric polymer piece placed against a glass coverslip," (Jeon et al., 2000), which can be used to successfully culture viable neurons separated by soma and axons for at least 2–3 weeks (Figure 1). The elastomeric polymer piece has two vertical channel molds (1.5 mm wide, 7 mm long, 100 mm high) connected by equally spaced, horizontally molded 450 μm long

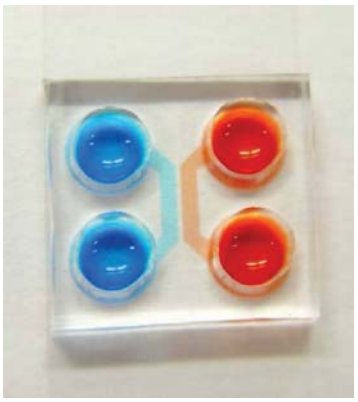


Figure 1

Optically transparent PDMS mold allows us to observe two different colored dyes in the chambers without mixing for over 20h. High fluidic resistance of the microgrooves produces a small but sustained flow between the compartments that counteracts diffusion.

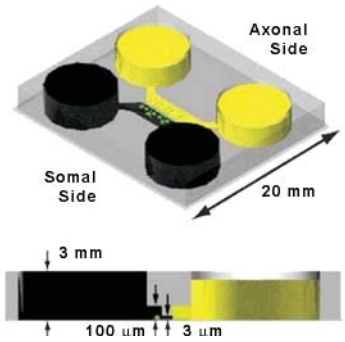


Figure 2

The culture chamber consists of an oxygen-permeable PDMS (polydimethylsiloxane) mold plasma bonded onto a PLL (polylysine)-coated coverslip. Inside this device are somal and axonal compartments connected by microgrooves. These microgrooves allow up to seven axons to grow into the axonal side.

microgrooves (10 µm wide, 3 µm high) (Figure 2), just long enough to allow enough axon ends to reach fresh media in the opposite channel. As a result, 3–4 days after embryonic day 18 rat neurons are seeded in the left channel, axons protrude through the microgrooves onto the adjacent channel, while the somas remain stationed in the channel, continuing to extend their dendrites (Figure 3). Using this method, we can accurately compartmentalize and determine the morphology of the soma and axons, which is imperative for observing the shape and transport behaviors of mitochondria in these two different neuron segments.

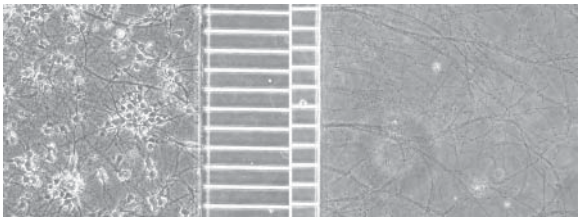
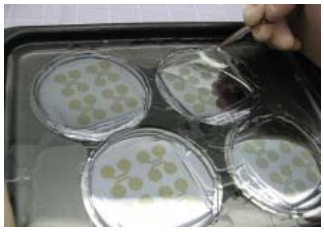


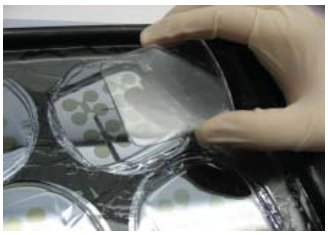
Figure 3

As depicted in this microscopic view of the platform, chambers allow isolated axon growth. Neurites grow through microgrooves from the somal compartment into the axonal compartment. These microgrooves allow neurites, but not the cell bodies, to enter the axonal side.

We chose to use a 450 µm microfluidic culture platform, not only because it separates the axons from the somas, but also because of its very small and compact size (20 mm x 20 mm), requiring a minimal amount of neurons and culture media for experimental analysis. The glass coverslip also allows for the use of time-lapse microscopy, which facilitates the biochemical and image analysis of mitochondrial transport patterns in the axon (Jeon et al., 2005). Using this method, we track mitochondrial trafficking patterns both in the soma and axons by transfecting the mitochondria with Mito-GFP and using time-lapse microscopy to analyze transport patterns and morphology.



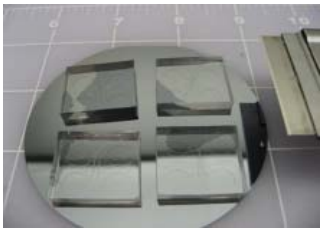
1. Bake PDMS



2. Cut Out

3. Device Mold

4. Cut Around



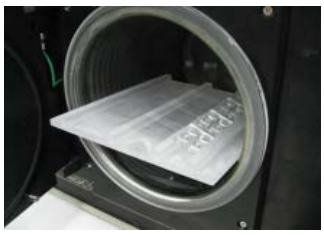
5. Four Devices

6. Punch Wells

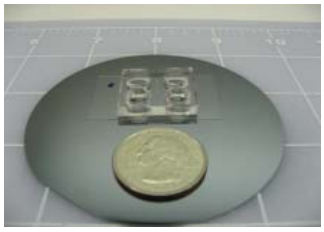
7. Blow Debris

8. Tape Debris

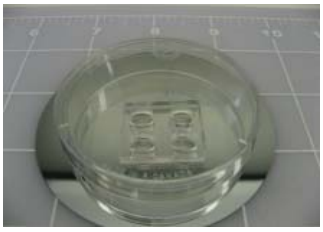
9. Autoclave



10. Plasma



11. Bond Faces



12. In Petri Dish

Soft Lithography Microfluidic Device
Polydimethylsiloxane (Sylgard, Midland, MI) was mixed with Silicon Elastomer (Dow Corning, Midland, MI) at a 10:1 ratio by mass for 5–10 minutes until thoroughly mixed. It was then placed in a vacuum container to eliminate bubbles. After the PDMS-Silicon Elastomer mixture was bubble-free, 7–10 g of the mixture was poured onto a silicon master wafer (University Wafer, South Boston, MA) engraved with the 450 μm groove microfluidic chamber design for neuron culture. They were then baked for at least 30 minutes in a 100 °C oven until solidified. Then the microfluidic chamber design on the baked soft lithography was cut and peeled off and the four wells in each design were punched out. Debris caught on the surface was either blown away with nitrogen gas spray or taped off.

PLL Coating of the Coverslips
One side of the coverslips was treated with Poly-L Lysine (PLL) (Sigma-Aldrich, St. Louis, MO) liquid concentration of 0.5 μg/μl, and kept in a 37 °C humidified incubator overnight. After the overnight incubation, PLL was aspirated and the cover slips were rinsed three times using autoclaved de-ionized (DI) water.

Plasma Treatment of the Autoclaved Devices
The bonding sides of the devices were plasma treated for 5 minutes in the Harrick Plasma Sterlilizer/Cleaner (Harrick Plasma, Ithaca, NY). These devices were bonded to the PLL-treated glass slides and kept in 5 cm Petri dishes.

Neuron Culture
Neurons from embryonic day 18 rat pups were cultured. Before loading the neurons, the devices were incubated in Neural Basal Media (NBM) (Invitrogen, Carlsbad, CA), which was made with 25 ml NBM, 250 μl 1% final concentration of Pen/Strep (Invitrogen, Carlsbad, CA), 500 μl 1:50 dilution of B-27 (Invitrogen, Carlsbad, CA), and 62.5 μl 0.25% of Glutamax (Invitrogen, Carlsbad, CA). NBM was added on the top two wells and was allowed to flow through the channels and microgrooves until it reached the bottom two wells. Waiting for the media to flow down to the bottom two wells eliminated the possibility of air bubbles getting trapped in the microgrooves, which then would oxidize and kill the cell. Once media flow was confirmed, the four wells were filled with additional NBM, and the device was incubated until the neurons were ready to be seeded.

Once the neurons were ready, media were aspirated from the wells away from the channels, just enough to keep the glass slide wet. Then the neurons were seeded only in the top left well, and the neuron flow through the channel was checked under the microscope. Next, the devices were incubated for 10 minutes to allow the neurons to stick to the PLL-treated glass slide before additional media was added.

Mitochrondrial-Green Fluorescent Protein (Mito-GFP)
2 μl of L2K with 50 μl of OptiMEM (Invitrogen, Carlsbad, CA) was briefly mixed in a microfuge tube. In a separate microfuge tube 0.8 μg of mito-GFP (Clonetech, Mountain View, CA), which is a green fluorescent protein with primers that tag cytochrome-c DNA sequence of mitochondria, was mixed with 50 μl of OptiMEM. Both tubes were incubated for 5 minutes at Room Temperature (RT), after which the two tubes were mixed together and incubated at RT for 20 minutes. Next, the media was removed from the neuron device and 100 μl of the transfection mixture was added to the soma side of the device. The device was incubated at 37 °C for 2.5 hours and rinsed twice with fresh NBM. After 48 hours of incubation, the neurons were ready to be imaged.

Time-Lapse Microscopy
An inverted Nikon fluorescent microscope (Nikon, Melville, NY) was used to take a time-lapse movie of the Mito-GFP tagged mitochondrial movement. The transfected neurons cultured in the 450 μm microfluidic device were placed on an inverted microscope and the whole stage was enclosed by a plastic chamber incubated at 37 °C. Time-lapse recordings were taken every 5 seconds at 600 ms exposure time for 30 minutes and the data was analyzed using MetaMorph imaging analysis software (Universal Imaging, Westchester, PA).

Please refer to these Web sites for visual procedures:

Fabrication of Microfluidic Device for the Compartmentalization of Neuron Soma and Axons:
http://www.jove.com/index/Details.stp?ID=261

Preparing E18 Cortical Rate Neurons for Comparmentalization in a Microfluidic Device:
http://www.jove.com/index/Details.stp?ID=305

Results

After detailed observations of mitochondrial trafficking patterns in the axons of approximately one hundred 450 μm microfluidic platforms, it was concluded that not all mitochondria are actively motile. Among the samples, around 30% of the neurons recorded had mitochondria that were all stationary, yet the neurons were still vital. Although we were highly interested in imaging neurons with active mito-

chondrial transport, neurons with inactive mitochondrial movements were not ignored.

Through the time-lapse recordings of active mitochondrial transport, we observed various kinds of mitochondrial movements in the axons. Motile mitochondria inconsistently moved with varying speeds that were fast and abrupt, slow and steady, or just fluctuated in their stationed location. In the sample shown, there were about five mitochondria that moved significantly with visible displacement, compared to the rest that fluctuated more than moving. Generally, mitochondrial movement in the axons was quick and abrupt. These active mitochondria were not in constant motion, but would rather manifest salutatory movement, where they would stop at random locations for varying periods of time before continuing their transport, or overlap with adjacent mitochondria for a short period of time before relocating to their favored direction. The directions of their movements were generally anterograde, towards the axon (Figure 4), or retrograde, towards the soma (Figure 5), with varying vertical leaps. In many cases, they would randomly disappear out of focus or reappear as they moved along the Z-axis,

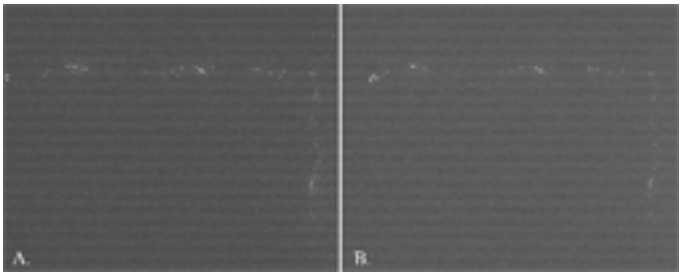


Figure 4

Seven days *in vitro* (DIV) anterograde mitochondrial movement. Mito-GFP transfected distal axon emitting green fluorescent light under FITC. (A&B) Motile mitochondria moving fast in anterograde direction after 110 seconds in comparison to the majority, which were fluctuating in this axon. Arrows indicate motile mitochondria.

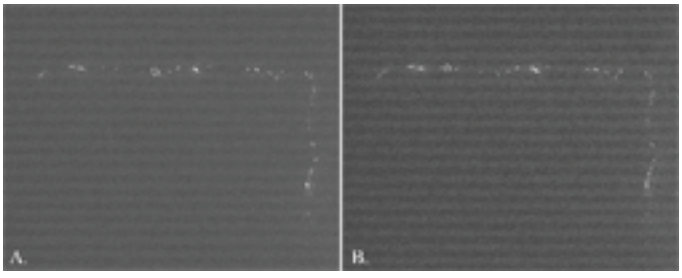


Figure 5

Seven-DIV retrograde mitochondrial movement. Mito-GFP transfected distal axon emitting green fluorescent light under FITC. (A&B) Motile mitochondria moving fast in retrograde direction after 160 seconds in comparison to the majority, which were fluctuating in this axon. Arrows indicate motile mitochondria.

observed as blinking mitochondria. As shown in Figure 6, we were able to track movement of every mitochondrion and numerically quantify its displacement, length, direction, and speed variability.

While observing mitochondrial movement in axonal transport, it was apparent that the morphology of each mitochondrion differed. Although they were generally round and robust, the size and shape was unique to each mitochondrion and many were found clustered near the axon tip (Figure 7). To see if mitochondrial morphology depended on their stationed location in the neuron, mitochondria in the dendrites were observed as well.

Mitochondrial morphology differed in the soma, supporting our hypothesis. They were generally 4–6 times the average length of mitochondria found in the axons, and their shapes were generally elongated or tubular. Also, their transport

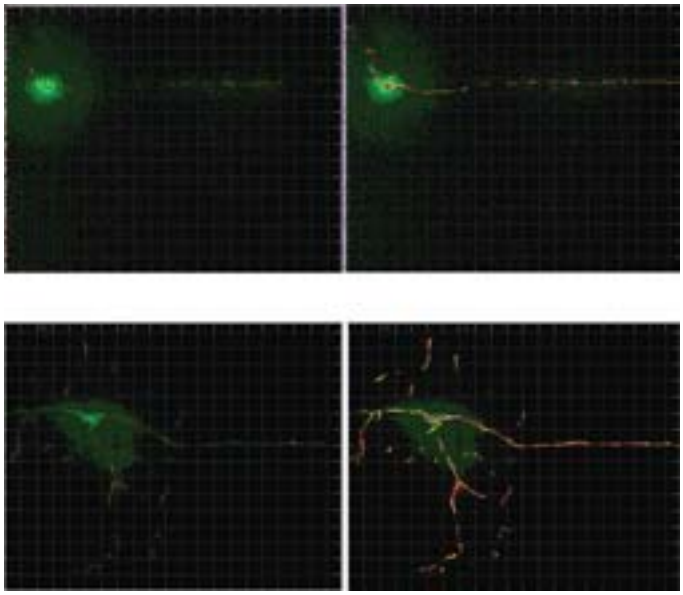


Figure 6

Two Imaris software images of axon (Left) Particle Tracking (Right) Particle tracking with dragon tail lines indicating direction and length traveled by each mitochondrion.

Figure 7

60x image of the fluorescent-tagged mitochondrial movement in the distal axon. (A) Bright Field (B) FITC at 600 ms exposure (C) Overlay.

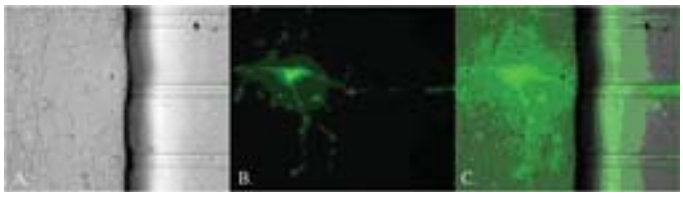


Figure 8

60x image of the fluorescent-tagged mitochondrial movement in the dendrites. (A) Bright Field (B) FITC at 600 ms exposure (C) Overlay.

patterns greatly differed from those of the mitochondria in the axons. Mitochondria located in the soma did not actively transport, but rather fluctuated in their stationed location. As depicted in Figure 8, this sample shows curvilinear-shaped mitochondria that curved back and forth without any noticeable displacement.

As our main interest was in the axonal transport, we looked for specific transport patterns or interactions among mitochondria. In all the samples observed, there was no specific transport pattern detected. Mitochondria were transported uniformly throughout the axon with specific anterograde and retrograde directions. However, there were interesting interactions among them. Occasionally, we observed mitochondrial fusion and fission. As displayed in Figure 9, a single mitochondrion would join another and fuse together or divide through fission. In this axon, mitochondrion (m1) moved in the retrograde direction for 55 seconds to join another mitochondrion for mitochondrial fusion, immediately followed by fission (Figure 9.a.). The mitochondrion 2 (m2) from mitofission, moved in the anterograde direction for 4 minutes and 20 seconds and merged with its adjacent mitochondrion (Figure 9.b). Another mitochondrion (m3) located to the right of fused m2 (m2+), moved anterograde for mitofusion with m2+ that occurred in 20 minutes and 15 seconds (Figure 9.c.).

Discussion

In this project we cultured neurons in a microfluidic platform that separates axons from the soma. As a result, we were able to gain a better understanding of axonal transport by observing mitochondrial trafficking in the axons. We analyzed mitochondrial trafficking based on the neurons' physiological properties. Dysfunctional mitochondria, whether in transport or mutation mitochondrial DNA, can trigger neuronal apoptosis (Wallace, 2001). Observing mitochondrial trafficking in embryonic neurons can serve as a tool to understand cellular transport patterns in healthy axons, so that it can be compared with neurons with axonal transport dysfunctions observed in many neurodegenerative diseases.

Our findings of mitochondrial morphology, movement behaviors, transport direction, and interactions are consistent with recent studies (Hollenbeck, 1995; Steward and Ligon, 2000; Reynolds and Chang, 2006). It has been suggested that mitochondrial clustering in the axon tip in embryonic neurons, but not in older neurons, shows that developing neurons require large amounts of ATP near the axon tips, or growth cones, for proper growth and development. This data supports our hypothesis that mitochondrial morphology depends on their location in the soma verses the axon. We observed elongated, tubular mitochondria that were grouped together, fluctuating in the dendrites; while in the axons, the mitochondria were rapidly moving

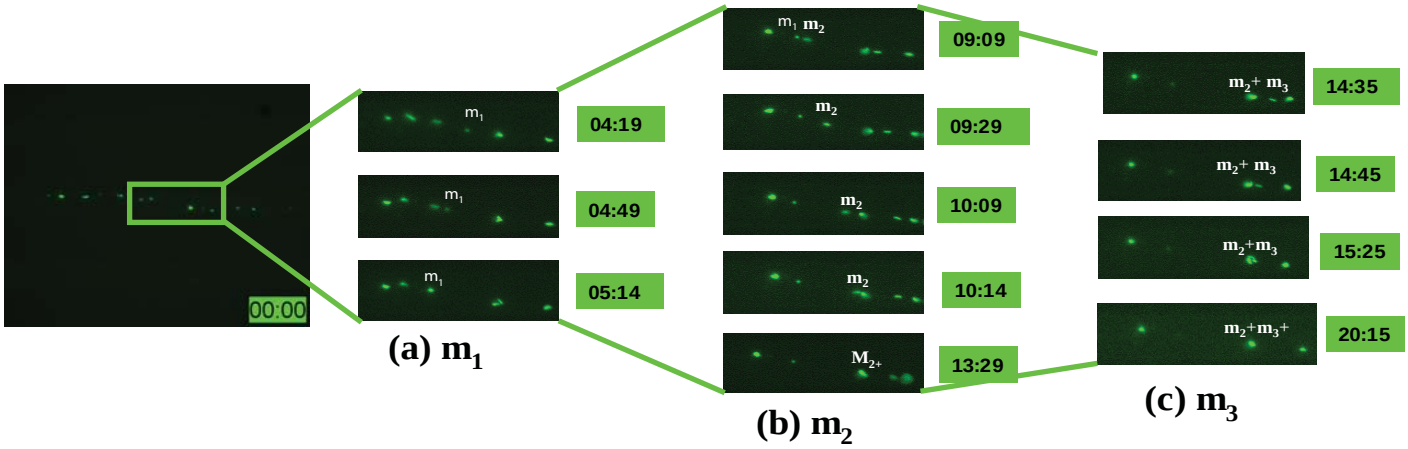


Figure 9

Mitochondrial fusion and fission changed the mitochondrial morphology. (a) Mitochondrial (m1) fusion occurs retrograde direction in 55 sec. (b) Mitochondrial (m2) fission occurs from (a) and fusion occurs anterograde direction in 4 min and 20 sec. (c) Mitochondrian m3 moves anterograde and mitochondrial fusion occurs with m2 in 20 min and 15 sec.

relative to those in the soma. It has also been suggested that mitochondria in the soma elongate during development and cluster near the dendritic membrane without any significant displacement. The reason is that dendrites constantly receive chemical signals from the synapse through their active sites, and to have efficient signal processing to the axon, mitochondria in the dendrites elongate themselves in order to be in contact with as many active sites as possible (Reynolds and Chang 2006). In the case of our mitochondrial fusion and fission recordings, mitochondria have proteins Mitofusins 1 and 2 and OPA1 localized on their outer membrane that are essential for mitochondrial fusion. A recent study suggests that mitochondrial fission may facilitate cellular apoptosis, whereas mitochondrial fusion may protect the neuron from cellular dysfunction (Chan and Chen, 2005).

Future analysis of mitochondrial displacement, length, velocity, and speed variability would provide a clearer model for axonal transport. It needs to be determined whether mitochondrial movement speeds are due to intracellular cues or because there are just different groups of mitochondria that are motile at different rates. Once a control pattern is obtained, the next step would be to expose the axons to Oxygen-Glucose Deprivation and observe changes in mitochondrial movement in the axons, as well as in the synapse, and determine the minimal and maximal exposure time for environmental insults and recovery of the mitochondria. Once this is achieved, it would be ideal to compare their mitochondrial trafficking to those in genetically manipulated neurons that resemble diseased neurons. Ultimately, we hope to develop a platform that leads to a quantitative functional assay for testing drugs that affect the axonal transport. Understanding mitochondrial trafficking may lead to elucidating the pathology of neurodegenerative diseases such as Multiple Sclerosis, Parkinson's, and Alzheimer's.

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