

Journal of Visualized Experiments

Analyzing mitochondrial transport and morphology in human induced pluripotent stem cells-derived neurons in hereditary spastic paraplegia --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60548R2
Full Title:	Analyzing mitochondrial transport and morphology in human induced pluripotent stem cells-derived neurons in hereditary spastic paraplegia
Section/Category:	JoVE Neuroscience
Keywords:	Mitochondrial transport, mitochondrial morphology, forebrain neurons, induced pluripotent stem cells, axonal degeneration, hereditary spastic paraplegia
Corresponding Author:	Xuejun Li UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	xjli23@uic.edu
Order of Authors:	Yongchao Mou Sukhada Mukte Eric Chai Joshua Dein Xue-Jun Li
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Rockford, Illinois, 61107, USA



Dr. Xue-Jun Li, Associate Professor
Department of Biomedical Sciences
Regenerative Medicine & Disability Research Lab
College of Medicine at Rockford
Department of Bioengineering
University of Illinois at Chicago
Phone: 815-395-5882
Email: xjli23@uic.edu

October 16, 2019

Dear Dr. Bajaj,

Thank you very much for your suggestions on our revised manuscript. We have addressed all the questions and included a revised manuscript with comments and our answers. In addition, we included a clean version which incorporates all the changes but without all the comments and answers. Please feel free to let me know if any other information is needed.

Best regards,

A handwritten signature in black ink, appearing to be "Xue-Jun Li".

Xue-Jun (June) Li, PhD

TITLE:

Analyzing Mitochondrial Transport and Morphology in Human Induced Pluripotent Stem Cell-Derived Neurons in Hereditary Spastic Paraplegia

AUTHORS AND AFFILIATIONS:

Yongchao Mou^{1,2}, Sukhada Mukte¹, Eric Chai¹, Joshua Dein³, Xue-Jun Li^{1,2}

¹Department of Biomedical Sciences, University of Illinois College of Medicine Rockford, Rockford, IL, USA

²Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, USA

³MD Program, University of Illinois College of Medicine Rockford, Rockford, IL, USA

Corresponding Author:

Xue-Jun Li (xjli23@uic.edu)

Email Addresses of Co-Authors:

Yongchao Mou (ycmou@uic.edu)

Sukhada Mukte (smukte2@uic.edu)

Eric Chai (echai4@uic.edu)

Joshua Dein (jdein2@uic.edu)

KEYWORDS:

mitochondrial transport, mitochondrial morphology, forebrain neurons, induced pluripotent stem cells, axonal degeneration, hereditary spastic paraplegia

SUMMARY:

Impaired mitochondrial transport and morphology are involved in various neurodegenerative diseases. The presented protocol uses induced pluripotent stem cell-derived forebrain neurons to assess mitochondrial transport and morphology in hereditary spastic paraplegia. This protocol allows characterization of mitochondrial trafficking along axons and analysis of their morphology, which will facilitate the study of neurodegenerative disease.

ABSTRACT:

Neurons have intense demands for high energy in order to support their functions. Impaired mitochondrial transport along axons has been observed in human neurons, which may contribute to neurodegeneration in various disease states. Although it is challenging to examine mitochondrial dynamics in live human nerves, such paradigms are critical for studying the role of mitochondria in neurodegeneration. Described here is a protocol for analyzing mitochondrial transport and mitochondrial morphology in forebrain neuron axons derived from human induced pluripotent stem cells (iPSCs). The iPSCs are differentiated into telencephalic glutamatergic neurons using well-established methods. Mitochondria of the neurons are stained with MitoTracker CMXRos, and mitochondrial movement within the axons are captured using a live-cell imaging microscope equipped with an incubator for cell culture. Time-lapse images are analyzed using software with "MultiKymograph", "Bioformat importer", and

“Macros” plugins. Kymographs of mitochondrial transport are generated, and average mitochondrial velocity in the anterograde and retrograde directions is read from the kymograph. Regarding mitochondrial morphology analysis, mitochondrial length, area, and aspect ratio are obtained using the ImageJ. In summary, this protocol allows characterization of mitochondrial trafficking along axons and analysis of their morphology to facilitate studies of neurodegenerative diseases.

INTRODUCTION:

Mitochondrial motility and distribution play a vital role in fulfilling variable and specialized energetic demands in polarized neurons. Neurons can extend extremely long axons to connect with targets through the formation of synapses, which demand high levels of energy for Ca^{2+} buffering and ion currents. Transport of mitochondria from soma to axon is critical for supporting axonal and synaptic function of neurons. Spatially and temporally dynamic mitochondrial movement is conducted by fast axonal transport at rates of several micrometers per second¹.

Specifically, motor or adaptor proteins, such as kinesin and dynein, participate in the fast organelle transport along microtubules to control the movement of mitochondria^{2,3}. Normal neuronal activity requires proper transport of newly assembled mitochondria from neuronal soma to distal axon (anterograde axonal transport) and reverse transport of mitochondria from the distal axon back to the cell body (retrograde transport). Recent studies have indicated that improper mitochondrial allocation is strongly associated with neuronal defects and motor neuron degenerative diseases^{4,5}. Therefore, to dissect the role of mitochondria in neurodegeneration, it is important to establish methods for examining mitochondrial movement along axons in live cultures.

There are two main challenges in examining and analyzing the tracking of mitochondria: (1) identifying mitochondria from the background in every frame, and (2) analyzing and generating the connections between every frame. In resolving the first challenge, a fluorescence labeling approach is used widely to distinguish mitochondria from the background, such as MitoTracker dye or transfection of fluorescence-fused mitochondrial targeting protein (e.g., mito-GFP)⁶⁻⁸. To analyze the association between frames, several algorithms and software tools have been described in previous studies⁹. In a recent paper, researchers compared four different automated tools (e.g., Volocity, Imaris, wrMTck, and Difference Tracker) to quantify mitochondrial transport. The results showed that despite discrepancies in track length, mitochondrial displacement, movement duration, and velocity, these automated tools are suitable for evaluating transport difference after treatment¹⁰. In addition to these tools, an integrated plugin “Macros” for ImageJ (written by Rietdorf and Seitz) has been widely used for analyzing mitochondrial transport¹¹. This method generates kymographs that can be used to analyze mitochondrial movement, including velocity in both anterograde and retrograde directions.

Mitochondria are highly dynamic organelles that constantly change in number and morphology

Page 1 of 15

in response to both physiological and pathological conditions. Mitochondrial fission and fusion tightly regulate mitochondrial morphology and homeostasis. The imbalance between mitochondrial fission and fusion can induce extremely short or long mitochondrial networks, which can impair mitochondrial function and result in abnormal neuronal activities and neurodegeneration. Impaired mitochondrial transport and morphology are involved in various neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and hereditary spastic paraplegia (HSP)¹²⁻¹⁵. HSP is a heterogeneous group of inherited neurological disorders characterized by the degeneration of the corticospinal tract and subsequent failure to control lower limb muscles^{16,17}. In this study, iPSC-derived forebrain neurons are used to assess mitochondrial transport and morphology in HSP. This method provides a unique paradigm for examining mitochondrial dynamics of neuronal axons in live cultures.

PROTOCOL:

1. Generation of telencephalic glutamatergic neurons from iPSCs

NOTE: The detailed protocol for maintaining iPSCs and their differentiation into telencephalic glutamatergic neurons are similar to those described previously¹⁸. Here, the critical process during the differentiation of human pluripotent stem cells is introduced and highlighted.

1.1. Culture iPSCs on mouse embryonic fibroblast (MEF) feeders in human embryonic stem cell (hESC) medium supplemented with fibroblast growth factor (bFGF, 4 ng/mL).

1.2. After incubation with dispase (1 mg/mL) for 3 min, dissociate the iPSCs into small clumps. Then, culture the iPSC aggregates in suspension in the hESC medium for 4 days. Refer to this timepoint, when the iPSCs are starting as the suspension culture, as day 1 (D1). Change the medium daily for 4 days.

1.3. At D5, collect the iPSC aggregates after centrifugation at 200 x *g* for 2 min and culture in neural induction media (NIM) for 3 days. Culture the cell aggregates in suspension for 3 days by changing half of the media every 2 days. Add DMH-1 (2 μ M) and SB431542 (2 μ M) to NIM medium to increase neural induction efficiency.

1.4. At D8, collect the iPSC aggregates after centrifugation at 200 x *g* for 2 min and let them adhere to 6 well plates (around 20–30 aggregates per well of the plate) by culturing in NIM with 10% FBS (or with laminin-coated plates) overnight. On the following day, remove the old medium and change to NIM every 2 days until D17.

NOTE: The generation of neuroepithelial cells, which is indicated by the formation of columnar cells with rosette structures, is observed in this period.

1.5. At D17, mechanically isolate the neuroepithelial cells in the center of colonies (lifting off directly by applying a small pressure to the center of the colonies) or enzymatically (treated

with dispase). Transfer the isolated neuroepithelial cells to non-treated tissue culture T25 flask containing 8 mL of NIM with addition of B27 (1x), cAMP (1 μ M), and IGF (10 ng/mL).

NOTE: The suspension culture allows cells to form neurospheres that are enriched with cortical projection neural progenitors.

1.6. After D35, plate the neurospheres on the poly-ornithine and LDEV-free reduced growth factor basement membrane matrix (**Table of Materials**)-coated 35 mm glass bottom dishes to generate telencephalic glutamatergic neurons (cortical projection neurons). Before plating, dissociate the neurospheres to small clusters by incubating with 1 mg/mL cell detachment solution for 2 min at 37 $^{\circ}$ C.

1.7. Remove the cell detachment solution by centrifugation and resuspend in 1 mL of neural differentiation medium (NDM). Plate cells on the glass bottom dish (around five clusters in 100 μ L of medium per dish) to let them attach. Then, add NDM (1 mL per dish) and culture the cells in NDM containing B27 (1x), cAMP (1 μ M), IGF (10 ng/mL), hBDNF (10 ng/mL), and GDNF (10 ng/mL).

NOTE: Small clusters are plated because they survive well and give rise to long projection neurons. Alternatively, cells can be dissociated and plated at a density around 20,000 cells per dish for better separation.

1.8. Characterize the telencephalic glutamatergic neurons by immunostaining the Tbr1 and β III-tubulin markers.

2. Examination of mitochondrial transport along axons of telencephalic glutamatergic neurons

2.1. Warm up the NDM and turn on the incubator for the fluorescence microscope set at 37 $^{\circ}$ C and 5% CO₂.

2.2. To visualize mitochondria along the axons of forebrain neurons, incubate the neurons with 50 nM red fluorescent dye to stain mitochondria in live cells (e.g., MitoTracker CMXRos) in NDM for 3 min at 37 $^{\circ}$ C. Next, wash cells 2x with warmed NDM. The neurons are incubated in NDM.

2.3. In order to record the mitochondrial transport along the axons, take time-lapse images of mitochondrial movement using the 40x objective with a fluorescence microscope.

NOTE: To stabilize the culture, perform the live cell imaging when the cells are incubated in the incubator for 20 min following mitochondrial staining.

2.4. Under phase field, identify the axons based on morphological characteristics (directly emerge from neuronal cell body, constant thin long neurites with no branching). Mitochondria

move in anterograde (from cell body to distal axon) and retrograde directions (from axonal terminal to cell body). To distinguish the direction of mitochondrial movement along axons, clearly identify the cell bodies of neurons. In this step, focus axons under phase field to reduce photobleaching.

2.5. After distinguishing the cell body and axon, adjust the exposure time and focus of mitochondria in axons. Then, capture the transport of mitochondria within axons every 5 s for a total duration of 5 min, yielding 60 frames. Randomly capture at least five locations for each dish and repeat 3x for each group.

3. Data analysis of mitochondrial transport and morphology in cortical neurons

NOTE: Analyze the collected data on mitochondrial transport using an image analysis software (e.g., ImageJ or MetaMorph¹⁹). Since ImageJ is readily available, perform the analysis of mitochondrial transport and morphology using ImageJ with the **MultiKymograph**, **Macros**, and **Analyze particles** plugins.

3.1. Analyzing mitochondrial transport using ImageJ

3.1.1. After capturing the time-lapse images of mitochondrial motility, analyze the velocity and movement of mitochondria using the **MultiKymograph** plugin. The images are saved as .tiff format files, which are analyzed by Fiji software following a previously reported method²⁰.

3.1.2. Download the Fiji software. Download plugins that will be needed for analyzing mitochondrial moving velocity from kymographs. Download the **Bio-formats Package** and **Kymograph Plugin** along with these four .class plugins, including: **MultipleKymograph.class**, **MultipleOverlay.class**, **StackDifference.class**, and **WalkingAverage.class** (**Table of Materials**). Move these files to the Fiji plugins folder. Download “tsp050706.txt” plugins to the plugins folder. Restart the Fiji software when the files are moved to the plugins folder.

3.1.3. Open Fiji software. Choose **plugins**, then import images of the .tiff series through **Bio-Formats Importer** under **Bio-Formats**. Make sure to choose **Standard ImageJ** in **Stack viewing**, choose **Open all series**, check **Autoscale**, then click **OK**. Take note of the frame number and size of images in pixels, which are displayed on the top of the image.

NOTE: Take 60 frames per image.

3.1.4. Consider making the images clearer by adjusting **brightness/contrast** under the **Image** menu for all 60 frames.

3.1.5. Right-click the line tool to choose **segmented line** and draw a segmented line starting from the cell body and ending at the terminal axon. Generate the kymograph by choosing **MultipleKymograph** under **Plugins**. The selection of line width is prompted after choosing the **MultipleKymograph**. Ensure that this is an odd number. Choose **1** for the line width. A

kymograph is generated after this step.

NOTE: Several important pieces of information can be read from the kymograph. The y-axis of kymograph is the time of duration (5 min) for the 60 frames. The x-axis represents the position of the selected axons.

3.1.6. Use a diagonal line in the kymograph to determine the movement direction of mitochondria (anterograde, retrograde, or stable). For example, a line going down to the right along the y-axis indicates anterograde movement, and a line going down to the left along the y-axis indicates retrograde movement. A vertical line indicates that there was no movement in the mitochondrion.

3.1.7. Measure the distance, time values, and velocity for the moving mitochondria using the **Macros** plugin in Fiji software. Go to **Plugins | Macros | Install | tsp050607.txt**. Draw a segmented line over the trace of mitochondrial movement on the kymograph, and always draw the line from the superior to inferior region (y-axis).

3.1.8. After drawing the line, go to **Plugins | Macros | read velocities from tsp**. Since a segmented line along the trace is drawn, the plugin reads segmented velocities corresponding to the line.

NOTE: The unit for all data is pixels. **dy sum** is the time consumed from the starting point, and **dx sum** indicates the distance of the mitochondrion moving in the x-axis. **dy now** and **dx now** shows the period time and movement distance for every segment, respectively. **actual speed** shows the speed for every segment ($dx\ now/dy\ now$). **average speed** indicates the average speed for the mitochondrial moving ($dx\ sum/dy\ sum$).

3.1.9. Change the unit of **dx now** from pixel to μm as the ratio of pixel in μm by measuring the scale bar. Convert the unit for distance from pixel to μm by measuring scale bars in images. Use the line tool to draw a line along the scale bar and measure the length of the line by choose **Measure' under "Analyze**. Change the time from pixel to seconds, as 1 pixel=5 seconds in this experiment.

3.1.10. In the spreadsheet file, calculate the average velocity and correspondingly label retrograde or anterograde movement. Average the anterograde or retrograde movement velocity.

3.1.11. Determine the percentage of stationary and motile mitochondria from the kymograph. The anterograde or retrograde moving mitochondria are defined as moving $5\ \mu m$ forward or backward from the origin during the entire period²¹. Mitochondria are considered stationary if they did not move more than $5\ \mu m$ during the 5 min.

3.2. Analysis of mitochondrial morphology using ImageJ

NOTE: Determine mitochondrial morphology by measuring mitochondrial length, area, and aspect ratio using ImageJ. To do so, follow the steps below.

3.2.1. In order to analyze the mitochondrial length and area within axons, download the **Straighten_.jar** plugin from ImageJ website (see **Table of Materials**) and move it to the folder of **plugins**. Restart ImageJ software.

3.2.2. Open the picture through the **open** function under **File** and convert the 32 bit image to 8 bit using **Type** under **Image**.

3.2.3. Draw a **segmented line** along the axons. Choose **Straighten** under **Plugins** and set 50 pixels for **Width of Filament/Wide Line**. This will generate a straightened axon.

3.2.4. Adjust the threshold under **Image** and set the measurement under **Analyze** by choosing "**Area | Perimeter | Fit ellipse | Shape descriptors**."

3.2.5. Measure the scale bar using the line function and set the scale under **Analyze** by filling the **distance in pixel**, **know distance**, and **the unit of length**. Then, choose **Global** to set this scale setting.

3.2.6. Use **Analyze Particles** under **Analyze** to determine the area. The prompt parameters are size (pixel²) = 0.20–infinity, circularity = 0.00–1.00, and show = ellipses. Choose **Display results**.

NOTE: The measurement will yield the results of multiple mitochondrial morphology parameters including area, perimeters, length (major), width (minor), and aspect ratio (AR). The corresponding mitochondrial number is also listed.

3.2.7. Calculate the mitochondrial number per axon (μm) using the mitochondrial number divided by axonal length.

REPRESENTATIVE RESULTS:

Here, human iPSCs were differentiated into telencephalic glutamatergic neurons, which were characterized by immunostaining with Tbr1 and βIII tubulin markers (**Figure 1A**). To examine the axonal transport of mitochondria, these cells were stained with red fluorescent dye, and time-lapse imaging was performed. Since ImageJ is readily available and easier to obtain, mitochondrial transport was further analyzed with the "MultiKymograph" and "Macros" ImageJ plugins, shown in **Figure 1**.

There are three respective states of mitochondrial motion, including static, anterograde, and retrograde movement (**Figure 1B,C**). A single mitochondrion can remain static or move in anterograde or retrograde direction within an axon, and here, a segmented line was drawn along the track of mitochondrial movement to determine velocity (**Figure 1D**). The velocity of

mitochondrial movement along the axon is shown in **Figure 1E** and corresponds to the segmented lines in **Figure 1D** after reading by “Macros” in ImageJ. Similar to the MetaMorph software, ImageJ can be used to determine the mitochondrial velocity and percentage of motile mitochondria based on the kymograph generated by ImageJ (**Figure 1F**). Using commercially available analysis software (e.g., MetaMorph), previous data showed that the percentage of motile mitochondria was significantly reduced in SPG3A neurons compared to normal neurons, while velocity was not altered¹⁹. To evaluate the ImageJ software, the percentage of motile mitochondria was examined, and a similar reduction in the percentage of motile mitochondria in SPG3A neurons compared to control wild-type (WT) neurons was observed (**Figure 1G**).

Regarding analysis of mitochondrial morphology, the mitochondrial area, length, and AR were analyzed using the “analyze particles” function in ImageJ. Axons were straightened using the “straighten” plugin (**Figure 2A,B**). Mitochondria were chosen clearly by adjusting the threshold (**Figure 2C,D**). Finally, the mitochondrial area, length (major), width (minor), aspect ratio, and perimeter were obtained from the straightened axon using the “Analyze Particles” plugin (**Figure 2E,F**). Abnormal mitochondrial morphology was (and has previously been) observed in HSP iPSC-derived telencephalic glutamatergic neurons, including SPG15 cells (**Figure 2G,H,I**)^{13,22}. Both the mitochondrial length and aspect ratio were significantly reduced in SPG15 neuron axons compared to control WT neuron axons (**Figure 2G,H,I**).

FIGURE AND TABLE LEGENDS:

Figure 1: Analysis of mitochondrial transport using ImageJ. (A) Immunostaining showing the generation of telencephalic glutamatergic neurons. Tbr1 (red), β III tubulin (green), and Hoechst (blue). Scale bars = 50 μ m. This figure has been modified from a previous study¹⁹. (B) Mitochondrial transport within axons of neurons. Anterograde mitochondrial movement is defined as the mitochondria moving from the cell body to distal axon, and retrograde mitochondrial movement originates from the distal axon and extends to the neuronal cell body. The segment line is drawn along axons from neuronal cell body to distal axonal terminal in order to generate kymographs. (C) Kymograph is generated using ImageJ; x-axis is the axonal position and (B) y-axis is time. One continuous white line is the mitochondrion moving in anterograde direction (pink arrowhead), retrograde direction (blue arrowhead), or static state (yellow arrowhead). (D) The segmented line is drawn to read the velocity using the Macros plugin. Numbers 1–8 describe the segment of the line. Anterograde movement (1–4, 6, 8) and static state (5, 7) can be distinguished from this magnified kymograph. (E) Time and moving distance for each segment, actual and average velocity (in pixels). (F) Kymograph of mitochondrial transport for WT and SPG3A cortical PNs using ImageJ. Scale bar = 10 μ m. (G) Motile mitochondrial ratio in WT and SPG3A cortical PNs using ImageJ (*p < 0.05).

Figure 2: Analyzing mitochondrial morphology using ImageJ. (A) Parameters for straightening the axon. (B) The representative straightened axon. (C,D) Adjustment of threshold to choose mitochondria. (E,F) The measured mitochondrial area, perimeter, length (major), width (minor), and aspect ratio (AR) corresponding to the selected mitochondria in (E). (G) Representative pictures of mitochondria in WT and SPG15 telencephalic glutamatergic neurons. Scale bars = 20

μm. (H) Mitochondrial length in WT and SPG15 neurons. (I) Aspect ratio of mitochondria in WT and SPG15 neurons (**p < 0.01) vs. WT. (G), (H), and (I) are modified from a previous study¹³.

Table 1: Advantages and disadvantages of some fluorescent tools for mitochondrial labeling.

DISCUSSION:

This article describes a method to analyze mitochondrial transport and morphology in neuronal axons using red fluorescent dye and ImageJ software, both of which provide a unique platform to study axonal degeneration and mitochondrial morphology in neurodegenerative disease. There are several critical steps in the protocol, including staining of mitochondria, live cell imaging, and analyzing the images. In this method, a fluorescent dye was used to stain mitochondria. Since human iPSC-derived neurons are easily detached from the dish, it is important to leave some solution in the dish and gently add neurobasal medium. The washing can be performed three or four times to remove the dye. In addition, mitochondria can be labeled with other reporters to measure their transport along axons, such as fluorescent protein fused mitochondria targeting proteins⁶. In long-term tracking, several probes (i.e., NPA-TPP, 2,1,3-benzothiadiazole [BTD] fluorescent derivatives, and a specific Raman probe) showed great potential in long-term mitochondrial tracking and mitochondrial dynamics analysis²³⁻²⁵. The advantages and disadvantages of various probes can be found in the **Table 1**.

After mitochondrial staining, live cell imaging is performed using a microscope equipped with an incubator. To effectively focus the neurons during imaging, neural samples should be kept in the 37 °C incubator with 5% CO₂ and in a humid environment for at least 15 min. To minimize the out-of-focus effects, images at different Z-positions can be taken to make a Z-stack, or the auto-focus function can be utilized. Importantly, to identify the direction of mitochondrial transport (anterograde or retrograde), phase images are taken to distinguish the neuronal cell body and axons. Another critical issue is photobleaching of fluorescent samples, which must be prevented to obtain efficient mitochondrial transport time-lapse images. An effective method to minimize photobleaching is to focus samples through the eyepiece and set all imaging parameters under the phase channel, except for exposure time. Moreover, the automatic scaling pattern can decrease photobleaching of fluorescence.

Mitochondria are highly dynamic organelles and can move in both anterograde and retrograde directions. In neurons, a few mitochondria within axons stay stationary during the recording. Among the moving mitochondria, motion status can vary over time. This phenomenon raises the important question of which mitochondria type is considered stationary or moving. This can be resolved by setting the threshold during mitochondrial axonal transport analysis. To distinguish the static mitochondria, Neumann et al. used the mitochondrial track center, which is defined as the mean of its position coordinates over time, then set the threshold to 350 nm/s so that the maximum deviation distance of the mitochondrion from its track center is in the first frame²⁶.

In another study, the authors set 50 nm/s as the threshold to distinguish stationary status from

the moving status²⁷. A 300 nm/s threshold was used here to distinguish the microtubule-based transport as done in previous reports^{28,29}. Although the threshold for stationary and moving mitochondria is different, setting the threshold can provide important relative information on mitochondrial movement within axons between wild-type and degenerative neurons.

Most protocols involving mitochondrial transport analysis have used kymograms, which are two-dimensional representation of positions versus time. Multiple automated tools have been developed for the analysis of particle tracking^{26,30-34}. These can accurately separate each frame. In addition to the velocity and motile percentage that ImageJ can measure, this method can measure motile events accurately. However, these are not free to use. Here, the analysis of mitochondrial transport was performed using ImageJ with the “Multikymograph” and “Macros” plugins. These plugins can effectively measure mitochondrial movement. The advantage of these plugins is their ease of use and ability to indicate alterations in mitochondrial axonal transport in the form of kymographs and velocity over time.

Motile mitochondria were analyzed in SPG3A and control neurons. A similar reduction in the percentage of motile mitochondria was observed using two different analysis methods, confirming the usefulness of ImageJ to analyze axonal transport. In addition, mitochondrial morphology can be analyzed using the same set of images, which provides important readouts for studying mitochondrial dysfunction in various neurological diseases. Since axonal degeneration and mitochondrial dysfunction usually occur during earlier stages, before neurons die, this method can be used to examine early pathological changes to help identify molecular pathways and screen therapeutics to rescue neurodegeneration.

ACKNOWLEDGMENTS:

This work was supported by the Spastic Paraplegia Foundation and the NIH (R21NS109837).

DISCLOSURES:

The authors declare no competing financial interests.

REFERENCES:

- 1 Brown, A. Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *Journal of Cell Biology*. **160** (6), 817-821 (2003).
- 2 Morris, R. L., Hollenbeck, P. J. Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons. *The Journal of Cell Biology*. **131** (5), 1315-1326 (1995).
- 3 Schwarz, T. L. Mitochondrial trafficking in neurons. *Cold Spring Harbor Perspectives in Biology*. **5** (6), (2013).
- 4 Magrane, J., Cortez, C., Gan, W. B., Manfredi, G. Abnormal mitochondrial transport and morphology are common pathological denominators in SOD1 and TDP43 ALS mouse models. *Human Molecular Genetics*. **23** (6), 1413-1424 (2014).
- 5 Alami, N. H. et al. Axonal transport of TDP-43 mRNA granules is impaired by ALS-causing mutations. *Neuron*. **81** (3), 536-543 (2014).
- 6 Wang, X., Schwarz, T. L. Imaging axonal transport of mitochondria. *Methods in*

441 *Enzymology*. **457**, 319-333 (2009).

442 7 Course, M. M. et al. Live Imaging Mitochondrial Transport in Neurons. *Neuromethods*.
443 **123**, 49-66 (2017).

444 8 Chazotte, B. Labeling mitochondria with fluorescent dyes for imaging. *Cold Spring*
445 *Harbor Protocols*. **2009** (6), pdb prot4948 (2009).

446 9 Meijering, E., Dzyubachyk, O., Smal, I. Methods for cell and particle tracking. *Methods in*
447 *Enzymology*. **504**, 183-200 (2012).

448 10 Bros, H., Hauser, A., Paul, F., Niesner, R., Infante-Duarte, C. Assessing Mitochondrial
449 Movement Within Neurons: Manual Versus Automated Tracking Methods. *Traffic*. **16**
450 (8), 906-917 (2015).

451 11 J. Rietdorf, A. S. <http://www.embl.de/eamnet/html/kymograph.html>. (2015).

452 12 Calkins, M. J., Manczak, M., Mao, P., Shirendeb, U., Reddy, P. H. Impaired mitochondrial
453 biogenesis, defective axonal transport of mitochondria, abnormal mitochondrial
454 dynamics and synaptic degeneration in a mouse model of Alzheimer's disease. *Human*
455 *Molecular Genetics*. **20** (23), 4515-4529 (2011).

456 13 Denton, K. et al. Impaired mitochondrial dynamics underlie axonal defects in hereditary
457 spastic paraplegias. *Human Molecular Genetics*. **27** (14), 2517-2530 (2018).

458 14 Kim-Han, J. S., Antenor-Dorsey, J. A., O'Malley, K. L. The parkinsonian mimetic, MPP+,
459 specifically impairs mitochondrial transport in dopamine axons. *Journal of Neuroscience*.
460 **31** (19), 7212-7221 (2011).

461 15 Shirendeb, U. P. et al. Mutant huntingtin's interaction with mitochondrial protein Drp1
462 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic
463 degeneration in Huntington's disease. *Human Molecular Genetics*. **21** (2), 406-420
464 (2012).

465 16 Lo Giudice, T., Lombardi, F., Santorelli, F. M., Kawarai, T., Orlacchio, A. Hereditary spastic
466 paraplegia: clinical-genetic characteristics and evolving molecular mechanisms.
467 *Experimental Neurology*. **261**, 518-539 (2014).

468 17 Blackstone, C. Cellular pathways of hereditary spastic paraplegia. *Annual Review of*
469 *Neuroscience*. **35**, 25-47 (2012).

470 18 Boisvert, E. M., Denton, K., Lei, L., Li, X. J. The specification of telencephalic
471 glutamatergic neurons from human pluripotent stem cells. *Journal of Visualized*
472 *Experiments*. 10.3791/50321 (74), (2013).

473 19 Zhu, P. P., Denton, K. R., Pierson, T. M., Li, X. J., Blackstone, C. Pharmacologic rescue of
474 axon growth defects in a human iPSC model of hereditary spastic paraplegia SPG3A.
475 *Human Molecular Genetics*. **23** (21), 5638-5648 (2014).

476 20 Marra, M. H., Tobias, Z. J., Cohen, H. R., Glover, G., Weissman, T. A. In Vivo Time-Lapse
477 Imaging in the Zebrafish Lateral Line: A Flexible, Open-Ended Research Project for an
478 Undergraduate Neurobiology Laboratory Course. *Journal of Undergraduate*
479 *Neuroscience Education*. **13** (3), A215-224 (2015).

480 21 Kang, J. S. et al. Docking of axonal mitochondria by syntaphilin controls their mobility
481 and affects short-term facilitation. *Cell*. **132** (1), 137-148 (2008).

482 22 Mou, Y., Li, X. J. Rescue axonal defects by targeting mitochondrial dynamics in
483 hereditary spastic paraplegias. *Neural Regeneration Research*. **14** (4), 574-577 (2019).

484 23 Huang, S. et al. New photostable naphthalimide-based fluorescent probe for

- mitochondrial imaging and tracking. *Biosensors & Bioelectronics*. **71**, 313-321 (2015).
- 24 Carvalho, P. H. et al. Designed benzothiadiazole fluorophores for selective mitochondrial imaging and dynamics. *Chemistry*. **20** (47), 15360-15374 (2014).
- 25 Yamakoshi, H. et al. A sensitive and specific Raman probe based on bisarylbutadiyne for live cell imaging of mitochondria. *Bioorganic & Medicinal Chemistry Letters*. **25** (3), 664-667, (2015).
- 26 Neumann, S., Chassefeyre, R., Campbell, G. E., Encalada, S. E. KymoAnalyzer: a software tool for the quantitative analysis of intracellular transport in neurons. *Traffic*. **18** (1), 71-88 (2017).
- 27 Chen, M. et al. A new method for quantifying mitochondrial axonal transport. *Protein & Cell*. **7** (11), 804-819 (2016).
- 28 De Vos, K. J., Sheetz, M. P. Visualization and quantification of mitochondrial dynamics in living animal cells. *Methods in Cell Biology*. **80**, 627-682 (2007).
- 29 Denton, K. R., Xu, C. C., Li, X. J. Modeling Axonal Phenotypes with Human Pluripotent Stem Cells. *Methods in Molecular Biology*. **1353**, 309-321 (2016).
- 30 Andrews, S., Gilley, J., Coleman, M. P. Difference Tracker: ImageJ plugins for fully automated analysis of multiple axonal transport parameters. *Journal of Neuroscience Methods*. **193** (2), 281-287 (2010).
- 31 Reis, G. F. et al. Molecular motor function in axonal transport in vivo probed by genetic and computational analysis in *Drosophila*. *Molecular Biology of the Cell*. **23** (9), 1700-1714 (2012).
- 32 Broeke, J. H. et al. Automated quantification of cellular traffic in living cells. *Journal of Neuroscience Methods*. **178** (2), 378-384 (2009).
- 33 Welzel, O., Knorr, J., Stroebel, A. M., Kornhuber, J., Groemer, T. W. A fast and robust method for automated analysis of axonal transport. *European Biophysics Journal : EBJ*. **40** (9), 1061-1069 (2011).
- 34 Mukherjee, A. et al. Automated kymograph analysis for profiling axonal transport of secretory granules. *Medical Image Analysis*. **15** (3), 354-367 (2011).
- 35 Klionsky, D. J. et al. Guidelines for the use and interpretation of assays monitoring autophagy. *Autophagy*. **12**, 1-222 (2016).
- 36 Metivier, D. et al. Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. *Immunology Letters*. **61** (2-3), 157-163 (1998).
- 37 Scaduto, R. C., Jr., Grotyohann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysical Journal*. **76** (1 Pt 1), 469-477 (1999).
- 38 Liu, X., Yang, L., Long, Q., Weaver, D., Hajnoczky, G. Choosing proper fluorescent dyes, proteins, and imaging techniques to study mitochondrial dynamics in mammalian cells. *Biophysics Reports*. **3** (4), 64-72 (2017).
- 39 Zhou, B., Lin, M. Y., Sun, T., Knight, A. L., Sheng, Z. H. Characterization of mitochondrial transport in neurons. *Methods in Enzymology*. **547**, 75-96 (2014).

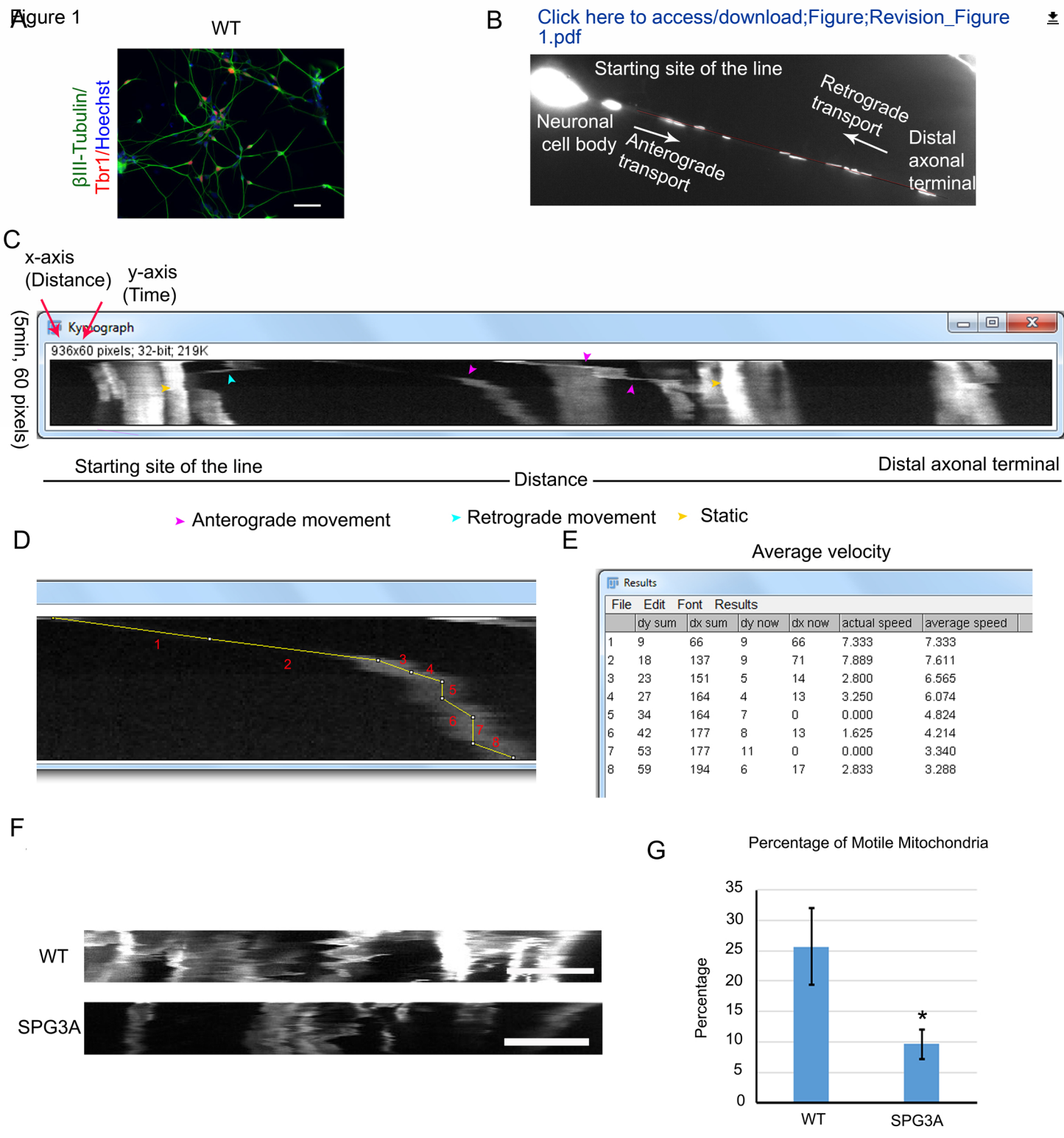
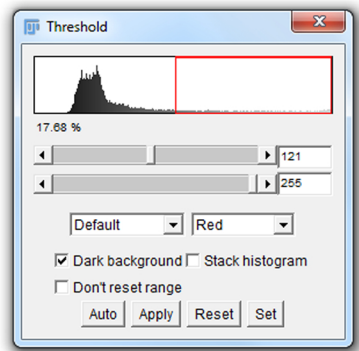


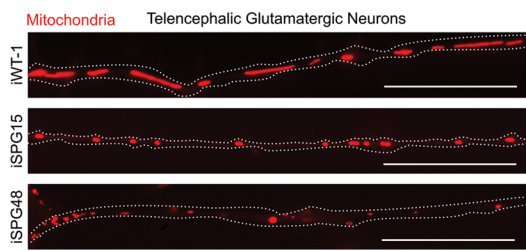
Figure 2



C



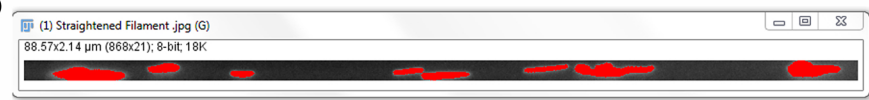
G



B



D



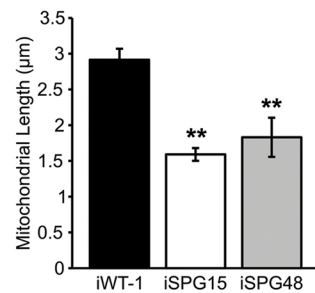
E



F

Results							
File	Edit	Font	Results				
	Area	Perim.	Major	Minor	Angle	Circ.	AR
1	1.260	7.038	3.327	0.482	2.180	0.320	6.899
2	1.135	5.321	2.531	0.571	179.360	0.504	4.432
3	6.456	15.723	6.903	1.191	0.956	0.328	5.798
4	1.416	6.774	3.353	0.538	2.038	0.388	6.234
5	5.362	12.323	5.425	1.258	177.437	0.444	4.311
6	6.081	18.918	8.085	0.958	0.070	0.214	8.442
7	1.645	9.631	4.845	0.432	4.503	0.223	11.206
8	2.561	10.955	5.058	0.645	179.747	0.268	7.845

H



I

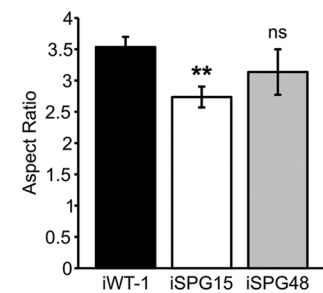


Table 1: Advantages and disadvantages of some fluorescent tools for mitochondrial labeling

Fluorescent Tools	Advantages	Disadvantages	References
MitoTracker	MitoTracker is mitochondrial potential-independent and can be used to analyze the colocalization of mitochondria with autophagosome or lysosome.	MitoTracker is not photostable enough for long-term research.	35
NPA-TPP	This dye is a novel photostable mitochondrial labeling reagent.	The synthesis and purification process of this dye is time-consuming and costly.	23
MitoBADY	This dye can be used for mitochondrial visualization using Raman microscopy with high sensitivity and specificity.	Using this dye requires Raman microscope.	25
TMRE	This dye is a non-toxic, specific mitochondrial staining dye with low concentration and no quenching effect.	TMRE labeling mitochondria depends on the mitochondrial membrane potential.	36, 37
Mitochondria-targeted fluorescent proteins	Mitochondria-targeted fluorescent proteins are more specific and stable.	This method needs transfection and transfection efficiency is different for different cell types.	38, 39

Name of Material/ Equipment	Company	Catalog Number
Accutase Cell Detachment Solution	Innovative Cell Technologies	AT104
Biosafety hood	Thermo Scientific	1300 SERIES A2
Bovine serum albumin (BSA)	Sigma	A-7906
Brain derived neurotrophic factor (BDNF)	Peprotech	450-02
Centrifuge	Thermo Scientific	Sorvall Legend X1R/ 75004261
Coverslips	Chemiglass Life Sciences	1760-012
Cyclic AMP (cAMP)	Sigma-Aldrich	D0627
Dispase	Gibco	17105-041
Dorsomorphin	Selleckchem	S7146
Dulbecco's modified eagle medium with F12 nutrient mixture (DMEM/F12)	Corning	10-092-CV
FBS	Gibco	16141-002
Fibroblast growth factor 2 (FGF2, bFGF)	Peprotech	100-18B
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Gibco	A1413201
Gem21 NeuroPlex Serum-Free Supplement	Gemini	400-160
Glass Bottom Dishes	MatTek	P35G-0.170-14-C
9" glass pipetes	VWR	14673-043
Glial derived neurotrophic factor (BDNF)	Sigma-Aldrich	D0627
GlutaMAX-I	Gibco	35050-061
Heparin	Sigma	H3149
Insulin growth factor 1 (IGF1)	Invitrogen	M7512

Knockout Serum Replacer	Gibco	A31815
Laminin	Sigma	L-6274
2-Mercaptoethanol	Sigma	M3148-100ML
MitoTracker CMXRos	Invitrogen	M7512
Neurobasal medium	Gibco	21103-049
Non Essential Amino Acids	Gibco	11140-050
N2 NeuroPle Serum-Free Supplement	Gemini	400-163
Olympus microscope IX83	Olympus	IX83-ZDC2
PBS	Corning	21-031-CV
Phase contrast microscope	Olympus	CKX41/ IX2-SLP
6 well plates	Corning	353046
24 well plates	Corning	353047
Poly-L-ornithine hydrobromide (polyornithine))	Sigma-Aldrich	P3655
SB431542	Stemgent	04-0010
Sterile 50ml Disposable Vacuum Filtration System 0.22 µm Millipore Express® Plus Membrane	Millipore	SCGP00525
Stericup 500/1000 ml Durapore 0.22 µm PVDF	Millipore	SCGVU10RE
Tbr1 antibody (1:2000)	Chemicon	AB9616
Trypsin inhibitor	Gibco	17075029
50 ml tubes	Phenix	SS-PH50R
15 ml tubes	Phenix	SS-PH15R
T25 flasks (untreated)	VWR	10861-572
Plugins for softwares		
Bio-formats Package	http://downloads.openmicroscopy.org/bio-formats/5.1.0/	

Fiji software	https://fiji.sc/	
Kymograph Plugin	https://www.embl.de/eamnet/html/body_kymograph.html	
MultipleKymograph.class	https://www.embl.de/eamnet/html/body_kymograph.html	
MultipleOverlay.class	https://www.embl.de/eamnet/html/body_kymograph.html	
WalkingAverage.class	https://www.embl.de/eamnet/html/body_kymograph.html	
StackDifference.class	https://www.embl.de/eamnet/html/body_kymograph.html	
Straighten_.jar	https://imagej.nih.gov/ij/plugins/straighten.html	
tsp050706.txt	https://www.embl.de/eamnet/html/body_kymograph.html	



Dr. Xue-Jun Li, Associate Professor
 Department of Biomedical Sciences
 Regenerative Medicine & Disability Research Lab
 College of Medicine at Rockford
 Department of Bioengineering
 University of Illinois at Chicago
 Phone: 815-395-5882
 Email: xjli23@uic.edu

September 29, 2019

Dear Dr. Bajaj,

We would like to thank you and the reviewers for the positive feedback and constructive suggestions, which were helpful for improving our manuscript. We have addressed the editorial comments and reviewers' concerns in the revised manuscript, as detailed in the following section and the manuscript file (major changes are labelled in blue color in the manuscript file).

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- The spelling and grammars have been checked and corrected in the text.

2. Please provide an email address for each author.

- The email address has been added.

3. Please provide at least 6 keywords or phrases.

- 6 Keywords have been provided.

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

- Formatted.

5. The current Long Abstract is over the 150-300-word limit. Please rephrase the Long Abstract to more clearly state the goal of the protocol

- The goal of the protocol has been added. We have 212 total words for the long abstract.

6. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

- Yes, it is within the word limit.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

- These have been checked.

8. Please revise the Introduction to include all of the following parts:

- The introduction has been modified.

9. Please move the hyperlinked text to the reference section or materials table.

- Corrected.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

- This has been adjusted.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

- These have been modified.

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

- Modified

13. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

- The related info has been added.

14. 2: please include how the steps are performed. Please include all the button clicks in the software, all the knob turns etc. How do you distinguish the cell body?

- The cell bodies were distinguished by the corresponding phase image that was taken under a microscope.

15. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

- Revised.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- These have been highlighted.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- Per the policy of the Journal (Human Molecular Genetics), "Copyright of any article published in HMG will belong to the author or their designee". "As part of the licence agreement, authors may use their own material in other publications provided that the Journal is acknowledged as the

original place of publication and Oxford University Press as the Publisher". We have attached the related policy and also acknowledged the Journal and the publisher in the manuscript.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- This has been revised.

19. Please do not abbreviate the journal titles in the references section.

- Corrected.

20. Please sort the materials table in alphabetical order.

- We have updated the table.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The article describes a method to analyze mitochondrial axonal transport and morphology using Fiji software with several available plugins including MultiKymograph, Bioformat importer and Macros. This method can make it easy to analyze mitochondrial transport and morphology in an automatic way, and will be useful for studying mitochondrial deficits in neurodegenerative diseases.

Major Concerns:

The method is described in detail, however, **the efficacy of the method is not well demonstrated**. In figure 1, **the results of mitochondrial movement and velocity are missing**, eg **the percentage of motile mitochondria versus static mitochondria**, and **the frequency histogram of mitochondrial velocity**. It is not shown whether there is any difference of mitochondrial transport between WT and HSP iPSC-derived neuron that can be detected using this method. In both figure 1 and figure 2, to show the efficacy of this method, the results should be compared to manual analysis and other automatic method for example metamorph.

- We thank the reviewer for bringing up this critical point. Using MetaMorph analysis, our previous work showed a significant reduction in the percentage of motile mitochondrial in SPG3A neurons compared with normal neurons. The velocity did not change in SPG3A neurons, suggesting that the transport machinery may not be changed. Therefore, to evaluate the ImageJ software, we examined the percentage of motile mitochondria and found a similar reduction in SPG3A neurons, confirming the effectiveness of this method. As for the analysis of mitochondrial morphology, our published paper used the ImageJ for analysis and found a significant alteration in mitochondrial length and aspect ratio in SPG3A neurons compared with control neurons (Fig.2), confirming the effectiveness of the ImageJ method.

Minor Concerns:

It would be better to test this analysis on live imaging of mitochondrial tagged fluorescent protein and living imaging of mitochondrial dye, and see whether it works well in both cases and how is the difference.

-This is a good suggestion. Though we have not used other probes, they have been used by other studies. We have cited the related references in Table 1.

Reviewer #2:

Manuscript Summary:

How to visualize and quantify mitochondrial transport in hiPSC-derived neurons

Major Concerns:

None

Minor Concerns:

Almost nothing is said about the pros and cons of different mitotrackers or other fluorescent dyes for mitochondria such as TMRE or tagged protein expression. Each has advantages and disadvantages. Some are specific for healthy mitochondria, for example, and are sensitive to pH. I would insist that the authors add a substantial paragraph and a table on pros and cons of the various choices. It would be so helpful to have that coalesced in one place.

-Thanks for the great suggestion. We have discussed this and added a table to the revised manuscript.

Reviewer #3:

The authors, Drs. Mou Y. et al., described a protocol for analyzing mitochondrial transport and mitochondrial morphology in human induced pluripotent stem cells (iPSCs) derived forebrain neuron axons. This protocol can characterize mitochondrial trafficking along axons and analyze their morphology to facilitate the study of neurodegenerative diseases.

The paper contains interesting novel findings, although it is too long and redundant in some parts. Peripheral material should be deleted. Finally, the quality of written English should be meliorated.

- We thank the reviewer for these good points. We have re-organized the introduction and discussion to make them more concise. The English has also been checked.

HUMAN MOLECULAR GENETICS

Instructions to authors

Please note that HMG now encourages authors to complete their copyright licence to publish form online.

[Open Access Option for Authors](#)

Please read these instructions carefully and follow them strictly to ensure that the review and publication of your paper is as efficient and quick as possible. The Editors reserve the right to return manuscripts that are not in accordance with these instructions.

All material to be considered for publication in *Human Molecular Genetics* should be submitted in electronic form via the [journal's online submission system](#). Once you have prepared your manuscript according to the instructions below, instructions on how to submit your manuscript online can be found by [clicking on this page](#).

[Scope](#)

[Submission](#)

[Format-free submission](#)

[USA editorial office](#)

[UK editorial office](#)

[Review of manuscripts](#)

[Preparation of manuscripts](#)

[Accepted manuscripts](#)

[HMG advance access](#)

[Policy](#)

[Copyright](#)

[Proofs](#)

[Skip to Main Content](#)

[Open Access option for authors](#)

It is understood that by publishing a paper in Human Molecular Genetics, the authors agree to make available to colleagues in academic (non-profit) research any of the cells, nucleic acids, antibodies, etc. that are not available from commercial suppliers and are required to substantiate the scientific conclusions of the paper.

Copyright

Copyright of any article published in HMG will belong to the author or their designee.

However, it is a condition of publication in the journal that authors grant an exclusive licence to publish to Oxford University Press. This ensures that requests from third parties to reproduce articles are handled efficiently and consistently and allows the article to be as widely disseminated as possible. As part of the licence agreement, authors may use their own material in other publications provided that the Journal is acknowledged as the original place of publication and Oxford University Press as the Publisher. Upon receipt of accepted manuscripts at Oxford Journals authors will be invited to complete an online copyright licence to publish form.

Authors are reminded that it is their responsibility to comply with copyright laws. It is essential to ensure that no part of the text or illustrations have appeared or are due to appear in other publications, without prior permission from the copyright holder. Signed patient consent forms must be obtained for recognizable photographs. Submission of the manuscript will be taken to indicate the authors compliance with these conditions.

Proofs

Authors will receive a PDF file of the complete paper by email. In the interest of speed, corrections must be returned within 48 hours.

Open Access Option For Authors

HMG authors have the option to publish their paper [open access](#); whereby, for a charge, their paper will be made freely available online immediately upon publication. After your manuscript is accepted the corresponding author will be required to accept a mandatory licence to publish agreement. As part of the licensing process you will be asked to indicate whether or not you wish to pay for open access. If you do not select the open access option, your paper will be published with standard subscription-based access and you will not be charged.

[Skip to Main Content](#)

Open access articles are published under Creative Commons licences. Authors publishing in HMG can use the following Creative Commons licences for their articles:

- [Creative Commons Attribution licence \(CC BY\)](#)
- [Creative Commons Non-Commercial licence \(CC BY-NC\)](#)

Visit the OUP licensing website to find out more about [Creative Commons licences](#).

You can pay Open Access charges using our Author Services site. This will enable you to pay online with a credit/debit card, or request an invoice by email or post. The open access charges applicable are: £2625 / \$3665 / €3036.

Please note: the Open Access charge reflects the cost of publishing your paper Open Access in HMG, so colour figures are now included and additional charges will not apply for authors publishing under an open access license.

Orders from the UK will be subject to the current UK VAT charge. For orders from the rest of the European Union, OUP will assume that the service is provided for business purposes. Please provide a VAT number for yourself or your institution, and ensure you account for your own local VAT correctly.

Reuse of Open Access Content

Once published under the open access model, this article will be distributed under the terms of the [Creative Commons Attribution Non-Commercial Licence](#) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Analyzing mitochondrial transport and morphology in human induced pluripotent stem cells-derived neurons in hereditary spastic paraplegia

Author(s):

Yongchao Mou, Sukhada Mukte, Xue-Jun Li

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

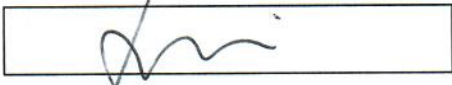
the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Xue-Jun Li	
Department:	Department of Biomedical Sciences	
Institution:	University of Illinois	
Title:	Associate Professor	
Signature:		Date: 7/10/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140