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## Assessment of Mitochondrial Trafficking as a Surrogate for Fast Axonal Transport in Human Induced Pluripotent Stem Cell–Derived Spinal Motor Neurons

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### Abstract

Axonal transport is essential for the development, function, and survival of the nervous system. In an energy-demanding process, motor proteins act in concert with microtubules to deliver cargoes, such as organelles, from one end of the axon to the other. Perturbations in axonal transport are a prominent phenotype of many neurodegenerative diseases, including amyotrophic lateral sclerosis. Here, we describe a simple method to fluorescently label mitochondrial cargo, a surrogate for fast axonal transport, in human induced pluripotent stem cell–derived motor neurons. This method enables the sparse labeling of axons to track directionality of movement and can be adapted to assess not only the cell autonomous effects of a genetic mutation on axonal transport but also the cell non-autonomous effects, through the use of conditioned medium and/or co-culture systems.

**Key words** Axonal transport, Mitochondria, Motor neuron, Human induced pluripotent stem cell

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### 1 Introduction

Axonal transport is a critical energy demanding cellular process describing the movement of organelles, vesicles, and macromolecules toward (retrograde) or away from (anterograde) the soma. Neurons, by virtue of their extraordinarily elongated polarization, are especially dependent on long-range cytoskeletal transport, and defective axonal transport has been implicated in the pathogenesis of a myriad age-related neurodegenerative diseases, including amyotrophic lateral sclerosis [1].

Here, we describe a simple method to fluorescently label mitochondrial cargo, a surrogate for fast axonal transport, in human induced pluripotent stem cell–derived motor neurons [2]. This method allows for sparse labeling of axons to track directionality of movement and can be adapted to assess not only the cell autonomous effects of a genetic mutation on axonal transport but also

the cell non-autonomous effects, through the use of conditioned medium and/or co-culture systems.

We first describe a method for the generation of spinal (lower) motor neurons (MNs) from human induced pluripotent stem cells (iPSCs) using an established protocol [3] with minor modifications, yielding a highly enriched and electrophysiologically active neuronal culture, devoid of glia, with circa 60% of cells being positive for islet-1 and islet-2 homeobox MN markers 1 week post plating [4]. We then describe a method for sparse labeling of neuronal mitochondria [2], allowing for the quantification of various parameters such as pausing, and speed, but also directionality of movement. It is thus advantageous over dye-based methods, which label all cells, such as MitoTracker [5].

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## 2 Materials

Prepare all solutions using sterile technique in a tissue culture hood.

### 2.1 Maintenance of Human Induced Pluripotent Stem Cells (iPSCs)

1. Matrigel Growth Factor Reduced Basement Membrane Matrix-coated plastic dishes (*see Note 1*).
2. Essential 8 medium (Gibco; A1517001) at 37 °C and 5% CO<sub>2</sub>.
3. iPSCs are passaged weekly using a 1:1 mixture of 1 mg/ml dispase and 2 mg/ml collagenase.

### 2.2 Motor Neuron (MN) Differentiation

1. Incubator set at 37 °C, 5% CO<sub>2</sub> with a mini orbital shaker (cat-code: 10309644)(*see Note 2*).
2. 9-cm nonadherent Petri dishes.
3. Accutase for single-cell dissociation of iPSCs.
4. Recommended stock concentrations of critical factors for aliquoting and storing at –20 °C, with freeze–thaw cycles minimized (*see Note 3*): 10 mM Activin inhibitor SB-431542 (AI) in DMSO, 50 mM ascorbic acid (light sensitive), 10 µg/ml recombinant human brain-derived neurotrophic factor (BDNF; we recommend cat code: 248-BDB) diluted in PBS + 0.1% BSA (carrier protein), 3 mM CHIR-99021 in DMSO, 10 µg/ml recombinant human ciliary neurotrophic factor (CNTF; we recommend cat code: 257-NT) diluted in PBS + 0.1% BSA (carrier protein), 10 mM DAPT in DMSO, 5 mg/ml DNase in 0.15 M NaCl (we recommend cat code: DN25, Sigma), 20 µg/ml FGF-2 (basic) diluted in PBS + 0.1% BSA (carrier protein), 10 µg/ml recombinant human glial-derived neurotrophic factor (GDNF; we recommend cat code: 212-GD) diluted in PBS + 0.1% BSA (carrier protein), 20 µg/ml recombinant human insulin-like growth factor-1 (IGF-1) diluted in PBS + 0.1% BSA (carrier protein), 100 µM

LDN-193189 in DMSO (light sensitive), 1 mM retinoic acid (RA; all trans) in DMSO (light sensitive), 1 mM smoothened agonist (SAG, light sensitive), 10 mM Y-27632 (ROCK inhibitor, RI).

5. MN Differentiation medium (“MN-Diff-Base”): 0.5× Advanced DMEM/F12, 0.5× Neurobasal medium, 1× Antibiotic–Antimycotic, 1× Glutamax, 100 μM beta-mercaptoethanol, 1× N-2 supplement, 1× B-27 supplement, 10 μM ascorbic acid. Filter units should be used and volumes adjusted accordingly to last no more than 7–10 days. Protect from light by covering with aluminum foil. Store at 4 °C and allow medium to naturally warm up toward ambient room temperature before using to feed cells.
6. Day 0 medium: MN-Diff-Base + 3i + 10 ng/ml FGF-2 + 10 μM RI, where “3i” refers to 20 μM SB-431542, 0.1 μM LDN-193189, and 3 μM CHIR-99021 (*see Note 4*).
7. Day 2/4 medium: MN-Diff-Base + 3i + 0.1 μM RA + 500 nM SAG (*see Note 5*).
8. Day 7 medium: MN-Diff-Base + 0.1 μM RA + 500 nM SAG + 10 ng/ml BDNF.
9. Day 9/11 medium: Day 7 medium + 10 μM DAPT.
10. Day 14 medium: MN-Diff-Base + 0.1 μM RA + 10 ng/ml BDNF + 10 μM DAPT + 10 ng/ml GDNF.

### **2.3 Motor Neuron Plating and Maintenance**

1. Cell strainers (40 μm).
2. μ-slide 8-well ibidi dishes (*see Note 6*).
3. Coating (store at –20 °C): poly-L-ornithine bromide, laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, fibronectin human plasma, and Matrigel.
4. Wash buffer: 1× DPBS (calcium and magnesium free), 0.45% glucose, 0.1% BSA, 2 mM MgCl<sub>2</sub>, 0.8 mM EDTA, 2.5% fetal bovine serum (FBS), 50 μg/ml DNase, 1× B-27 supplement, 1× N-2 supplement. Store at 4 °C and it can be prepared up to 1 week prior to MN dissociation (with DNase, B-27 and N-2 supplements being added to the buffer immediately prior to its use in the MN dissociation procedure).
5. Trypsin + DNase solution: 3 ml of 0.05% trypsin-EDTA (*see Note 7*) + 200 μl DNase (5 mg/ml stock; *see Note 8*) per 9-cm Petri dish.
6. “Stop” solution: FBS + DNase (3 ml per plate): 3 ml FBS + 60 μl DNase per 9-cm Petri dish.
7. MN Neurotrophic Factor medium (‘MN-NF’): 1× Neurobasal medium, 1× anti-anti/PSF, 1× MEM Non-Essential Amino Acids Solution, 1× Glutamax, 1× B-27 supplement, 1× N-2

supplement, 100  $\mu\text{M}$  beta-mercaptoethanol, 2.5  $\mu\text{M}$  ascorbic acid, 1  $\mu\text{M}$  retinoic acid, 10 ng/ml recombinant human BDNF, 10 ng/ml recombinant human CNTF, 10 ng/ml recombinant human GDNF, and 10 ng/ml recombinant human IGF-1. Filter units should be used and volumes adjusted accordingly to last no more than 7–10 days. Protect from light by covering with aluminum foil. Store at 4 °C and allow medium to naturally warm toward ambient room temperature before using to feed cells.

8. 25  $\mu\text{M}$  L-glutamic acid (GluE) and 1  $\mu\text{M}$  uridine/5-fluoro-2'-deoxyuridine (U/FDU), stored at  $-20$  °C, are added to MN-NF in the initial phases of MN culture (L-glutamic acid just at platedown).

### **2.4 Mitochondrial Labeling with Mito-DsRed2**

1. To label individual mitochondria in a variable proportion of MNs, a lentivirus expressing a mitochondrial targeting sequence fused to the N-terminal region of DsRed2 protein under CMV promoter should be generated. Lentiviral titers for each batch should be empirically calculated.
2. Phenol red-free Neurobasal medium to address the high visible light absorption extinction coefficient of phenol red and thus increase the signal-to-noise ratio by minimizing the source of autofluorescence (although this is not essential for imaging DsRed2 when compared to imaging GFP/cyan spectrum, as phenol red is particularly fluorescent when excited at 440 nm).
3. A live-imaging compatible widefield microscope with Cy3 filter set, a 63 $\times$  oil 1.4 N.A. objective, and an incubation chamber maintained at 37 °C and 5% CO<sub>2</sub>.
4. Fiji software [6] for image analysis.

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## **3 Methods**

### **3.1 Motor Neuron Differentiation from Human iPSCs (Summarized Workflow in Table 1)**

1. Dissociate iPSCs into single cells using Accutase, and neuralize as a suspension culture using “3i” providing dual-SMAD inhibition (via SB-431542 and LDN-193189) and potentiation with the Wnt-agonist (CHIR-99021) in “Day 0” medium.
  - (a) Add 1 ml of Accutase per well (iPSCs at ~80% confluency) and incubate at 37 °C for no longer than 5–10 min. Keep checking until condensed iPSC colonies start to loosen. Pipette up and down to remove difficult areas.
  - (b) During the incubation time, prepare 15 ml Falcon tubes by labeling tubes and adding 2–3 ml of PBS.
  - (c) Add 1 ml of PBS to each well (to dilute Accutase 1:1) and pipette cells up and down to break up the colonies into

**Table 1**

**Summary timeline and feeding schedule from iPSC to MN. “3i” refers to SB-431542, LDN-193189, and CHIR-99021. OCT4 is an iPSC marker; OLIG2 is a marker of the pMN domain that gives rise to MNs (and oligodendrocytes); and HB9 is a marker of spinal motor neurons**

Day	Medium for feeding	Marker	
Day 0	MN-diff-base +3i+FGF+RI (single-cell iPSC dissociation with Accutase)	OCT4	3i + FGF+RI Day 0–1
Day 1	No feeding		
Day 2	MN-diff-base +3i +RA+SAG		3i+ RA+SAG Day 2–6
Day 3	No feeding		
Day 4	MN-diff-base +3i +RA+SAG		
Day 5	No feeding		
Day 6	No feeding		
Day 7	MN-Diff Base +RA+SAG+BDNF		RA+SAG+BDNF Day 7–8
Day 8	No feeding		
Day 9	MN-Diff Base +RA+SAG+BDNF+DAPT	OLIG2	RA + SAG + BDNF Day 9–13
Day 10	No feeding		+DAPT
Day 11	MN-Diff Base +RA+SAG+BDNF+DAPT	HB9	
Day 12	No feeding		
Day 13	No feeding		
Day 14	MN-Diff Base +RA+BDNF+DAPT+GDNF		RA+BDNF+DAPT Day 14–16
Day 15	No feeding		+GDNF
Day 16	Dissociation and platedown		

single cells (N.B. cells that form thread-like structures are dying).

- (d) Add the cells to the prepared 15-ml Falcon tubes.
  - (e) Centrifuge at 400 rcf for 4 min.
  - (f) Aspirate supernatant and add 5 ml of PBS, resuspend and centrifuge again at 400 rcf for 4 min.
  - (g) Aspirate supernatant and add an appropriate volume of “Day 0” medium depending on the size of the pellet (1–3 ml).
  - (h) Count cells and plate  $2 \times 10^6$  cells per 9-cm Petri dish in “Day 0” medium.
  - (i) Place labeled Petri dish on a mini orbital shaker set at 50 rpm within an incubator set at 37 °C, 5% CO<sub>2</sub>.
2. On day 1, check plate to see if small embryoid bodies (EBs) have formed. If there are only single cells, then there is likely a problem. If EBs are already forming big clusters visible by eye, then decrease the starting cell number (from  $2 \times 10^6$  cells) or make sure you spread the cells in the dish when you place the dish back on the shaker in the incubator.
  3. On day 2, neural spheres are simultaneously patterned to spinal cord identity by treating with RA and SAG, promoting

caudalization and ventralization, respectively, along with 3i for an additional 5 days.

- (a) Gently swirl Petri dish to collect EBs (spheres) into the middle of the plate.
  - (b) Transfer spheres into 15-ml Falcon tube (*see Note 9*).
  - (c) Re-use plates by washing petri dish with PBS, aspirate PBS from the Petri dish and lid if any condensation has formed (*see Note 10*).
  - (d) Centrifuge at 200 rcf for 4 min (*see Note 11*).
  - (e) Add 10 ml of “Day 2/4” medium using a 10-ml pipette to gently dislodge the pellet and add to Petri dish. Care should be taken not to dissociate into single cells.
  - (f) Re-place the Petri dish on the mini orbital shaker set at 50 rpm within the incubator (*see Note 12*).
  - (g) On day 4, repeat the above, but it may now be possible to swirl the plate so as to move the spheres into the center of the plate and then aspirate the surrounding medium without the need for collecting the cells in a Falcon and centrifuging (which is more disruptive).
4. On day 7, spheres are maintained in culture with “Day 7” medium containing RA and SAG, with the addition of BDNF, to generate MN progenitors. By this stage, all feeding should be done by swirling the plate and aspirating the surrounding medium and replacing with 10 ml of fresh medium.
  5. From day 9, MN progenitors are cultured in “Day 9/11” medium, which has DAPT, an inhibitor of Notch signaling (*see Note 13*), for an additional two feeds (day 9 and day 11). If the medium in the plates is yellowing prior to the day of feeding, we recommend splitting the plate into two plates (each fed with 10 ml of medium).
  6. At day 14, the spheres should be fed with “Day 14” medium, which has GDNF, but lacks SAG (which is now no longer needed).

**3.2 Motor Neuron  
Dissociation  
and Platedown (from  
Day 16 Onwards, See  
Note 14)**

1. At day 14, pre-treat  $\mu$ -slide eight-well ibidi dishes with 100  $\mu\text{g}/\text{ml}$  poly-L-ornithine bromide by overnight coating at room temperature, followed by washing with tissue-culture grade water three times and then air drying and UV treatment inside a tissue culture hood.
2. Next, on day 15, coat the plate with laminin from 5  $\mu\text{g}/\text{ml}$  Engelbreth-Holm-Swarm murine sarcoma basement membrane, 10  $\mu\text{g}/\text{ml}$  fibronectin human plasma, and Matrigel (1:20) (“LMF” coating) (*see Note 15*) and leave overnight at 4 °C (*see Note 16*).

3. Between days 14 and 16, MN spheres are dissociated using 0.05% trypsin–EDTA as follows (*see Note 17*):
  - (a) Take LMF plates out of the fridge and place in incubator.
  - (b) Collect spheres in a 15-ml Falcon tube. Discard Petri dishes.
  - (c) Wash twice with 5 ml of calcium/magnesium-free DPBS (*see Note 18*).
  - (d) Aspirate DPBS.
  - (e) Add 3 ml of pre-warmed 0.05% trypsin/DNase per 15-ml tube.
  - (f) Incubate for 10–15 min, occasionally inverting a few times to separate the EBs.
  - (g) Stop the trypsin enzymatic reaction by adding 1 volume (3 ml) of “Stop” solution and then add 4 ml of cold Wash Buffer.
  - (h) Allow spheres to settle to the bottom of the Falcon tube.
    - (i) Aspirate the supernatant.
    - (j) Add 1 ml of cold Wash Buffer.
  - (k) Dissociate spheres with P1000 pipette, pipetting up and down 10–12 times, avoiding the generation of air bubbles.
    - (l) Add 2 ml of cold Wash Buffer, shake the tube and let non-dissociated spheres settle down over 4–5 min.
  - (m) Collect supernatant (approximately 2.5 ml) and transfer to a new 15-ml tube containing 2 ml of cold Wash Buffer.
  - (n) Add 0.5 ml of cold Wash Buffer and dissociate for a second time. Add a further 2 ml of cold Wash Buffer. Leave to settle and take off supernatant and pool.
  - (o) Repeat **Step n** one more time.
  - (p) Filter pooled supernatants through 40  $\mu$ m cell strainer filter into a new 50-ml Falcon tube that already contains 2–4 ml of cold Wash Buffer.
  - (q) Once filtered, transfer this to a new, labeled 15-ml Falcon tube.
  - (r) Centrifuge at 400 rcf for 5 min.
  - (s) Aspirate supernatant.
  - (t) Resuspend in 3–4 ml of MN-NF, supplemented with GluE & U/FDU, depending on the size of the pellet (aiming for 1–5 million cells/ml) (*see Note 19*).
  - (u) Aspirate the LMF coating from  $\mu$ -slide 8-well ibidi dishes.



- (v) Plate cells at a density of 50,000–70,000 cells per well. 50% of the MN-NF medium should be replaced every 2–3 days. The medium should be supplemented with 1  $\mu$ M U/FDU for at least 1 week post platedown to remove residual proliferating progenitor cells. If cell non-autonomous effects on axonal transport are to be assessed, one can use conditioned medium (from, for example, astrocyte monocultures) or plate the MNs in direct contact with different cell types.

### 3.3 Labeling of Mitochondria

1. Sparsely transduce the cells at platedown with lentivirus expressing mitoDsRed2. Lentivirus stored at  $-80^{\circ}\text{C}$  should be thawed on ice and the appropriate volume of lentivirus (*see Note 20*) should be added to the cell suspension (total volume: 300  $\mu$ l) for 10 min prior to platedown onto a  $\mu$ -slide 8-well ibidi dish. The virus is washed off at the next routine replacement of the cell culture medium after 2–3 days.
2. At the chosen time point for imaging, such as 21 days post platedown, change the medium 30 min prior to imaging to phenol red-free Neurobasal supplemented with  $1\times$  Glutamax (*see Note 21*).
3. Heat the microscope stage for at least 30–60 min prior to experimentation to minimize focal drift.
4. Perform fluorescence live-cell imaging of mitochondrial axonal transport. Typical settings to capture mitochondrial motility are a 0.2 Hz capture of a  $\sim 100$   $\mu$ m stretch of axon and a small z-stack (because the axons are not homogeneously in the same plane) for a 5 min time lapse. Guard against unnecessary excitation/laser power to minimize bleaching.
5. Image at least 4 axons ( $n$ ) per line per differentiation ( $N$ , where  $N = 3$ ). “Proximal” axon measurements are acquired from the axon immediately adjoining the cell body, and “distal” were measured from the distal tip. Notably, this method sparsely labels only a few neurons in a highly dense culture, thus permitting the assessment of the directionality of mitochondrial movement (and hence velocity) within the axon. Maximum intensity projections can be computed in Fiji [6].
6. Generate kymographs (space–time plots) to determine the numbers of stationary ( $\leq 0.1$   $\mu\text{m/s}$ ) versus bidirectionally motile mitochondria (either predominantly toward or away from the soma). Kymographs can be generated and analyzed using a number of softwares, including KymoToolBox (*see Note 22*) [7, 8] in Fiji.

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## 4 Notes

1. Matrigel is thawed at 4 °C for 1–2 h. While thawing, chill a 10-ml stripette, 1.5-ml tubes, and 1-ml pipette tips. Once thawed, dilute 1:2 with cold Advanced DMEM/F12 using a cold stripette. Create 1-ml aliquots using cold tips and tubes for storage at –20 °C. For coating iPSC plates, use aliquots at 1:30 (i.e., 1:60 final concentration) Matrigel, leaving plates overnight at 4 °C, or 30 min at 37 °C, or room temperature for 2 h. Plates can be kept for up to 7 days at 4 °C.
2. We recommend the shakers to be on a backup battery power supply.
3. Light-sensitive components should be wrapped with aluminum foil when exposed to light. Aliquots once thawed can be kept at 4 °C for 7–10 days before discarding. N.B. for LDN-193189, the solubility on the specification sheet should be checked for each batch, since it differs.
4. ROCK (rho kinase) inhibitor (10 μM) is added to the initial feed (days 0 medium) to improve iPSC survival following Accutase dissociation to single cells, together with basic fibroblast growth factor (FGF-2) (10 ng/ml) to promote proliferation.
5. The original analysis by Maury et al. [3] showed no statistical difference in the proportion of OLIG2-positive cells between use of either 1 μM or 0.1 μM RA.
6. We recommend the use of μ-slide 8-well ibidi dishes (80826, ibidi GmbH) because of their high optical quality. The volume of media per well for this plate is 300 μl.
7. 0.25% Trypsin–EDTA is diluted in calcium- and magnesium-free Earle’s Balanced Salt Solution (EBSS).
8. Since DNase may lose <10% of its activity when stored for a week in aliquots at –20 °C, we recommend using the stocks up in 4–5 weeks, otherwise consider doubling the volume added in dissociation buffers. In any event, one may need to adjust the DNase concentration, since some lines tend to be more difficult to dissociate than others. In general, this protocol generates large, sticky EBs; thus, it is better to start out with a higher concentration of DNase and optimize accordingly.
9. Check the volume, since some evaporation can occur; if there is less than 8 ml of medium present, then ensure that the incubator has enough water in the RH pan.
10. Avoid cross-contamination by using a different aspirator pipette for each plate.

11. This is a lower rcf centrifugation than for day 0, so that single cells remain in the supernatant (to be aspirated).
12. Always mark on the lid of the Petri dish the details of the date of feeding and composition of the medium.
13. Inhibition of the Notch pathway reduces progenitor cell proliferation and differentiates OLIG2 positive progenitors to neuronal, i.e., MN, fate.
14. We recommend dissociation at day 16 (but it can be done from day 14 onwards; the earlier dissociation will have more MN progenitors and fewer post-mitotic MNs); however, half of the spheres can be dissociated and half kept back and fed with “day 14” medium every other day, with a second platedown procedure planned 5–7 days later (in case the dissociation process fails the first time).
15. Make up in fresh cold Advanced DMEM/F12. From the working stock aliquots as described in **Note 1**, Matrigel is used at 1:10 (i.e., 1:20 final concentration).
16. Plates are best left overnight at 4 °C, or, if they are needed sooner, leave them at 37 °C for 30–60 min prior to platedown. Plates can be kept for up to 10 days at 4 °C.
17. Speed of platedown is critical to success. As such, we do not recommend handling more than six different cell lines at ones.
18. Invert the Falcon tubes several times and let the spheres settle for 2 min.
19. Adding too little MN-NF will lead to clumping of cells. Ensure that the pellet is resuspended fully and invert the tubes regularly and be vigilant for clumping. Plate cells as quickly as possible.
20. We recommend that the transduction multiplicity of infection (MOI) is optimized to visualize 1–10% of cells labeled with mito-dsRed2. Such sparse labeling permits the visualization of labeled mitochondria in single axons without distraction from the visualization of overlapping axons from neighboring cells.
21. Supplements such as B-27 are phototoxic.
22. Refer to [https://github.com/fabricecordelieres/IJ\\_KymoToolBox](https://github.com/fabricecordelieres/IJ_KymoToolBox) for a detailed description of the plugin.

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