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TITLE:

Axonal Transport of Organelles in Motor Neuron Cultures using Microfluidic Chambers System

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SUMMARY:
Axonal transport is a crucial mechanism for motor neuron health. In this protocol we provide a detailed method for tracking the axonal transport of acidic compartments and mitochondria in motor neuron axons using microfluidic chambers.

ABSTRACT:
Motor neurons (MNs) are highly polarized cells with very long axons. Axonal transport is a crucial mechanism for MN health, contributing to neuronal growth, development, and survival. We describe a detailed method for the use of microfluidic chambers (MFCs) for tracking axonal transport of fluorescently labeled organelles in MN axons. This method is rapid, relatively inexpensive, and allows for the monitoring of intracellular cues in space and time. We describe a step by step protocol for: 1) Fabrication of polydimethylsiloxane (PDMS) MFCs; 2) Plating of ventral spinal cord explants and MN dissociated culture in MFCs; 3) Labeling of mitochondria and acidic compartments followed by live confocal imagining; 4) Manual and semiautomated axonal transport analysis. Lastly, we demonstrate a difference in the transport of mitochondria and acidic compartments of HB9::GFP ventral spinal cord explant axons as a proof of the system validity. Altogether, this protocol provides an efficient tool for studying the axonal transport of various axonal components, as well as a simplified manual for MFC usage to help discover spatial experimental possibilities.

KEYWORDS:
microfluidic chambers, motor neurons, spinal cord explants, axonal transport, mitochondria, acidic compartments

INTRODUCTION:

MNs are highly polarized cells with long axons, reaching up to one meter long in adult humans. This phenomenon creates a critical challenge for the maintenance of MN connectivity and function. Consequently, MNs depend on proper transport of information, organelles, and materials along the axons from their cell body to the synapse and back. Various cellular components, such as proteins, RNA, and organelles are shuttled regularly through the axons. Mitochondria are important organelles that are routinely transported in MNs. Mitochondria are essential for proper activity and function of MNs, responsible for ATP provision, calcium buffering, and signaling processes[1,2]. The axonal transport of mitochondria is a well-studied process[3,4]. Interestingly, defects in mitochondrial transport were reported to be involved in several neurodegenerative diseases and specifically in MN diseases[5]. Acidic compartments serve as another example for intrinsic organelles that move along MN axons. Acidic compartments include lysosomes, endosomes, trans-Golgi apparatus, and certain secretory vesicles[6]. Defects in the axonal transport of acidic compartments were found in several neurodegenerative diseases as well[7], and recent papers highlight their importance in MN diseases[8].

To efficiently study axonal transport, microfluidic chambers that separate somatic and axonal compartments are frequently used[9,10]. The two significant advantages of the microfluidic system, and the compartmentalization and the isolation of axons, render it ideal for the study of subcellular processes[11]. The spatial separation between the neuronal cell bodies and axons can be used to manipulate the extracellular environments of different neuronal compartments (e.g., axons vs. soma). Biochemical, neuronal growth/degeneration, and immunofluorescence assays all benefit from this platform. MFCs can also assist in studying cell-to-cell communication by coculturing neurons with other cell types, such as skeletal muscles[12–14].

Here, we describe a simple yet precise protocol for monitoring mitochondria and acidic compartment transport in motor neurons. We further show the use of this method by comparing the relative percentage of retrograde and anterograde moving organelles, as well as the distribution of transport velocity.

PROTOCOL:

The care and treatment of animals in this protocol were performed under the supervision and approval of the Tel Aviv University Committee for Animal Ethics.

1. **MFC preparation**

1.1. PDMS casting in primary molds (**Figure 1**)

1.1.1. Purchase or create primary molds (wafers) following a detailed protocol[9].

1.1.2. Use pressurized air to remove any type of dirt from the wafer platform before proceeding to the coating step. The surface of the wafers should look smooth and clear.

1.1.3. Fill a container with 50 mL of liquid nitrogen. Prepare a 10 mL syringe and 23 G needle.

NOTE: All procedures from this step forward must be performed in a chemical hood.

1.1.4. In the chemical hood, use the syringe and needle to pool 2 mL of liquid nitrogen. Though it may seem like air was drawn, the syringe is filled with nitrogen (**Figure 1A**). Place the wafer-containing plate in a sealable container.

1.1.5. Screw open a chlorotrimethylsilane bottle, pierce the rubber cap using the nitrogen-filled syringe, and inject the entire contents of the syringe into the bottle. Without pulling out the needle, turn the bottle upside down and draw back 2 mL of chlorotrimethylsilane.

NOTE: Because of the syringe pressure, a small amount of chlorotrimethylsilane is sprayed out of the needle. To avoid a hazard, point the needle toward the inner wall of the hood (**Figure 1B**).

1.1.6. Spread chlorotrimethylsilane uniformly in the container (from step 1.1.3), but not directly on the wafer or wafer-containing plate. Close the container and incubate for 5 min per wafer.

NOTE: If this is the first time the wafer is coated with chlorotrimethylsilane, a 1 h incubation should be allowed for each wafer.

1.1.7. Do not take the wafers and container out of the chemical hood for 30 min.

CAUTION: Chlorotrimethylsilane is highly volatile.

1.1.8. Weigh the PDMS base (see **Table of Materials**) in a 50 mL tube and add PDMS curing agent at a ratio of 17:1 respectively (e.g., 47.05 g of base and 2.95 g of curing agent). Mix for 10 min using a low speed rotator.

1.1.9. Pour PDMS into each wafer-containing plate to the desired height (**Figure 1C**).

NOTE: Using thin microfluidic chambers (up to 3–4 mm) improves adherence to the culture dish and prevents leakage.

1.1.10. Place all plates together inside a vacuum desiccator for 2 h (**Figure 1E**). This process removes the air trapped within the PDMS, thus eliminating air bubbles and forming a clear, uniform mold.

1.1.11. Place the plates inside an oven for 3 h (or overnight) at 70 °C (**Figure 1E**).

NOTE: The plates should be level when placed in the oven.

1.2. PDMS casting in epoxy molds

NOTE: Because wafer preparation is expensive, requires special equipment, and may damage the fragile wafers, it is possible to generate epoxy replicas of wafers. The replicas are cheaper, more durable, and can be used for mass production of microfluidic chambers.

1.2.1. Cast and cure PDMS (as described in 1.1.8–1.1.11) into the original wafer.

1.2.2. Remove and cut off excess parts of PDMS leaving only the microfluidic elements and the functional area required for processing them into microfluidic chambers.

1.2.3. Immediately wrap the PDMS with thick, sticky tape to prevent it from accumulating dust.

1.2.4. Choose a tissue culture grade plastic dish that fits the entire PDMS inside and leave room for epoxy around it. The distance from the PDMS to the plastic dish should be less than 5 mm.

1.2.5. Prepare a small amount of PDMS mixed in a ratio of 10:1 (base:curing agent). The fresh liquid PDMS will be used to glue the solid PDMS onto the bottom of the plastic plate.

1.2.6. Apply a minimal amount of the liquid PDMS to the center bottom of the plastic plate and then remove the sticky tape from the PDMS and adhere it to the plastic dish bottom. Make sure that the microfluidic elements are facing upwards.

1.2.7. Let the PDMS cure for 30 min in a 70 °C oven.

1.2.8. Prepare the epoxy resin by mixing the base and curing agent in a ratio of 100:45 respectively in a test tube. Different epoxy resins may have different mixing ratios. The required volume for a regular 100 mm plate is approximately 40 mL.

1.2.9. Let the epoxy mix well for 10 min in a rotator until the mixture becomes visibly homogenous (i.e., there are no visible fiber-like artifacts in the liquid).

1.2.10. Centrifuge the epoxy mixture at 400 x *g* for 5 min to remove air bubbles caught inside.

1.2.11. During centrifugation, spread a thin layer of silicone grease around the walls and all other exposed plastic parts of the culture dish. This will prevent the epoxy from polymerizing with the dish plastic and will enable removal of the cured epoxy easily at the end of the protocol.

1.2.12. Pour the epoxy slowly into the dish until it completely covers the PDMS and goes beyond it by at least 5 mm. Prevent the formation of any bubbles within the epoxy by keeping zero distance between the tube and the plate. Place the plate in a secure place so it will not be moved for the next 48 h.

1.2.13. After 48 h the epoxy should be completely cured. Insert the plate into a preheated oven at 80 °C for 3 h for final curing.

1.2.14. Remove the cured epoxy from the plate and the original PDMS mold by gently yanking the plastic wall of the plate until it breaks. It should then easily separate from the epoxy and peel off.

1.2.15. Once extracted, wipe the remaining grease off the new epoxy replica and inset it upside down (i.e., with the replicated microfluidic elements facing up) into a new culture dish. The epoxy replica is now ready for PDMS casting.

1.2.16. Use pressurized air or N_2 to blow any remains of PDMS or dirt off the epoxy mold and rinse it 2x with isopropanol. Fill it a third time and incubate for 10 min on an orbital shaker plate. Rinse the mold again 3x with isopropanol and discard the remaining liquid. Blow dry with air or N_2 or place in a 70 °C oven until dry.

NOTE: Follow safety procedures when working with and discarding isopropanol.

1.2.17. Keep the mold plates closed until casting. Follow steps 1.1.8.–1.1.11.

1.3. Punching and sculpting the PDMS into an MFC (**Figure 2**)

1.3.1. Cut and remove the PDMS mold from the plate by following the (+) marks on the wafers using a scalpel. Do not use force, as the molds are fragile (**Figure 2A**).

1.3.2. Follow the instructions drawn on the sketch to punch and cut the chambers depending on the experimental setup (**Figure 2B**–**F**).

1.3.2.1. For spinal cord explant culture (**Figure 2C**, **Figure 2E**), punch two 7 mm wells in the distal side of a the large MFC. Locate the wells in a way that they will overlap with the channel edges. On the proximal side, punch one 7 mm well in the middle of the channel, with minimal overlap so that sufficient space will be left for the explants. Punch two additional 1 mm holes in the two edges of the proximal channel. Turn the MFC with the microfluidic elements facing upwards, and using a 20 G needle, carve three small explant caves on the punched 7 mm well.

1.3.2.2. For dissociated MN culture (**Figure 2D**, **Figure 2F**), punch four 6 mm wells in the edges of the two channels of a small MFC.

1.4. Sterilizing the MFC for tissue culture use

1.4.1. Spread 50 cm long sticky tape bands on the bench. Press and pull back the chamber to face the sticky tape (both upper and lower faces) and remove crude dirt. Place the clean chambers in a new 15 cm plate.

NOTE: Do not press directly on the microfluidic elements when these are facing upwards.

1.4.2. Incubate the chambers in analytical grade 70% ethanol for 10 min on an orbital shaker.

1.4.3. Dispose of the ethanol and dry the chambers in a tissue culture hood or in an oven at 70 °C.

1.5. Placing the MFC on a glass bottom dish

1.5.1. Place the chamber in the center of a tissue culture grade 35 mm/50 mm glass bottom dish and apply minor force on the edges to make the PDMS and dish bottom bind. To avoid breaking the glass bottom, always apply force on top of a solid surface.

1.5.2. Incubate 10 min in 70 °C. Press the chambers to strengthen adherence to the plate.

1.5.3. Incubate under UV light for 10 min.

1.6. Coating and culturing

1.6.1. Add 1.5 ng/mL poly-L-ornithine (PLO) to both compartments. Make sure the PLO is running through the channels by pipetting the coating media a few times directly in the channel entrance.

1.6.2. Examine the microfluidic chamber under a light microscope with 10x magnification to check for the presence of air bubbles. If air bubbles are blocking the microgrooves, place the MFC in a vacuum desiccator for 2 min. Later, remove the excess air that got caught in the channels by pipetting the coating media through them. Incubate overnight.

1.6.3. Replace PLO with laminin (3 µg/mL in DDW) for overnight incubation in the same manner.

1.6.4. Prior to plating, wash the laminin with neuronal culture medium.

2. **Neuronal culture plating**

2.1. Dissociated motor neuron culture

2.1.1. Using straight scissors and fine forceps, dissect a spinal cord out of an E12.5 ICR-HB9::GFP mouse embryo. Work in an 1X HBSS solution with 1% penicillin-streptomycin (P/S) (**Figure 3A–C**).

2.1.2. Using microdissection scissors, remove the meninges and the dorsal horns (**Figure 3D**).

2.1.3. Collect the spinal cord pieces and transfer to tube (**#1**) with 1 mL HBSS + 1% P/S.

2.1.4. Cut the spinal cords to small pieces using curved scissors and wait for the pieces to settle.

2.1.5. Add 10 µL of trypsin 2.5% and place in a 37 °C water bath for 10 min. After 5 min, mix by tapping the tube. The pieces should form a helix-like clump.

2.1.6. Transfer the clump into a new tube (**#2**) containing 800 µL of prewarmed L-15, 100 µL of BSA 4%, and 100 µL of 10 mg/mL DNase. Grind 2x, then wait for 2 min to let undissociated pieces settle. Transfer the supernatant to a new tube (**#3**)**.**

2.1.7. Add 100 µL of BSA 4%, 20 µL of 10 mg/mL DNase, and 900 µL of complete neurobasal medium (CNB, see **Table of Materials** and **Table 1**). Grind 8x and wait 2 min. Collect supernatant to tube **#3**.

2.1.8. Repeat step 2.1.7 and grind 10x. Collect supernatant to tube **#3**. A small amount of tissue should be left at the bottom of the tube.

NOTE: If a large clump still remains at the bottom of tube **#2**, repeat step 2.1.8.

2.1.9. Add 1 mL of BSA 4% cushion to the bottom of tube **#3**.

2.1.10. Centrifuge at 400 x *g* for 5 min. Discard the supernatant.

2.1.11. Resuspend the cell pellet by gently tapping the tube, then add 1 mL of CNB medium. Pipette 6x and add 20 µL of 10 mg/mL DNase.

2.1.12. Supplement with an additional 5 mL of CNB medium and transfer 3 mL to a new tube (**#4**)**.**

2.1.13. Add 1 mL of 10.4% density gradient medium (see **Table 2**) to the bottom of each tube (**#3** and **#4**). A sharp phase separation between the two interfaces should appear.

2.1.14. Centrifuge at 775 x *g* for 20 min at room temperature (RT). Centrifuge deceleration should be set to a low level to avoid breakdown of the phase separation.

2.1.15. Cells should be floating, appearing as a cloudy interphase between the media. Collect the cells from both tubes into a new tube (**#5**) already containing prewarmed 1 mL CNB medium.

2.1.16. Add an additional 4–6 mL of CNB medium.

2.1.17. Add 1 mL of BSA 4% cushion to the bottom of the tube.

2.1.18. Centrifuge at 400 x *g* for 5 min at RT. Discard the supernatant and resuspend the pellet gently with 1 mL of CNB medium.

2.1.19. Count the cells. A yield of 0.75–1 x 10^6 MN per spinal cord is expected.

NOTE: Ventral spinal cord also contains other neuronal subtypes besides motor neurons (e.g., interneurons). MN purity depends mostly on the removal of dorsal areas during dissection and the ability to reach MN enriched rostral areas. To ensure the imaged neurons are MNs, use of a

mice strain with an endogenous MN marker, such as HB9::GFP mice, is recommended. To achieve pure MN culture (but with decreased cell yield), use of FACS purification[15] is possible.

2.1.20. Plating dissociated MN culture in the MFC

2.1.20.1. Concentrate 150,000 MNs per chamber by centrifuging at 400 x *g* for 5 min.

2.1.20.2. Aspirate the supernatant and gently resuspend the cells in rich neurobasal medium (RNB) at 4 µL per MFC. RNB is CNB supplemented with additional 2% B27 and 25 ng/mL BDNF.

2.1.20.3. Remove the medium from both compartments, leaving a low volume equivalent to ~10 µL in the wells of the distal compartment. It appears as a thin ring of medium in the well perimeter.

2.1.20.4. Slowly load 4 µL of cells into the channel. Take out 4 µL of the well in the other side of the channel, and slowly load them back directly into the channel to reverse the current flow and maximize cell density in the channel.

2.1.20.5. Verify that the cells have entered the channel using a 10x light microscope and place the chamber in the incubator for 30 min without adding more media.

2.1.20.6. Slowly add ~10–15 µL of RNB into the proximal and distal wells and place the chambers in the incubator for another 15 min.

2.1.20.7. Following this incubation, slowly add ~75–80 µL of RNB into each well.

2.1.21. Dissociated MN culture maintenance

2.1.21.1. One day after plating (DIV1), replace medium with RNB supplemented with 1 µM cytosine arabinoside (Ara-C) to inhibit glial growth.

2.1.21.2. Two days after Ara-C application (DIV3) replace medium with fresh CNB medium in the proximal compartment (without Ara-C).

2.1.21.3. In order to enhance crossing of axons through the microgrooves, apply RNB supplemented with 25 ng/mL of glial cell-derived neurotrophic factor (GDNF) and 25 ng/mL of brain-derived neurotrophic factor (BDNF) only to the distal compartment. Maintain a volume gradient of at least 10 µL per well between the axonal distal wells (higher volume) and the proximal wells.

2.1.21.4. Refresh the medium every 2 days. It can take the axons up to 4–6 days to cross distally.

2.2. Spinal cord explant culture

2.2.1. Using straight scissors and fine forceps, dissect a spinal cord out of an E12.5 ICR-HB9::GFP mouse embryo. Work in an 1X HBSS solution with 1% penicillin-streptomycin (P/S) (**Figure 3A–3C**).

2.2.2. Using microdissection scissors, remove the meninges and the dorsal horns (**Figure 3D**).

2.2.3. Cut the spinal cord into 1 mm thick transverse sections (**Figure 3E**). Dispose of all medium from the proximal compartment of the MFC.

2.2.4. Pick up a single spinal cord explant with a pipette in a total volume of 4 µL. Inject the explant as close as possible to the cave and draw out any excessive liquid from the proximal well via the lateral outlets (1 mm punches). The explants should be sucked into the proximal channel.

2.2.5. Slowly add 150 µL of spinal cord explant medium (SCEX, see **Table of Materials** and **Table S3**) to the proximal well.

2.2.6. Spinal cord explant culture maintenance

2.2.6.1. Add SCEX medium in the proximal compartment, and rich SCEX medium (SCEX with 50 ng/mL of BDNF and GDNF) in the distal compartment. Maintain a volume gradient of at least 15 µL per well between the distal wells (higher volume) and the proximal well.

2.2.6.2. Refresh the medium every 2 days. It can take the axons up to 3–5 days to cross distally.

3. Axonal transport (**Figure 4A**)

3.1. Labeling of mitochondria and acidic compartments

3.1.1. Prepare fresh SCEX medium (or CNB for dissociated MN) containing 100 nM Mitotracker Deep Red FM and 100 nM LysoTracker Red. Incubate for 30–60 min at 37 °C. Other colors can be used as long as their fluorophores do not overlap.

3.1.2. Wash 3x with warm CNB/SCEX medium. The plates are ready for imaging.

3.2. Live imaging

3.2.1. Acquire 100 time-lapse image series of axonal transport at 3 s intervals, with a total of 5 min per movie.

NOTE: The imaging system used in this study included an inverted microscope equipped with spinning disc confocal, controlled via propriety cell imaging software, 60x oil lens, NA = 1.4, and an EMCCD camera. Movies were acquired in a controlled environment at 37 °C and 5% CO_2.

NOTE: Longer or shorter time-lapse movies can be imaged, dependent on the experiment. Even overnight movies can be recorded if needed. However, it is critical to try and reduce the exposure time and laser power, as well as the number of total images, to decrease phototoxicity and bleaching during movie acquisition.

4. Image analysis (**Figures 4**–**5**)

4.1. Analysis of particle transport distribution and density using kymograph analysis

4.1.1. Open the file in FIJI. Separate channels pressing **Image**|**Stacks**|**Tools**|**Deinterleave**.

4.1.2. Set image properties by pressing **Image**|**Properties**.

4.1.3. Choose the **Segmented Line Tool** by right-clicking the **Line Icon**. Set **Width** to 8–10 by double-clicking the **Line Icon**. Be consistent with the same line width throughout the entire analysis.

4.1.4. Mark a segmented line following the axon path from distal to proximal. Double-click to stop the line marking.

4.1.5. Click **t** to add a new line region of interest (ROI) to the **ROI Manager**. Add this to a spreadsheet analysis table.

4.1.6. Click **m** to measure area and length of the axon. Add this to the analysis table.

4.1.7. Generate a kymograph by clicking **Plugins**|**KymoToolBox**|**Draw Kymo**. Alternatively, other kymograph generation plugins available can be used.

4.1.8. Manually count moving (retrograde or anterograde) and nonmoving particles and add them to the table in the correct column.

NOTE: Particles are classified as moving anterograde (i.e., moving left in the kymograph) or retrograde (i.e., moving right) if their displacement is higher than 10 μm in the specific direction. It is possible to measure the displacement simply by marking a horizontal line with the **Line Icon** and pressing **m**. Immobile particles or those that do not meet the displacement criteria are defined as nonmoving (**Figure 4B**).

4.2. Single particle tracking: Manual tracking

4.2.1. Download the manual tracking plugin for FIJI software (developed by Fabrice P. Cordelière) from http://rsb.info.nih.gov/ij/plugins/track/track.htmL.

4.2.2. Open the file in **FIJI/ImageJ**. Use the **Rotate** option to align the MFC grooves horizontally.

4.2.3. To improve the signal-to-noise ratio if needed, click **Process**|**Subtract Background**.

4.2.4. Open the **Manual Tracking** plugin. Set the parameters (e.g., pixel size, time interval, etc.) according to the specific microscope used for imaging. For the results shown here, the microscope and lens the ratio was 0.239 µm/pixel and the frame interval was 3 s.

4.2.5. Obtain the tracks X and Y coordinates and save the results by copying the text to spreadsheet.

4.2.6. Analyze multiple-channel movies by clicking **Image**|**Color**|**Merge Channels** to merge the channels and then track only colocalized puncta.

4.3. Single particle tracking: Semi-automated tracking (**Figure 5**)

4.3.1. Open the analysis software. This study used Bitplane Imaris software version 8.4.1.

4.3.2. **Switch** to **Surpass** in the top menu.

4.3.3. Click **Image Processing**| **Swap Time** and **Z**|**Ok**.

4.3.4. Click **Edit**|**Image Properties** (**Ctrl+I**)|**Geometry**|**Voxel Size Row**. Set **Image Properties** according to the microscopy setup used.

NOTE: For the data displayed here, the microscope and lens ratio was 0.239 µm/pixel.

4.3.5. Click **All Equidistant** and change the **Time Interval**. For example, use 3 s as the interval. Click the **Reset** button on the right bottom or click **Ctrl+B**.

4.3.6. Add a layer of **Spots** in the top left by clicking an **Icon of Small Yellow Spots**. At the bottom left a new **Menu for Editing the Spots** is opened.

4.3.7. Press the **Right Blue Arrow** until the spot detection starts.

NOTE: It is important to filter out some of the dots using the filter on the bottom left of the window. Check the movie a few times to see that a sufficient number of dots is selected.

4.3.8. Verify or configure the parameters to fit the experimental needs. For example, **Max Distance** = 12 µm (The maximal allowed distance between two distinct spots to still include them in the same single track); **Max Gap Size** = 1 (The number of frames that a track is allowed to miss and still considered one track).

4.3.9. Click **Settings** and then **Track Style** = **Off**, **Points** = **Sphere**.

4.3.10. Using the **Filter Bar**, choose different filters for adjustment. For example, in the data supplied here, **Track Duration** = 9 removed all tracks with fewer than 3 frames. When all the parameters are set, click the **Right Green Arrow**. Further editing is not possible after this step.

4.3.11. Click the **Small Pencil with Dots** to manually edit all the tracks.

4.3.12. View the movie (**Figure 5B**). If an error occurs, there are several possible options:

4.3.12.1. To disconnect a track, click the **Object** option and choose the two spots that need to be disconnected holding **Ctrl**, and choose **Disconnect**.

4.3.12.2. To connect a track, click the **Object** option, choose the two spots that need to be connected holding **Ctrl** and choose **Connect**.

4.3.12.3. To delete a track or spot, with the right option (**Track/Object**), switch to the screen with the **Regular Pencil Icon**, and choose **Delete**.

4.3.12.4. To add spots manually, switch to the **Regular Pencil Icon Screen**. At the bottom of the screen there is a **Manual Tracking Mark**. Make sure the **Auto-Connect Checkbox** is **V**. On the movie itself, in order to add a spot, hold the **Shift** button and Left-Click.

4.3.13. To add a spot to an existing track, choose a desired track (yellow) and frame, switch to the **Regular Pencil Icon Screen** and add a spot manually. When the entire movie is finished, switch to the **Icon that Looks Like a Red Graph** (**Statistics**). It is possible to edit the analyzed parameters later. To edit, on the bottom left of the screen, press the **Swedish Key Icon**. For example: Position X, Position Y.

4.3.14. Press on the **Icon that Looks Like Several Floppy Disks**, **Export All Statistics**.

NOTE: The spreadsheet output can be either handled directly or further analyzed using the published code[9] used for this analysis, which will be shared upon demand. The following parameters are extracted from the analysis: Speed, Track Displacement, Run Length, Velocity (including Directionality), Stop Count, Average Stop Duration, Alpha, Direction Changes, and Instantaneous Velocity. A detailed explanation of each parameter is described in Gluska et al.[9].

REPRESENTATIVE RESULTS:

Following the described protocol, mouse embryonic HB9::GFP spinal cord explants were cultured in MFC (**Figure 4A**). Explants were grown for 7 days, when axons fully crossed into the distal compartment. Mitotracker Deep Red and Lysotracker Red dyes were added to the distal and proximal compartments in order to label the mitochondria and acidic compartments (**Figure 4C**). Axons in the distal grooves were imaged, and the movies were analyzed as follows: First, we compared the general movement distribution using kymograph analysis (**Figures 4B**, **4D**). This analysis revealed a bias in the retrograde direction only in acidic compartments (nonmoving = 77.1 ± 9.5%; retrograde = 16.9 ± 8.3%, anterograde = 6 ± 5%; **Figure 4E**) but not in mitochondrial

transport (nonmoving = 83.4 ± 6.8%; retrograde = 10.5 ± 6.9%; anterograde = 6.8 ± 5.1%; **Figure 4F**). Kymograph analysis was used to quantify the particle density, revealing a higher number of mitochondrial particles compared to acidic compartments in HB9::GFP spinal cord explant axons (Mitochondria = 0.46 ± 0.13; Acidic compartments = 0.3 ± 0.07 particles/µm axon, **Figure 4G**).

Next, single particle transport analysis was conducted using semiautomated software followed by in-house code (**Figure 5A–B**). This analysis revealed that despite having similar particle velocity in general (**Figure 5C**), when observing the distribution of velocities (**Figure 5D**) only acidic compartments but not mitochondria displayed a bias towards retrograde movement.

FIGURE LEGENDS:

Figure 1: Silicone mold preparation. Schematic drawing describing the procedure of chlorotrimethylsilane wafer cleansing. (**A**) First, 50 mL of liquid nitrogen were added to an appropriate container. Working in a chemical hood, a syringe and needle were used to draw 8 mL of liquid nitrogen. The entire content of the syringe was injected into the chlorotrimethylsilane bottle. The bottle was turned with the cap facing down and 8 mL of chlorotrimethylsilane were drawn back. (**B**) Chlorotrimethylsilane spread in the container (not directly on the wafer). The container needs to be closed, followed by 5 min incubation for each mold. (**C**) Liquid PDMS was poured into each wafer up to the desired height. (**D**) All plates were placed together inside a vacuum desiccator for 2 h, followed by 3 h–overnight in a 70°C oven.

Figure 2: MFC specialized design. (**A**) Polymerized PDMS template taken out of the mold using a metal scalpel. (**B**) Depending on the experimental setup either 6 mm, 7 mm, or 1 mm punchers were used for punching the PDMS templates. (**C**) For explant culture in the MFC, 7 mm and 1 mm punchers were used, and a 20 G syringe was utilized for making "caves" for easy explant insertion. (**D**) For dissociated MN culture MFC, a 6 mm puncher was used to create four wells at the channel edges. (**E–F**) Illustrations of the final MFC shapes described in **C** and **D**, respectively.

Figure 3: Neuronal culture. (**A**) E12.5 mouse embryo was placed in position after the head, tail, and skin were removed in order to expose the neural tube. (**B**) Dissection of the whole spinal cord. (**C**) Using gentle forceps, the meninges was peeled away from the spinal cord. (**D**) Left panel: Removal of the spinal cord lateral segments from the ventral spinal cord to yield better MN purification. Right Panel: Representative image of dissociated MN culture in the MFC. HB9::GFP axons crossed to the distal compartment (green). Hoechst staining indicates neuronal nuclei (blue). (**E**) Spinal cord explants generated by dissecting 1 mm thick transverse sections of the ventral spinal cord. Representative image of HB9::GFP (green) spinal cord explant axons in an MFC.

Figure 4: Axonal transport of mitochondria and acidic compartment in MNs. (**A**) Illustration of the axonal transport essay. Lysotracker Red and Mitotracker Deep Red were added to both the proximal and distal compartments of the MFC, containing HB9::GFP ventral spinal cord explant. (**B**) Kymograph analysis. Moving particles were defined as moving anterograde or retrograde following displacement of more than 10 µm in that direction. Rotating or immobile particles were counted as nonmoving. Scale bar = 10 µm. (**C**) First frame of a time-lapse movie displaying

primary HB9::GFP mouse spinal cord explant axons dyed with Lysotracker red to tag acidic compartments and Mitotracker Deep Red to tag mitochondria. Scale bar = 10 µm. (**D**) Representative kymographs displaying a typical axonal movement of acidic compartments and mitochondria. Scale bar = 10 µm. (**E**) Kymograph analysis of mitochondrial axonal transport, ****p < 0.0001, Anova with Holm-Sidak correction (n = 77 axons). Scale bar = 10 µm (**F**) Kymograph analysis of acidic compartment axonal transport, ** p < 0.01, ****p < 0.0001, Anova with Holm-Sidak correction (n = 77 axons). (**G**) Axonal particle density analysis of mitochondria and acidic compartments, ****p < 0.0001, Student's t-test (n = 77 axons). Error bars represent values with SD.

Figure 5: Semi-automated single particle analysis to measure organelle velocity. (**A**) Schematic workflow for semiautomated single particle transport analysis. (**B**) Ventral spinal cord explant axons were analyzed for single particle tracking. The analysis software is capable of tracking single axonal particles in time-lapse movies, as indicated for mitochondria (yellow dots, upper panel) and acidic compartments (green dots, lower panel). (**C**) The average velocity did not change between mitochondria and acidic compartments, Mann Whitney test (n = 417 mitochondria, n = 371 acidic compartments). Error bars represent values with SD. (**D**) Distribution of mitochondrial and acidic compartments retrograde and anterograde velocities.

Table 1: Recipe for preparation of complete neurobasal (CNB) solution.

Table 2: Recipe for preparation of density gradient medium solution.

Table 3: Recipe for preparation of spinal cord explant (SCEX) solution.

Table 4: Comparison between spinal cord explants and dissociated MN culture based on perimeters of speed, feasibility, glial presence, manipulation possibilities, immunostaining, and viral infection.

DISCUSSION:

In this protocol, we describe a system to track axonal transport of mitochondria and acidic compartments in motor neurons. This simplified in vitro platform allows precise control, monitoring, and manipulation of subcellular neuronal compartments, enabling experimental analysis of motor neuron local functions. This protocol can be useful for studying MN diseases such as ALS, to focus on understanding the underlying mechanism of axonal transport dysfunction in the disease[10,16]. Moreover, this system can also be applied for studying transport of trophic factors[9,16], microRNA[10], mRNA[8], and labeled proteins in healthy and diseased MNs or in other neurons, such as sensory[9] or sympathetic axons[14]. A similar method can also be applied to study organelle transport in a coculture system, such as MNs cultured with skeletal muscle cells[12] or sympathetic neurons cocultured with cardiomyocytes[14]. The MFC system can be utilized to generate active NMJs[17,18] and study the effect of synapse formation on axonal transport[16].

This protocol has several advantages compared to other protocols for culturing of neurons in MFC and using live imaging to analyze axonal transport: 1) MFCs are commercially available by

several manufacturers. However, self-manufacturing of the MFCs is extremely cost effective compared to the commercial alternative. A single PDMS wafer yields four large MFCs or nine small ones at the minimal expense of several US dollars for the PDMS resin itself. 2) The self-manufactured MFCs can be modified to answer specific experimental needs (e.g., changing the size and the location of the wells, increasing the thickness of the MFC PDMS). 3) The MFCs can be irreversibly attached to a plate (via plasma bonding) but can also be recycled multiple times to reduce the possibility of contamination. 4) The PDMS MFCs are transparent, making them ideal for live imaging by having reduced background, which is critical for axonal transport assays where signal-to-noise distinction could be a limiting factor. 5) Spinal cord explant culture is very efficient and fast. One embryonic spinal cord can yield up to 30 explants, enough for 10 MFCs. This can help to save time and materials, and ensures that even a pregnant mouse with few embryos can produce a successful experiment. See a detailed comparison of spinal cord explant and dissociated MN culture in **Table 4**.

This protocol is relatively simple and inexpensive. However, it requires expertise in several technical matters. The manufacturing and handling of the MFC needs to be accurate and gentle, to avoid structural defects and creation of air bubbles in the obligatory vacuum step (1.1.10), for example. During the optional vacuum step (1.6.2) it is important to clear all the air, or it will block axonal crossing, but also keep the vacuum short to avoid detachment of the MFC from the glass. Pay close attention to the dissection protocol steps, as it is important to properly remove the meninges and dorsal horns, as well as to try and cut the pieces to the right size in order to insert them to the MFC's cave without using physical force. Any excessive physical force applied to the MFC can easily detach the grooves or the entire MFC, thus the procedure should be done gently. Culturing of MNs and plating them in the MFC needs to be relatively swift, as MNs tend to aggregate and lose viability quickly under stress conditions such as the low medium volume of the plating step. The plating in the MFC should be done in low volume to allow proper attachment of the neurons in the MFC channel, and after no more than 30 min warmed medium should be added very gently to prevent detachment of the MNs.

After several days in culture, and once axons have extended to the distal compartment, the cultures are ready to undergo organelle staining followed by acquisition of axonal transport movies and finally a specific procedure for image analysis. The analysis can be performed either by single particle tracking over time, or by using an automated or semiautomated tracking algorithm. In our experience, automated methods are less time-consuming, but have several disadvantages. Mainly, automated tracking reliability is decreased, with crowded axons that cross paths. Furthermore, automated algorithms have a decreased ability to distinguish between overlapping particles. Consequently, performing manual or semiautomated tracking is recommended. In general, semiautomated tracking is preferable as it is less time-consuming, but manual tracking can be a good option when there is no available paid software. A thorough comparison between manual and automatic analysis can be found in Gluska et al.[9].

In conclusion, adopting this simple protocol can yield important data for the study of axonal transport of various cellular components. It can be adapted to fit a diversity of imaging setups and neuronal subtypes, as well as to cocultures of neurons with other cells. It also allows distinct

spatial pharmacological manipulation of either cell bodies or axons in order to understand basic mechanisms of neuronal health and to improve drug development for diseases with altered axonal transport.

ACKNOWLEDGEMENTS:

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DISCLOSURES:

The authors declare no conflict of interest.

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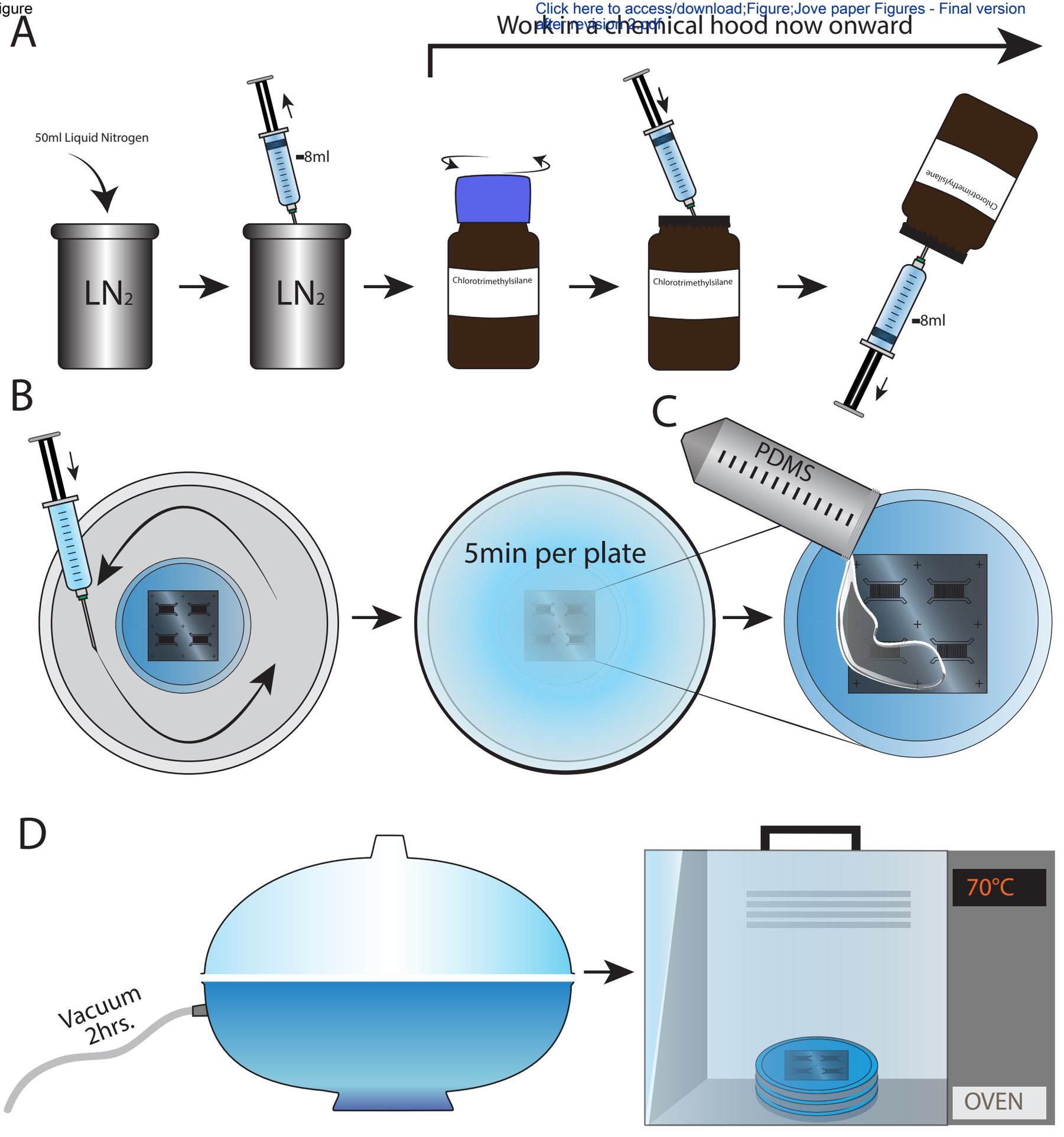
Figure



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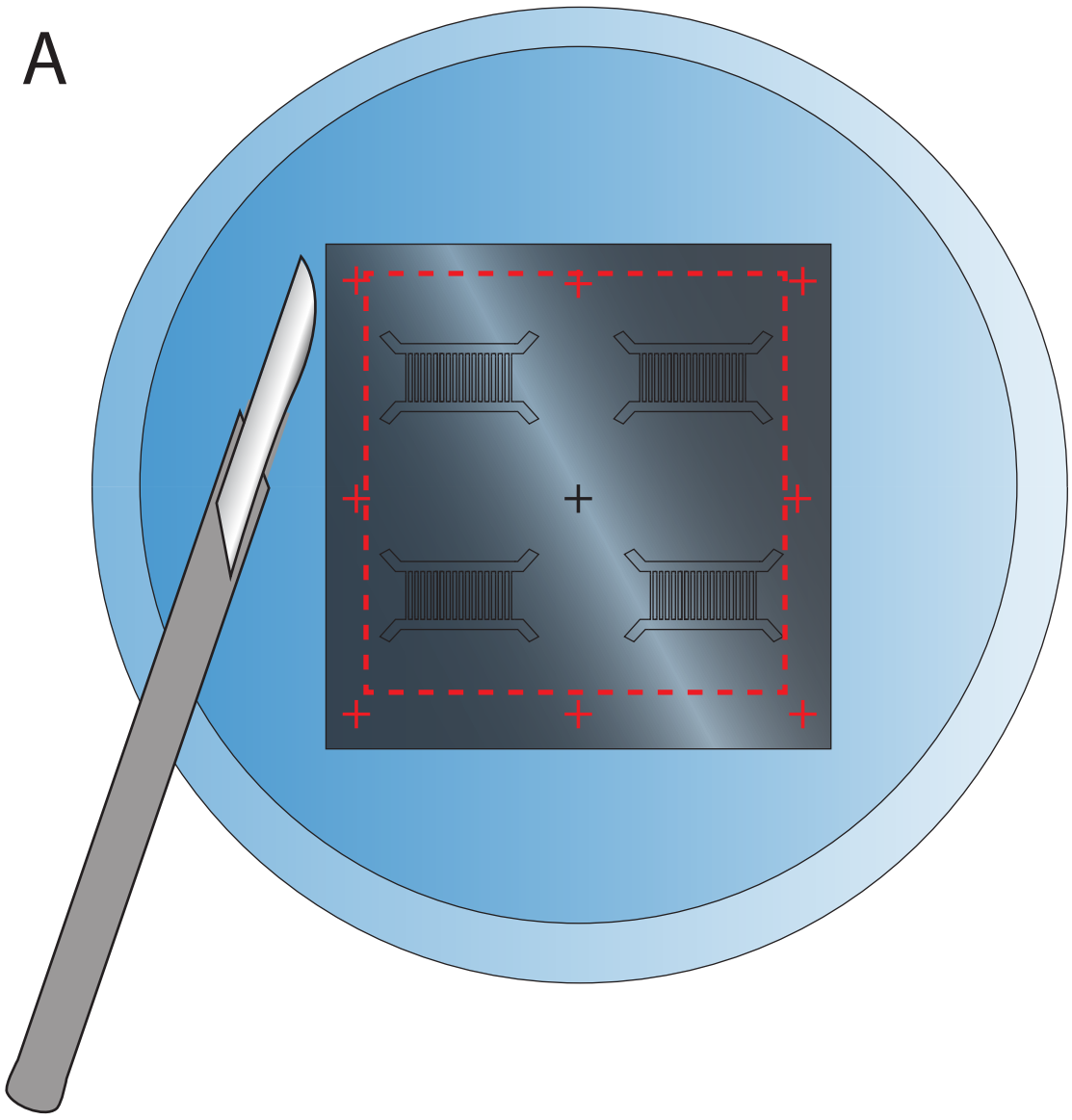

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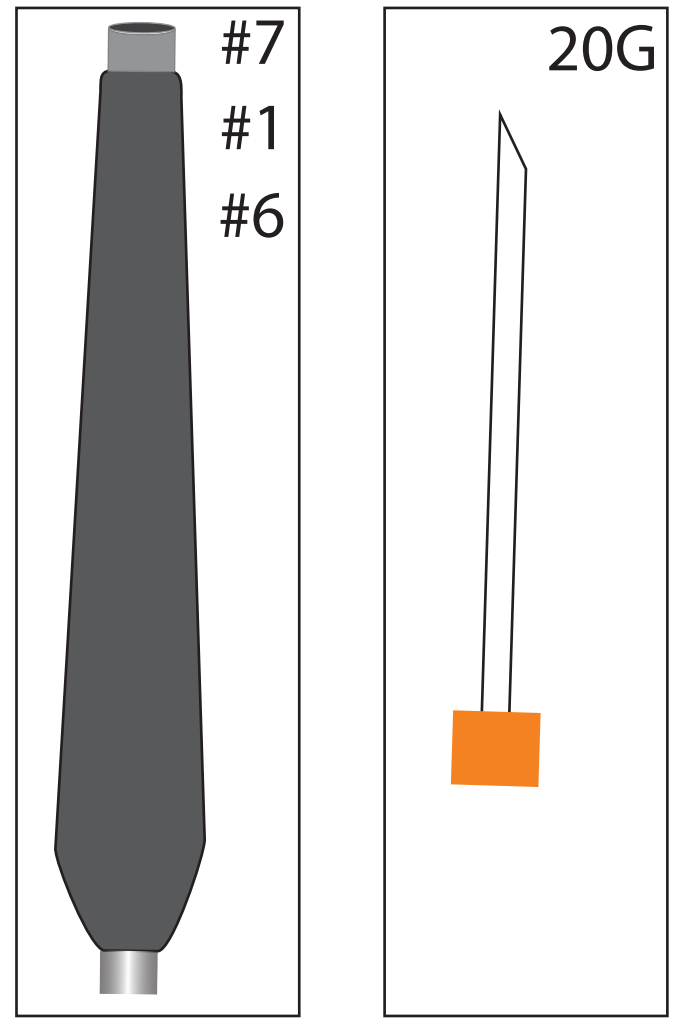
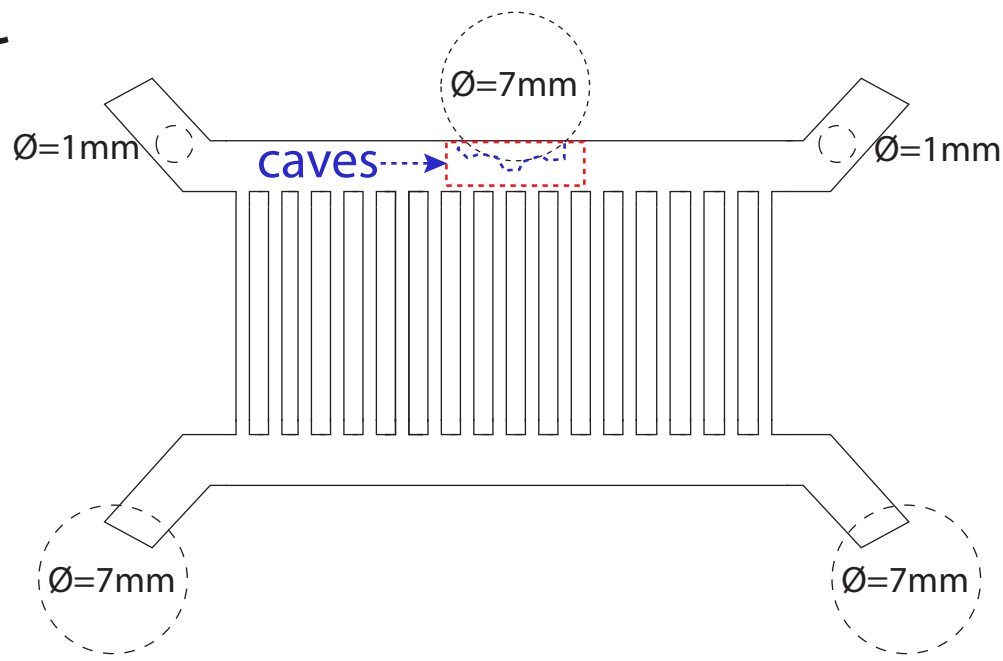

#7
#1
#6
20G

C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Ø=1mm
caves
Ø=1mm
Ø=7mm
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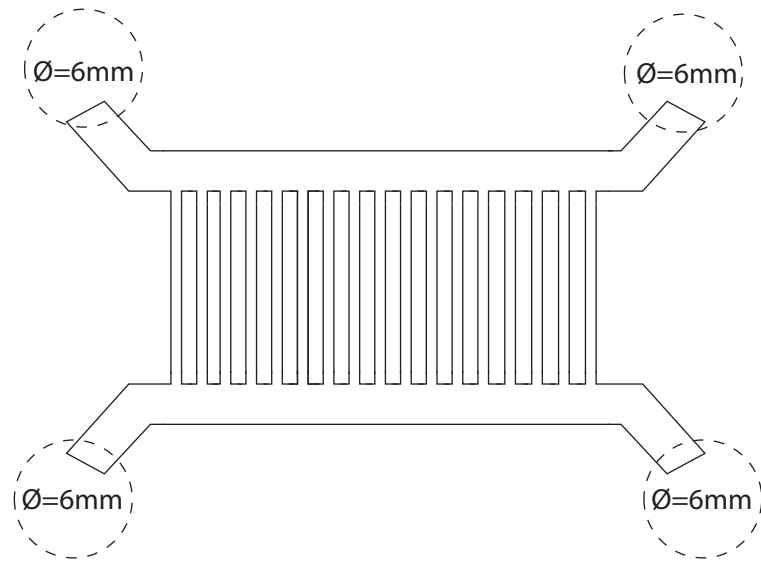
D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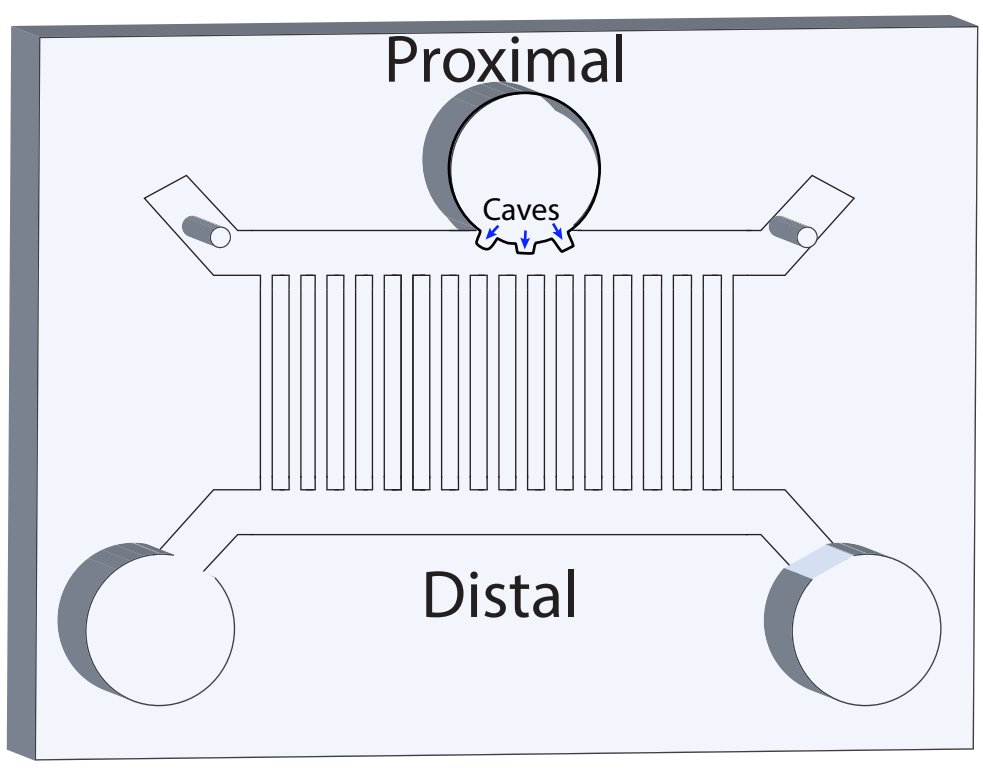
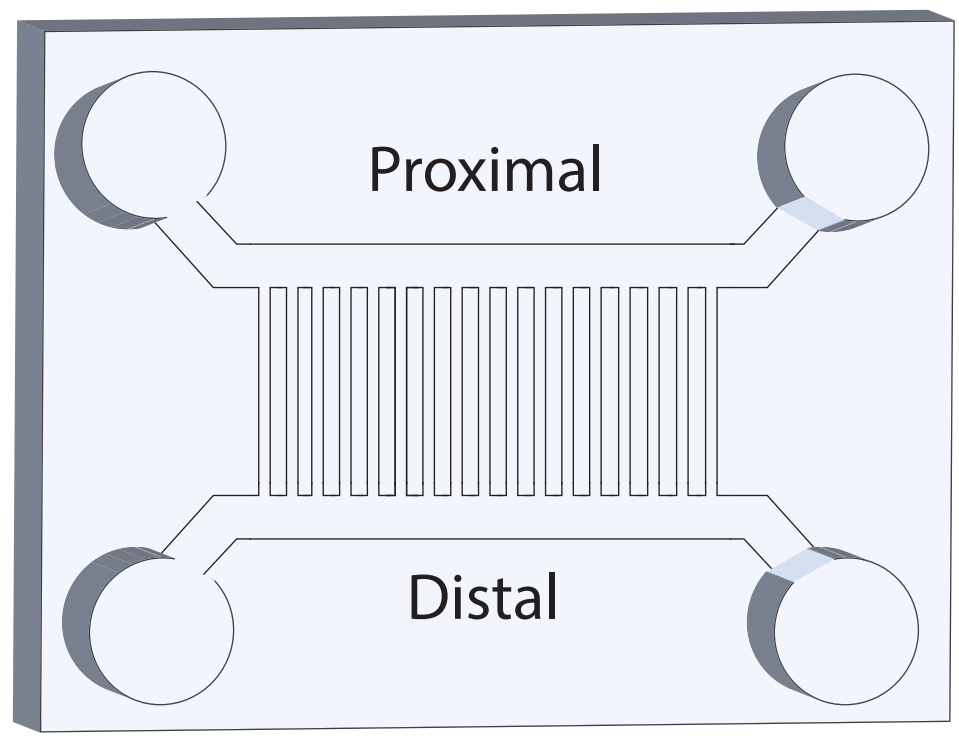
Ø=6mm
Ø=6mm
Ø=6mm

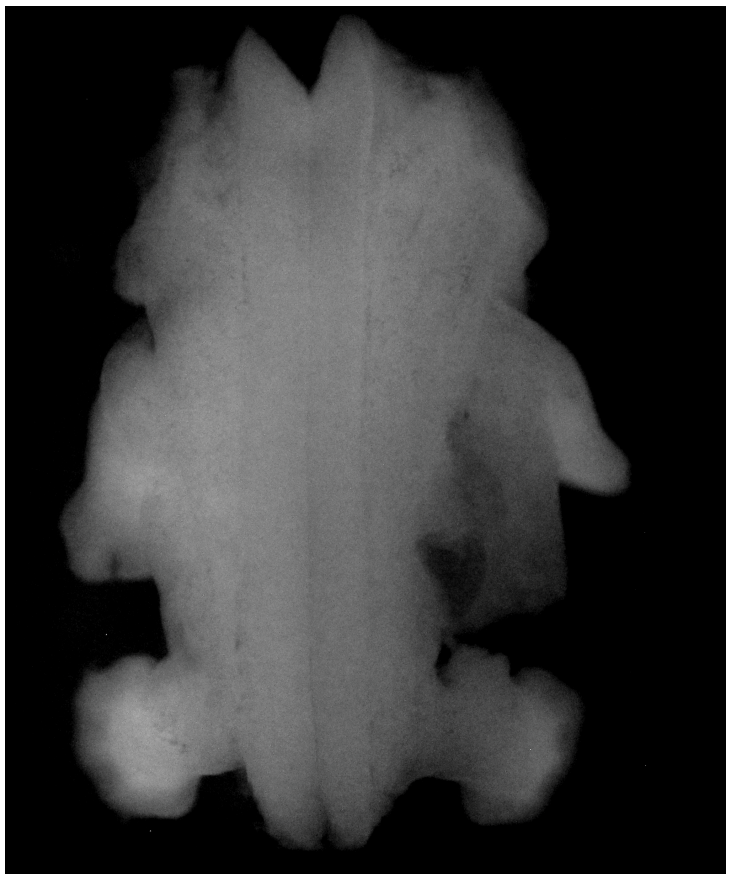
E


Proximal
Caves
Distal

F


Proximal
Distal

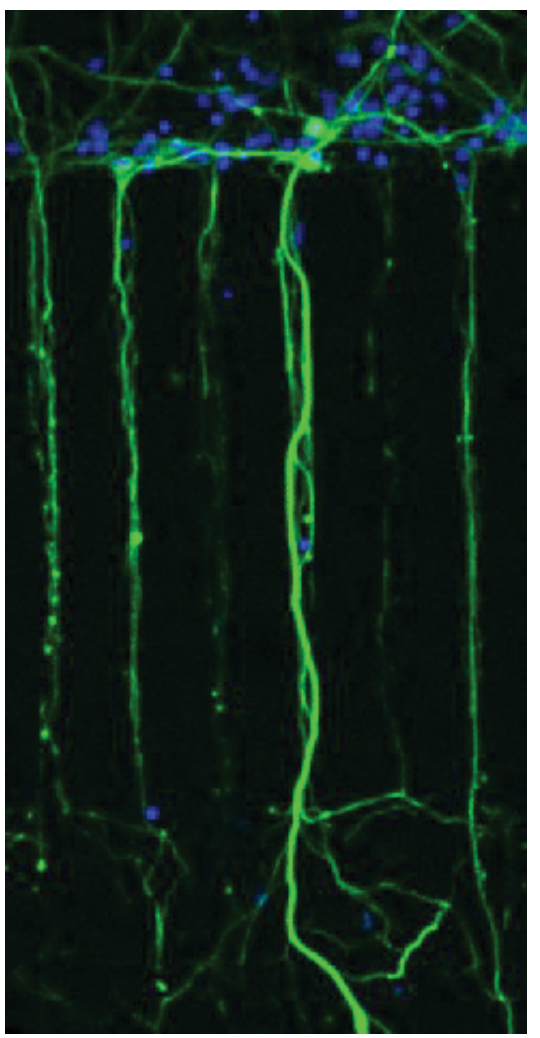
A



B

C

D Mass Culture



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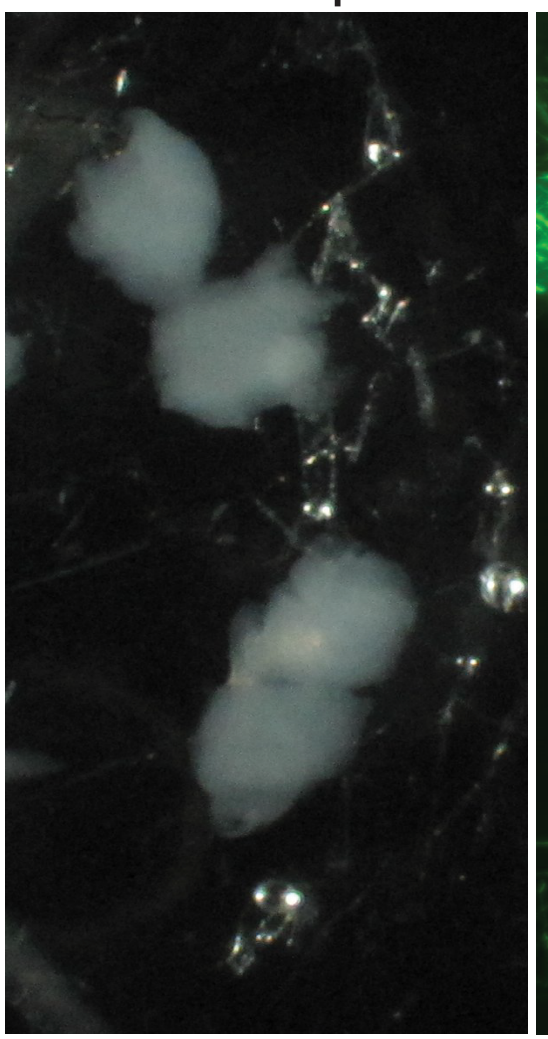

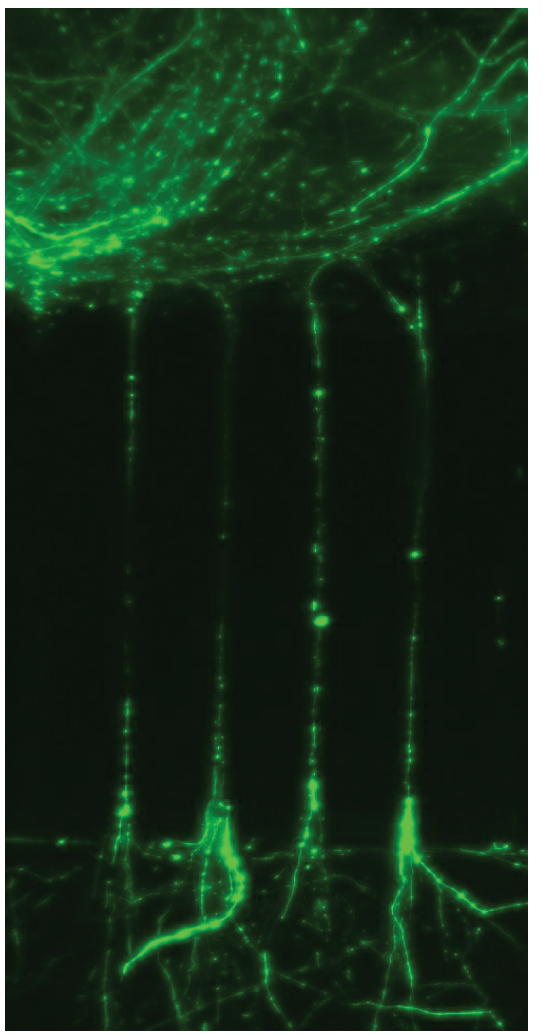

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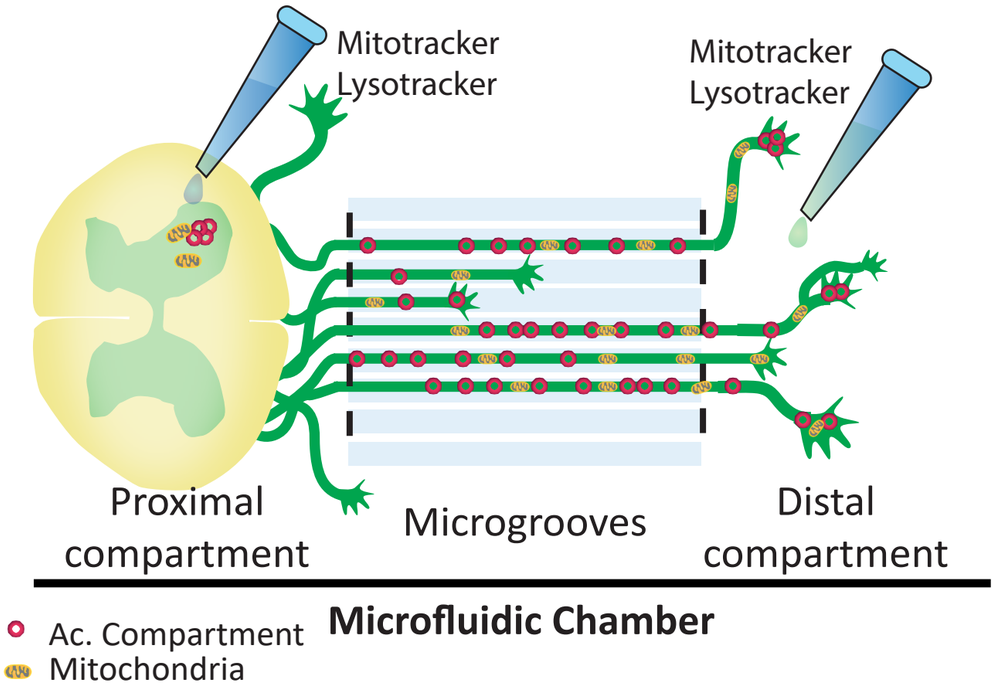
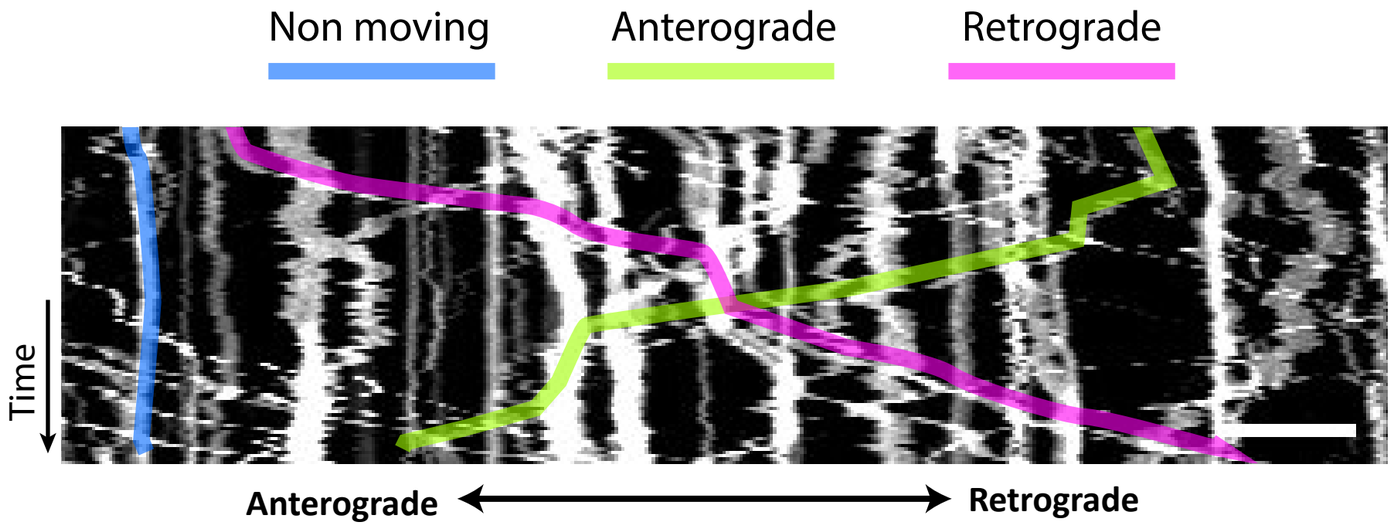
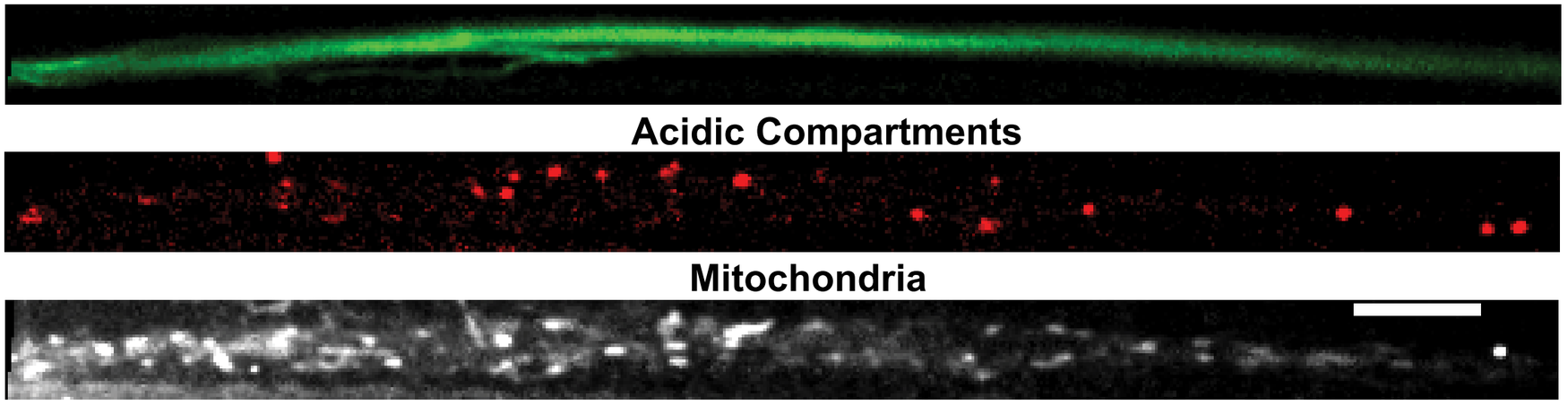

Mitotracker
Lysotracker
Mitotracker
Lysotracker
Proximal
compartment
Microgrooves
Distal
compartment
Microfluidic Chamber
Ac. Compartment
Mitochondria

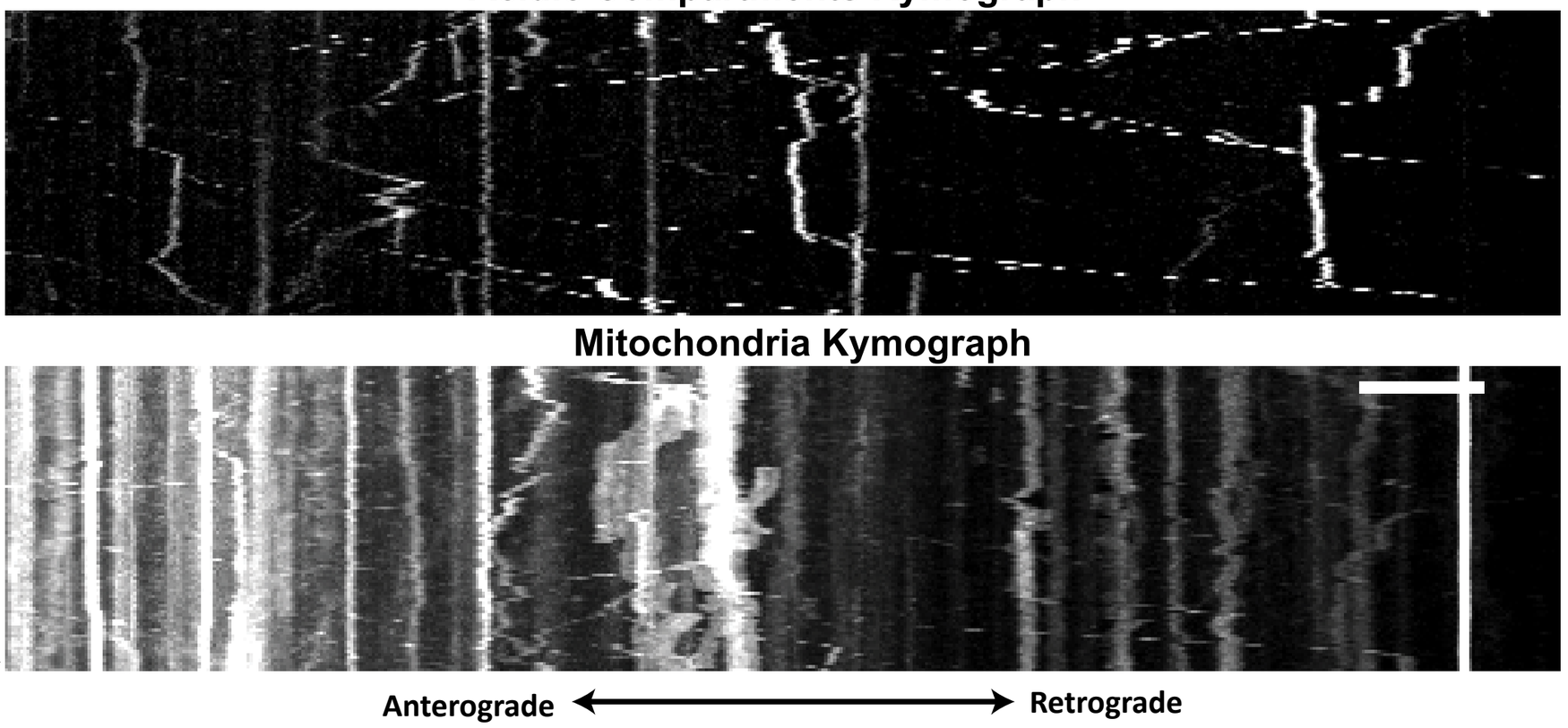
B


Non moving
Anterograde
Retrograde
Time
Anterograde
Retrograde

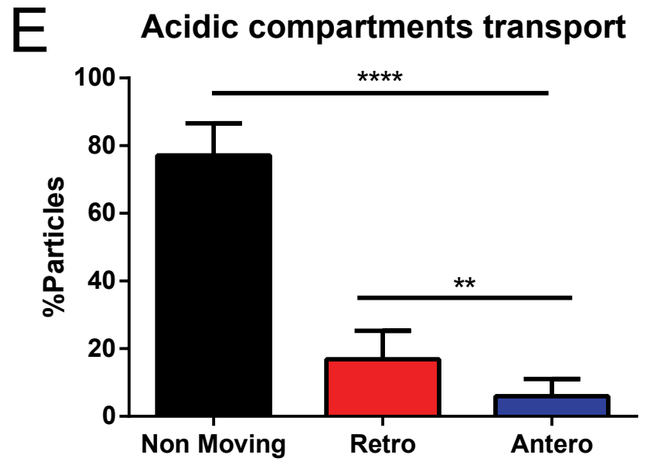
C


HB9::GFP
Acidic Compartments
Mitochondria

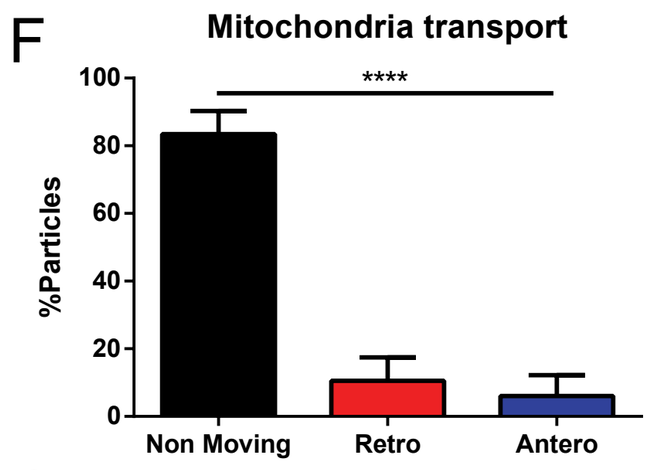
D


Acidic Compartments Kymograph
Mitochondria Kymograph
Time
Anterograde
Retrograde

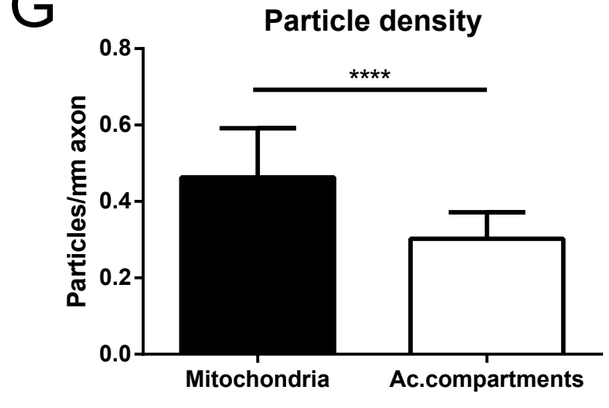
E


Acidic compartments transport
%Particles
100
80
60
40
20
0
****
**
Non Moving
Retro
Antero

F


Mitochondria transport
%Particles
100
80
60
40
20
0
****
Non Moving
Retro
Antero

G


Particle density
Particles/mm axon
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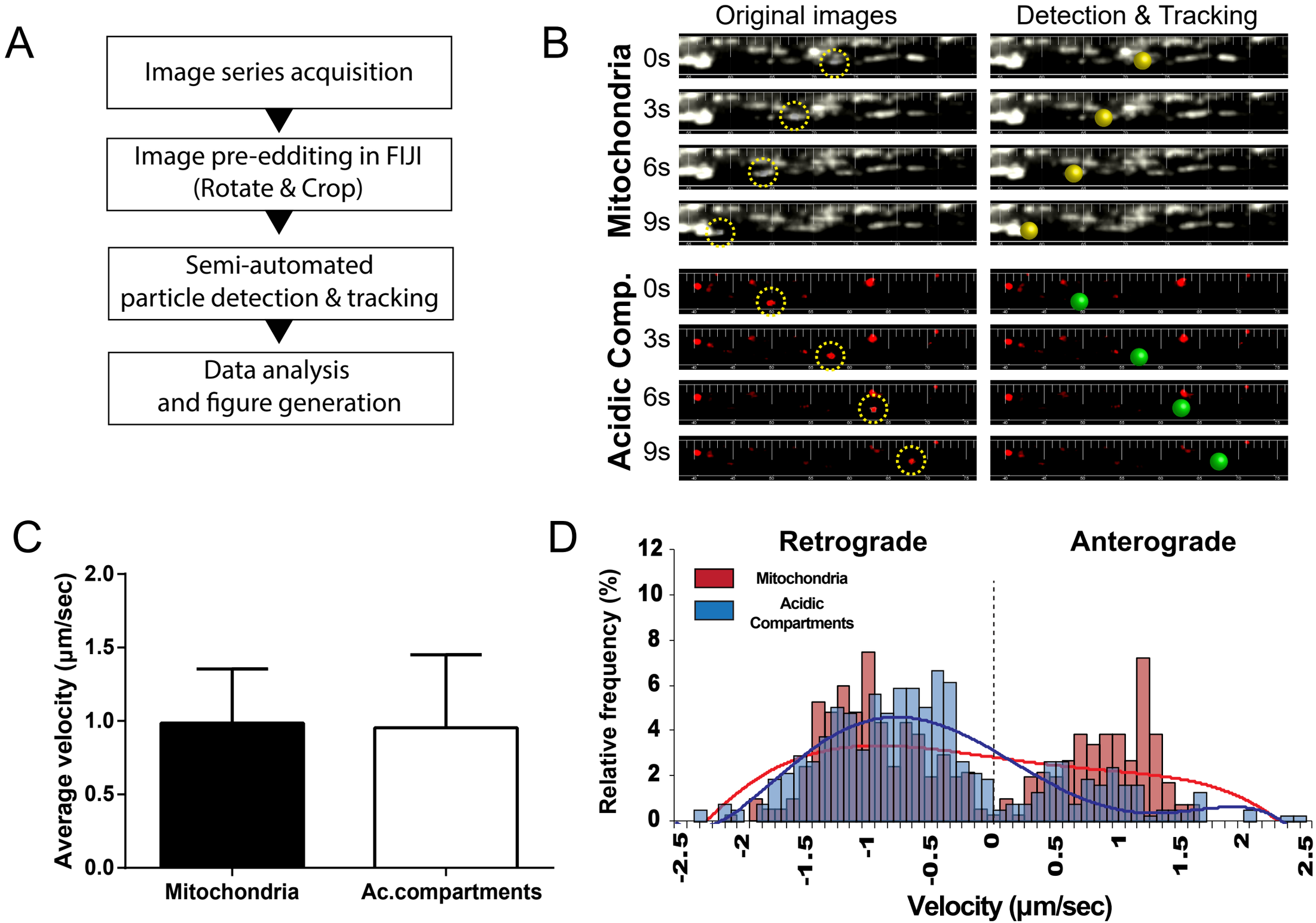
0.6
0.4
0.2
0.0
****
Mitochondria
Ac.compartments


A
Image series acquisition
Image pre-edditing in FIJI
(Rotate & Crop)
Semi-automated
particle detection & tracking
Data analysis
and figure generation
B
Original images
Detection & Tracking
Mitochondria
0s
3s
6s
9s
Acidic Comp.
0s
3s
6s
9s
C
Average velocity (µm/sec)
2.0
1.5
1.0
0.5
0.0
Mitochondria
Ac.compartments
D
Retrograde
Anterograde
Mitochondria
Acidic
Compartments
Relative frequency (%)
12
10
8
6
4
2
0
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Velocity (µm/sec)

Complete Neurobasal Medium – for 50n	
Ingredient	**Volume**
Neurobasal	47mL
B27	1 mL
Horse serum	1 mL
P/S	0.5 mL
L-Glutamine (Glutamax)	0.5 mL
Beta-Mercaptoethanol (50mM)	25 µL
BDNF (10ug/mL)	5 µL
GDNF (10ug/mL)	5 µL
CNTF (10ug/mL)	2.5 µL

nL
Concentration
2%
2%
1%
1%
25μM
1ng/mL
1ng/mL
0.5ng/mL

	Optiprep Solution - for 10mL
Ingredient	**Volume**
DDW	5.27 mL
Density Gradient Medium (Optiprep) 60%	1.73 mL
Tricine 100mM	1 mL
Glucose 20% (w/v)	2 mL

Concentration
10.4% (w/v)
2%
2%

Spinal Cord Explant Medium (SCX) – for 2(	
Ingredient	**Volume**
Neurobasal	19.5 mL
B27	200 µL
P/S	100 µL
L-Glutamine (Glutamax)	100 µL
BDNF	50 µL

)mL
Concentration
2%
1%
1%
25ng/mL

MN Culture
Longer Procedure prior to plating
Extra caution needed for plating in MFC
High concentration of motor neurons with no glial cells – more accurate
Unlimited manipulation possibilities on both Soma and axons
Easy immunostaining for both cell bodies and axons
High efficiency of viral infection

Spinal Cord Explants
Short procedure – Dissect & Plate
Easier to plate in the MFC
Presence of glial cells and other neuronal types – more physiological
Limited manipulation possibilities on cell bodies
Low efficiency during immunostaining of cell bodies within the explant.
Very low efficiency of viral infection

Name of Material/ Equipment	Company
35mm Fluodish – glass bottom dish	World Precision Instruments WPI
50mm Fluodish – glass bottom dish	World Precision Instruments WPI
Andor iXon DU-897 EMCCD camera	Andor
ARA-C (Cytosine β-D-arabinofuranoside)	Sigma-Aldrich
B-27 Supplement (50X)	Thermo Fisher
BDNF	Alomone Labs
Biopsy punch 1.25mm	World Precision Instruments WPI
Biopsy punch 6mm	World Precision Instruments WPI
Biopsy punch 7mm	World Precision Instruments WPI
Bitplane Imaris software - version 8.4.1	Imaris
Bovine Serum Albumine (BSA)	Sigma-Aldrich
Chlorotrimetylsilane	Sigma-Aldrich
CNTF	Alomone Labs
Density Gradient Medium - Optiprep	Sigma-Aldrich
Deoxyribonuclease I (DNAse) from bovine pancreas	Sigma-Aldrich
Dow Corning High-vacuum silicone grease	Sigma-Aldrich
DPBS 10X	Thermo Fisher
Dumont fine forceps #55 0.05 × 0.02 mm	F.S.T
Epoxy Hardener	Trias Chem S.R.L
Epoxy Resin	Trias Chem S.R.L
FIJI software	ImageJ
GDNF	Alomone Labs
Glutamax 100X	Thermo Fisher
HB9:GFP mice strain	Jackson Laboratories
HBSS 10X	Thermo Fisher
iQ software	Andor
Iris scissors, curved, 10 cm	AS Medizintechnik
Iris scissors, straight, 9 cm	AS Medizintechnik
Laminin	Sigma-Aldrich
Leibovitz's L-15 Medium	Thermo Fisher
LysoTracker Red	Thermo Fisher
Mitotracker Deep-Red FM	Thermo Fisher
Neurobasal medium	Thermo Fisher
Nikon Eclipse Ti micorscope	Nikon
Penicillin-Streptomycin (P/S) Solution	Biological Industries
Poly-L-Ornithin (PLO)	Sigma-Aldrich
Sylgard 184 silicone elastomer kit	DOW Corning Corporation
Trypsin from bovine pancreas	Sigma-Aldrich

Vannas spring microdissection scissors, 3 mm blade	F.S.T
Yokogawa CSU X-1	Yokogawa

Catalog Number	Comments/Description
FD35-100	
FD5040-100	
C1768	stock of 2mM in filtered DDW
17504044	
B-250	Dilute to 10µg/mL in filtered ddw wit
504530	For preperation of large MFC
504533	For preperation of small MFC
504534	For preperation of large MFC
#A3311-100G	5% w/v in ddw
#386529-100ML	
C-240	Dilute to 10µg/mL in filtered ddw wit
D1556	
DN-25	stock 10mg/mL in neurobasal
Z273554-1EA	For epoxy mold preperation
#14200-067	dilute 1:10 in ddw
1125520	
IPE 743	For epoxy mold preperation
RP 026UV	For epoxy mold preperation
G-240	Dilute to 10µg/mL in filtered ddw wit
#35050-038	
005029	
#14185-045-	Dilute 1:10 in ddw with addition of 1
11-441-10	
11-440-09	
#L-2020	
11415064	
L7528	
M22426	
21103049	
03-031-1	
#P8638	Dilute 1:1000 in flitered 1X PBS
#3097358-1004	
T1426	stock 25 mg/mL in 1XPBS

15000-00

h 0.01% BSA)

h 0.01% BSA)

h 0.01% BSA)

% P/S and filter

הפקולטה לרפואה ע"ש סאקלר
TEL AVIV UNIVERSITY אוניברסיטת תל אביב

September 26th 2019

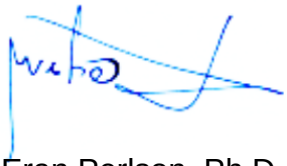
Dr. Alisha DSouza
Senior Review Editor
Journal of Visualized Experiments (JOVE)

Dear Dr. DSouza,

We are resubmitting our manuscript entitled: " **Axonal Transport of Organelles in Motor Neuron Cultures using Microfluidic Chambers System**", for publication in JOVE. Here we describe a detailed protocol for the fabrication of microfluidic chambers (MFCs), culturing and plating motor neurons or spinal cord explants in the MFCs, organellar staining and imaging and finally several methods for image analysis. Based on the editor and reviewer comments, we revised the manuscript and formatted the text according to the journal requirements. See below our detailed response in a point-by-point format.

We hope that you will agree with our answers supplied in response to the reviewers' comments, and that the modifications we did improved and strengthen this work. We are looking forward to your answer and will be glad if you find our modified work suitable for publication in JOVE.

Thank you for your consideration,
Sincerely,



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Editor comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We did a thorough reading of the manuscript and corrected all errors we found.

2. Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 415-420, 448-462, to avoid this overlap.

All the overlaps have been removed and new text was written instead.

3. Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in imperative voice: Lines 119, Lines 225-232

The text was edited to be written in imperative tense as needed in the specific locations suggested and in other locations as well. Several "Note" parts have been removed while others have been added or moved to the discussion.

4. Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Button clicks and specific numerical values were added every place it was possible.

Some examples:
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Ethics statement has been added.

2) Line 169: how is this done? If you have already described this, please reference the steps.

This section was edited to include more details regarding the mold preparation. The part including preparation of epoxy replicas was not included in previous publications but a reference to the primary molds preparation appears in point 1.1.1.

3) Line 270: what magnification?

The exact magnification was added.

4) Line 287: mention animal strain. Mention surgical tools used.

Details regarding animal strain were added, as well as description of the surgical tools. The exact surgical tool description appears in the materials table.

5. Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

All the numbering and indentations have been set as needed.

6. Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

All the noted points were acknowledged during the highlighting of the revised version.

7. Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1)

modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion was substantially extended to include all the points mentioned here.

8. Remove the embedded tables from the manuscript after lines 326, 265, 393. All tables must be numbered and upload the table as an individual excel files.

The embedded tables were removed and now appear as individual excel files.

9. Please spell out journal names.

Journal names have been spelled out in the reference section.

10. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are "Sylgard, scotch, Optiprep, Glutamax, Bitplane Imaris , MATLAB
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

All commercial names were removed wherever applicable and there was a generic name to replace the commercial name (i.e. PDMS base instead of Sylgard base). If needed (no generic name available), the name was addressed in parenthesis () and the reader was referred to the table of materials.

11. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All the figures are original and do not appear in any prior publication.

Reviewer 1:

Manuscript Summary:
This is a well described protocol for culturing motor neurons in microfluidic chambers and measuring axonal movements. All needed information is clearly presented, with one small caveat (see below).

1. The description of making a "cave" in the device to help when holding explants is not clear. Fig2 does not show where the "cave" is located. This can easily be improved.

The figure was corrected to show the exact location of the “caves”

2. There is no discussion/comparison of this protocol with already published ones. E.g., the authors do not use plasma bonding. Could they say why?

We shortly compared the protocol to other protocols in the introduction and discussion parts.
Regarding plasma bonding, we referred to its use in the main text, second paragraph of the discussion, point number 3. Briefly, we do not use plasma bonding as it is irreversible, thus not allowing to recycle and clean the chambers and glass plates in a through way as de-attachment allows. However, it is a valid option and we refer to it as such.

Reviewer 2:

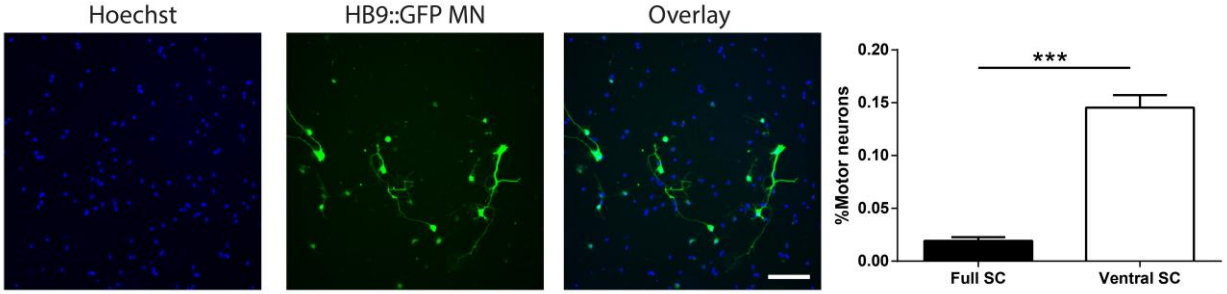
Altman et al described a method that enables monitoring of mitochondrial and acidic transport in motor neurons. The authors utilized a compartmentalized chamber to separate somatic and axonal regions of motor neurons. Then, they labeled mitochondria and lysosomes with Mitotrack and Lysotracker respectively. Lastly, they explained how to analyze the data from the culture. Overall, the figures are very clear and easy to follow, which will be appreciated by many readers. However, there is still room for improvement. The readers would have more advantage from the manuscript if it included more details.

1. Include the purity of the motor neurons which could be obtained by following the method. Although the manuscript mentioned to remove the dorsal horns, there must be partial contamination of other neurons.

A “note” comment regarding the culture purity was added to the main text (after point 2.1.20). The motor neuron culture indeed contains other cell types, mainly interneurons, and 15% of HB9+ motor neurons. To have a pure MN culture, you need methods such as FACS (Referenced in the paper. The exact method appears in Schaller et al, 2017). As we wrote in the main text, we recommend the use of a transgenic mouse strain such as HB9::GFP to validate that the imaged axons are motor neurons.

2. Include the data that demonstrate the isolated cells are motor neurons.

In this provided figure, we compared our HB9+ culture of spinal cord with or without dorsal horn removal. The HB9+ cell % is calculated as number of nuclei that are colocalized with HB9::GFP divided by the total nuclei. As you can see, we achieve ~15% motor neurons in our ventral spinal cord purification and culture. We know those are motor neurons because they are positive for HB9, a known MN differentiation factor (Wichterle et al, 2002) that is the common “gold standard” regarding MN identity in culture.



3. Fix the typo (37oC) at page 12, line 402.

The typo was fixed.

4. What is the maximal time which could be used for the time-lapse image? The manuscript described the time-lapse series of axonal transport for 5 minutes per movie. However, this time window will be too short for certain studies.

A “note” comment was added in the main text after point 3.2.1. As we write there, longer or shorter time-lapse movies can be imaged, dependent on the exact experiment. Even over-night movies can be imaged if needed. However, it is critical to try and reduce the exposure time and laser power, as well as the number of total images, to prevent phototoxicity and bleaching during movie acquisition.

5. Describe whether spinal cord explants or motor neuron cultures are used to produce the Figure 4 & 5. Although the authors used MNs in the figure legend, it can still confuse the readers.

We clarified this using an illustration in figure 4A, in the main text and in the figure legends.

6. Expand the discussion to emphasize the importance of this method.

The discussion was expanded to highlight the variety of experimental possibilities this method can allow the readers, in a simplified and relatively inexpensive approach.