Analysis of mitochondrial membrane potential in neuronal subcompartments using microfluidic devices

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Summary
This article describes the method for seeding and staining neuronal mitochondria in microfluidic chambers. The fluidic pressure gradient in these chambers allows the selective treatment of mitochondria in axons to analyze their properties in response to pharmacological challenges without affecting the cell body compartment.

Abstract
Mitochondrial dysfunction is a common phenotype in many neurodegenerative diseases. Given the elaborate architecture and extreme length of some axons, it is not surprising that mitochondria in axons can experience different environments compared to their cell body counterparts. Interestingly, dysfunction of axonal mitochondria often precedes effects on the cell body. In order to model axonal mitochondrial dysfunction *in vitro*, microfluidic devices allow treatment of axonal mitochondria without affecting the somal mitochondria. The fluidic pressure gradient in these chambers prevents diffusion of molecules against the gradient, thus allowing for analysis of mitochondrial properties in response to local pharmacological challenges within axons. Here, we describe the seeding of dissociated hippocampal neurons in microfluidic devices, staining with a membrane-potential sensitive dye, and treatment with a mitochondrial toxin as well as the subsequent microscopic analysis. This versatile method to study axonal biology can be applied to many pharmacological perturbations and imaging readouts and is suitable for several neuronal subtypes.

Introduction

Mitochondria are the main suppliers of ATP (adenosine triphosphate) in neurons. As neuronal health is intimately linked to mitochondrial function, it is not surprising that dysfunctional regulation of these organelles has been associated with the onset of various neurodegenerative

diseases including Parkinson's disease[1]. Furthermore, mitochondrial intoxication has successfully been used to model Parkinsonian symptoms in animals[2]. In both animal models and the human disease, the demise of neurons starts at the distal parts[3,4], hinting that axonal mitochondria might be more susceptible to insults. However, the biology of mitochondria specifically in axons is not well understood due to the difficulties associated with targeted treatment and analysis of axonal mitochondria without simultaneous disturbance of cell body processes.

Recent advances in culturing techniques of dissociated neurons *in vitro* now allow the fluidic separation of axons and cell bodies through microfluidic devices[5]. As depicted in figure 1, these devices feature four access wells (**a**/**h** and **c**/**i**), with two channels connecting each pair (**d** and **f**). The large channels are connected with each other by a series of 450 µm long microchannels (**e**). Intentional differences in the fill levels between the two chambers create a fluid pressure gradient (Fig. 1B) that prevents diffusion of small molecules from the channel with lower fluid level to the other side (Fig. 1C illustrated with Trypan blue dye).

We recently used microfluidic devices to study the requirement for local translation in axonal mitophagy, the selective removal of damaged mitochondria[6]. Presented here are the different steps that allowed us to induce local mitochondrial damage through selective treatment of axons using the mitochondrial complex III inhibitor Antimycin A.

Protocol

1. Preparation of microfluidic devices

1.1. Coat one 6-well glass bottom tissue culture plate with a final concentration of 20 µg/mL Poly-D-Lysine and 3.4 µg/mL Laminin in PBS (phosphate-buffered saline).

1.2. Incubate overnight in the dark at room temperature.

NOTE: Coating can also be performed at 37 °C for 1-2 hours.

NOTE: Choice of coating depends on the cell type used.

1.3. Bring the 6-well plate to the sterile hood.

1.4. Wash 2 times with sterile ddH_2O.

NOTE: Do not wash with salt containing buffers such as PBS, as salt crystals will interfere with seal formation.

1.5. Allow the plate to dry in a tilted position for 3-5 min.

1.6. Remove excess water by vacuum suction or pipetting.

1.7. Soak the microfluidic chamber in 80% ethanol.

1.8. Allow the microfluidic chamber to dry for 3-5 min in a tilted position.

1.9. Remove excess ethanol by vacuum, suction or pipetting.

1.10. When completely dry, place the microfluidic chamber in the center of the well.

NOTE: The formation of the seal can be observed as a change in reflective properties upon exclusion of air from the interface between the plate and the silicone device.

NOTE: Both the plate and microfluidic chamber need to be completely dry before assembly to create a good seal. However, drying time should be minimized as much as possible to ensure proper adherence of the cells. We therefore visually inspect the wells and chambers for remaining droplets of liquid. If no droplets are visible immediately assemble the chambers.

1.11. Gently tap the microfluidic chamber at its borders and microgroove section in the middle for proper attachment to the glass plate.

2. Seeding and maintaining of neurons.

2.1. Collect the desired number of dissociated hippocampal neurons per chamber in a 1.5 mL reaction tube. We usually seed $1.5x10^5$ neurons per device for imaging-based applications.

2.2. Centrifuge neurons at 1000 xg for 4 min.

2.3. Discard the supernatant and resuspend the pellet in 8 µL of B27-Neurobasal media.

2.4. Pipet the cell solution into the entrance of the channel (**b** in Fig. 1A).

2.5. Tap on the back of the plate to assist flow through the channel.

NOTE: Tapping can be done quite forcefully without worry that the seal will break.

2.6. Aspirate any remaining cell suspension at the exit of the channel (labelled **g** in Fig. 1A) to decrease number of cells outside the channel.

2.7. Incubate the microfluidic chamber with cells for 15-20 min at 37 °C and 5% CO_2.

2.8. Fill 50 µL of B27-Neurobasal media in the top axonal well (**c**).

2.9. Tap on the back of the plate to assist flow through the channel.

2.10. Fill wells on the soma side (**a** and **h**) with 150 µL B27-Neurobasal media each, creating a tension bubble on top. The volume of the bubble is about 20 µl.
NOTE: Creation of a tension bubble is not necessary, but it provides a clear visual of the higher volume on the soma side.

2.11. Fill both wells of the axonal side (**c** and **i**) with 100 µL B27-Neurobasal media each.

NOTE: To promote axonal growth through the microgrooves, it is recommended that the wells on the soma side contain more media than the wells on the axon side. However, axons will by chance grow through the microgooves without volume differences.

2.12. Incubate the microfluidic chamber at 37 °C and 5% CO_2 for 7-8 days.

NOTE: Growth of axons into the axonal chamber can usually be observed starting at *day in vitro* (DIV) 5. Longer culturing times are possible if more mature cultures are desired.

2.13. Feed neurons every 2-3 days by removing medium from the two upper wells (**a** and **c**) and replacing it with fresh B27-Neurobasal medium until a tension bubble is formed.

3. Staining with mitochondrial membrane potential sensitive dye

3.1. At DIV7-8, assess that the axons have grown through the microgrooves and extend into the axonal compartment underneath a light microscope.

NOTE: Different DIV stages are also possible depending on the desired age.

3.2. Perform 2 washes with pre-warmed imaging medium (37 °C) by removing the B27-Neurobasal medium from all wells and adding about 100 µl of the imaging medium to the top wells of both the axonal and soma channels (**a** and **c**) and let the medium flow through to the lower wells (**h** and **i**). Remove the medium from the lower wells as well as any leftover medium in the top wells, and repeat with fresh medium, leaving the wells empty at the end of the wash.

NOTE: Due to the high capillary force in the channels (**d** and **f**) there will be remaining wash medium in the channels which should not be removed to prevent the neurons from drying.

NOTE: Any phenol-red free imaging medium can be used here, as for instance Hibernate E. If the microscope is not set up with a CO_2 supply, ensure that the imaging medium uses an alternative buffering system to carbonate/CO_2 (E.g. phosphate).

3.3. Dilute a 1 mM tetramethylrodamine ethyl ester (TMRE, red) stock to 5 nM in imaging medium.

NOTE: Do not exceed 1% DMSO in the final dilution to avoid toxicity.

3.4. Add 100 µL of the 5 nM TMRE dilution to both the somatic and axonal top wells (**a** and **c**) and let the medium flow through both the somatic- and axonal compartments until there is an equal volume in all wells. Fill up with TMRE-containing medium.

3.5. Place the neurons back into a controlled environment chamber (37 °C and 5% CO_2) for 25 min.

3.6. Perform 2 washes with pre-warmed imaging medium (37 °C) as described before. On the last wash, fill up with 100 µL in each well and create a tension bubble of medium on the somatic wells (**a** and **c**).

4. Live-cell imaging

NOTE: still images shown were acquired on a spinning disk confocal, using a 40X NA 1.25 immersion objective. 200 ms exposure time and 10% laser power for the red channel and 500 ms exposure time for brightfield were chosen. However, also regular confocal or widefield inverted microscopes can be used to study TMRE intensity.

4.1. Image the cells with an inverted microscope.

4.2. Ensure that the microscope is equipped with a stage incubator to maintain the neuronal culture at 37 °C throughout the experiment.

4.3. Select a region of interest in the somatic compartment, and follow it through the microgrooves into the axonal compartment. Ensure that the microgrooves imaged matches those in the somatic as well as the axonal compartment (for easier baseline and post-treatment comparison).

4.4. Capture fluorescence images using excitation at 561 nm and emission at 625 nm for red fluorescence signal.

4.5. Acquire the images.

4.6. To induce mitochondrial depolarization, add 20 µM Antimycin A in imaging medium to the axonal compartment only. To ensure a volume difference between the soma and axonal chambers, check the presence of a tension bubble on the somatic side (equals volume > 110 µl). Remove all imaging medium from the lower well of axonal side (**i**), except for the medium inside the channel (**f**). Add 160 µL 20 µM Antimycin A to the top axonal well (**c**) and let it flow through the channel until there is an equal volume in both axonal wells (Volume in axonal wells roughly 80µl per well).

NOTE: Make sure that the volume in the axonal wells are smaller than in the somatic wells to ensure proper fluid pressure. We recommend a difference of at least 10 µl (10% of the well volume), but also higher differences are possible.

4.7. Incubate neurons in the controlled environment chamber (37 °C) for 20-30 min.

4.8. Repeat imaging as described above. Make sure that you image the same positions (easily identified by the microgrooves) as during baseline acquisition.

Representative Results

Primary hippocampal neurons were grown in microfluidic devices for 7-8 days before mitochondria were stained with the membrane sensitive dye TMRE for 25 min in both channels. As shown in figure 2A, this yielded a homogenous staining of mitochondria on both sides of the microgrooves, yet was not sufficient to equilibrate the staining into the middle of the microgrooves. Upon addition of Antimycin A to the axonal side, somal mitochondria retained the TMRE signal (Fig. 2B and Video 1), whereas TMRE fluorescence was lost from axonal mitochondria (Fig. 2C and Video 1).

Figure and video legends

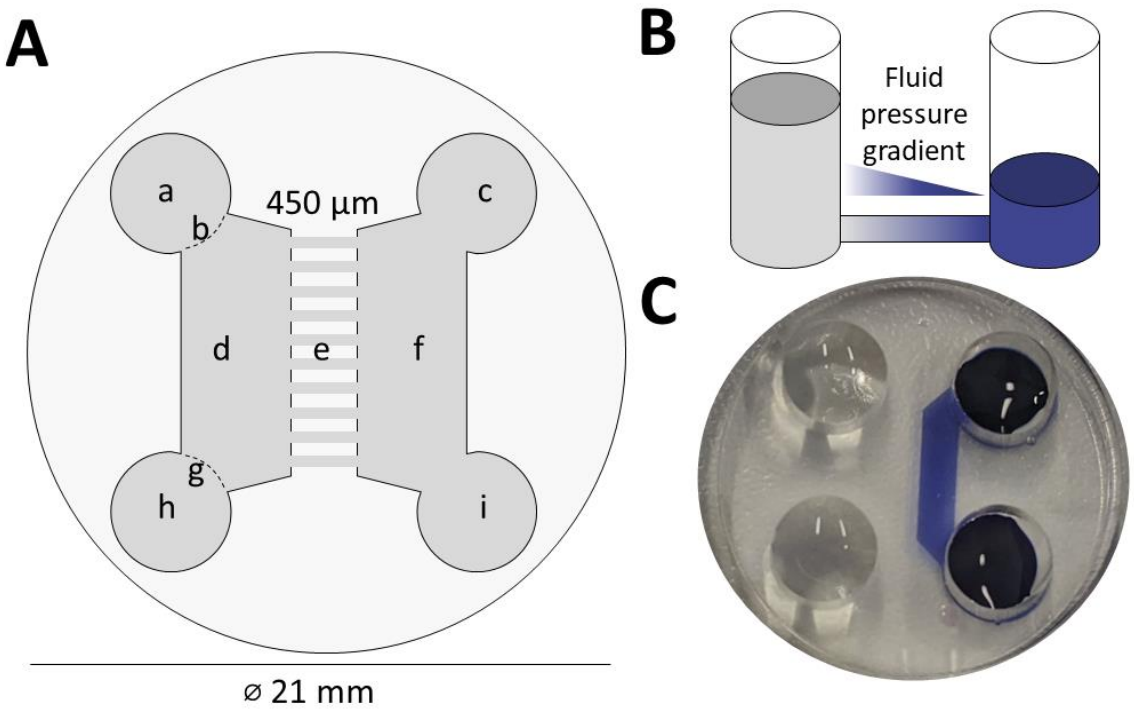

Figure 1: Microfluidic devices allow the fluidic isolation of axons. (**A**) Schematic of microfluidic device used in this experiment. The silicone disk (diameter 21 mm) fits easily into a 6 well plate. a) Well on soma side, b) Entrance of channel on soma side, c) Well on axon side, d) Channel on soma side, e) Microgrooves, f) Channel on axon side, g) Exit of channel on soma side, h) Well on soma side, i) Well on axon side. (**B**) Schematic detailing how the different fluid levels create a fluidic pressure gradient across the microchannels. (**C**) Demonstration of fluidic isolation by addition of Trypan blue to one side of the chamber. Note that due to the fluidic pressure gradient the blue dye does not equilibrate across the microchannels.

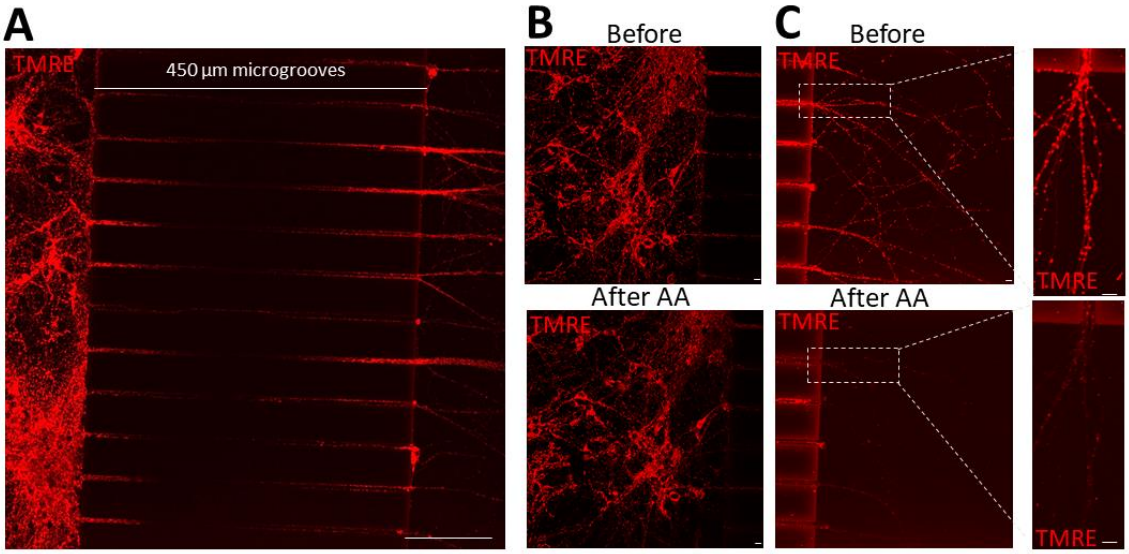

Figure 2: Selective treatment of axons depolarizes axonal but not cell body mitochondria. (**A**) Representative micrograph presenting an overview of the effectivity of TMRE staining in

microfluidic devices. Scale bar 100 µm. (**B-C**) Representative micrograph showing the same area before and after addition of 20 µM Antimycin A (AA) in the cell body and the axonal compartment, respectively. Scale bar: 10 µm.

Video 1: Effect of Antimycin A treatment on the mitochondrial membrane potential in axons and cell bodies. Live cell imaging of the loss of TMRE fluorescence upon addition of Antimycin A, imaged on an EVOS M5000 widefield microscope.

Discussion

Here, we describe a method to seed and culture dissociated hippocampal neurons in a microfluidic device in order to separately treat axonal mitochondria. We are showcasing the utility of this approach with the membrane sensitive dye TMRE and the complex III inhibitor Antimycin A (as previously demonstrated[7]), but this method can be easily adapted to other mitochondrial dyes or genetically encoded sensors of mitochondrial functions that allow local, microscopy based readouts[8]. Other neuronal cell types can also be grown in microfluidic chambers, such as primary cortical neurons[9] or iPSC-derived motorneurons[10], making this platform a versatile tool to study mitochondrial function in neurodegeneration in the neuronal cell type of interest. The assembly of microfluidic devices is a crucial step to achieve an efficient seal and is most easily explained by watching an experienced researcher perform the assembly. The downstream labelling and treatment procedure described here are meant to only be exemplary and may be adjusted to fit the protocols currently established in the respective labs, while maintaining the fluidic pressure gradient.

The seeding technique we describe here differs from published protocols and the manufacturer's description as we skip the suggested washes of the assembled device and rather directly seed the dissociated neurons into the dry chamber (Step 2). We have found that this reduces the number of neurons needed, as it increases the density of neurons within channel **e** and limits the spread of neurons into the wells far away from the microchannels. The dry seeding is aided by tapping of the bottom of the plate (Step 2.5), which may or may not be necessary depending on the force applied previously when assembling the device. There is some variability to the tightness of the seal due to differences in force applied during assembly that may lead to restricted growth through the microgrooves. Also, remaining moisture can disturb the seal formation and allow axonal growth or even cell migration underneath the device. Both problems can easily be spotted prior to staining (compare step 3.1), leading to the exclusion of faulty chambers from the experiment.

Disclosures

The authors declare no competing interests.

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References

1. Murali Mahadevan, H., Hashemiaghdam, A., Ashrafi, G. & Harbauer, A. B. Mitochondria in Neuronal Health: From Energy Metabolism to Parkinson's Disease. *Adv. Biol.* **5**, 2100663 (2021).
2. Dauer, W. & Przedborski, S. Parkinson's disease: mechanisms and models. *Neuron* **39**, 889–909 (2003).
3. Moratalla, R. *et al.* Differential vulnerability of primate caudate-putamen and striosome-matrix dopamine systems to the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3859–3863 (1992).
4. Cheng, H.-C., Ulane, C. M. & Burke, R. E. Clinical progression in Parkinson disease and the neurobiology of axons. *Ann. Neurol.* **67**, 715–725 (2010).
5. Taylor, A. M. *et al.* A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nat. Methods* **2**, 599–605 (2005).
6. Harbauer, A. B. *et al.* Neuronal mitochondria transport Pink1 mRNA via synaptojanin 2 to support local mitophagy. *Neuron* (2022). doi:10.1016/j.neuron.2022.01.035
7. Ashrafi, G., Schlehe, J. S., LaVoie, M. J. & Schwarz, T. L. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. *J. Cell Biol.* **206**, 655–70 (2014).
8. Harbauer, A. B., Schneider, A. & Wohlleber, D. Analysis of Mitochondria by Single-Organelle Resolution. *Annu. Rev. Anal. Chem. (Palo Alto. Calif).* 1–16 (2022). doi:10.1146/annurev-anchem-061020-111722
9. Taylor, A. M. *et al.* Axonal mRNA in uninjured and regenerating cortical mammalian axons. *J. Neurosci.* **29**, 4697–707 (2009).
10. Altman, T. *et al.* Axonal TDP-43 condensates drive neuromuscular junction disruption through inhibition of local synthesis of nuclear encoded mitochondrial proteins. *Nat. Commun.* **12**, 6914 (2021).