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Use of pre-assembled plastic microfluidic chips for compartmentalizing primary murine neurons

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Alisha Dsouza, Ph.D.
Senior Review Editor, JoVE

Dear Alisha,

We are pleased to re-submit our manuscript entitled “Use of pre-assembled plastic microfluidic chips for compartmentalizing primary murine neurons” to JoVE. We are encouraged by the reviewer’s comments and are happy to note that they did not have any major concerns. I believe that our current revision addresses all their minor concerns.

As requested we have included a detailed response to all the points raised by the editor and reviewers. We also tracked our changes to the manuscript file. As suggested by one reviewer, we have now added a table comparing the plastic multi-compartment chip with traditional PDMS-based devices. We also added an additional figure showing side-by-side neuronal growth within these two types of multi-compartment devices. Overall, I believe the additions strengthen the manuscript substantially.

I appreciate your time and effort working on this manuscript and I look forward to publishing with JoVE.

Sincerely,

Anne Marion Taylor, Ph.D.
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TITLE:

Use of Pre-Assembled Plastic Microfluidic Chips for Compartmentalizing Primary Murine Neurons

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Microfluidic chamber, neuron culture, hippocampal neurons, XonaChip, microfluidic chip, cultured neurons, compartmentalized chamber, primary neurons, chip, axon isolation

SHORT ABSTRACT:

This protocol describes the use of plastic chips to culture and compartmentalize primary murine neurons. These chips are preassembled, user-friendly, and compatible with high-resolution, live, and fluorescence imaging. This protocol describes how to plate rat hippocampal neurons within these chips and perform fluidic isolation, axotomy and immunostaining.

LONG ABSTRACT:

Microfabricated methods to compartmentalize neurons have become essential tools for many neuroscientists. This protocol describes the use of a commercially available pre-assembled plastic chip for compartmentalizing cultured primary rat hippocampal neurons. These plastic chips, contained within the footprint of a standard microscope slide, are compatible with high-resolution, live, and fluorescence imaging. This protocol demonstrates how to retrograde label neurons via isolated axons using a modified rabies virus encoding a fluorescent protein, create isolated microenvironments within one compartment, and perform axotomy and immunocytochemistry on-chip. Neurons are cultured for >3 weeks within the plastic chips, illustrating the compatibility of these chips for long-term neuronal cultures.

INTRODUCTION:

Traditional neuron culture approaches result in random outgrowth of axons and dendrites, which prevent the study of neurons in their unique polarized morphology. Microfabricated

multicompartment devices have become well-established and well-used research tools for neuroscientists in the last 10–15 years (selected high-profile publications are referenced[1-17]). These devices compartmentalize neurons and provide a method to physically and chemically manipulate subcellular regions of neurons, including somata, dendrites, axons, and synapses[18,19]. They also provide multiple experimental paradigms that are not possible using random cultures, including studies of axonal transport, axonal protein synthesis, axon injury/regeneration, and axon-to-soma signaling. The basic 2-compartment configuration consists of two parallel microfluidic channels separated by a series of smaller perpendicular microgrooves. Primary or stem cell-derived neurons are plated into one of the microfluidic channels, settle and attach to the bottom surface of the device, and extend neurites over the course of days. Many growth cones find their way into the microgrooves, which are small enough that they prevent cell bodies from entering. Because growth cones are physically restricted and unable to turn around within the microgrooves, they grow straight into the adjacent compartment (axonal compartment) where they are isolated.

Historically, these devices have been molded using poly(dimethylsiloxane) (PDMS) from a photolithographically patterned master mold and are either made in-house in investigators' laboratories or purchased commercially. One of the main drawbacks of using PDMS is its hydrophobicity[20]. PDMS can be made hydrophilic temporarily, but then quickly becomes hydrophobic within hours in a non-aqueous environment[20]. Because of this, the devices must be attached to a glass coverslip or other suitable substrate at the time of use. Pre-assembled plastic multicompartment chips are now commercially available (*e.g.*, XonaChips) in injection molded plastic. These chips are made permanently hydrophilic, simplifying device wetting and allowing the pre-assembly of the chip with a thin film of cyclic olefin copolymer (COC) enclosing the microfluidic channels on the bottom. These chips are fabricated in an optically transparent plastic suitable for high-resolution fluorescence imaging.

The purpose of this protocol is to demonstrate the use of the pre-assembled plastic microfluidic chips for multiple experimental paradigms performed using murine hippocampal or cortical neurons. This protocol describes how to retrograde label neurons using a modified rabies virus within the chip. Axotomy for studies of axon injury and regeneration are also described. Lastly, this protocol shows how to perform fluorescence immunostaining with the device.

PROTOCOL:

Note: A schematic of the plastic multicompartment chip is shown in **Figure 1A, B**. The chip is the size of a standard microscope slide (75 mm × 25 mm). The features of the chip, including main channels or compartments, wells, and microgrooves are labeled and are provided for future reference. **Figure 1C** is a photograph of the chip demonstrating the fluidic isolation of the compartments.

1. **Preparation and Coating of the Multicompartment Chips**

1.1 In a bio-safety cabinet, place the chip into a Petri dish or other suitable sterile container.

1.2 Add 100 µL of pre-coating solution to the upper left well of the chip and allow it to flow through the main channel into the adjoining well.

Note: The pre-coating solution is used to pre-coat the microfluidic channels to eliminate the potential for trapping air bubbles within the chip.

1.3 Fill the lower left well with 100 µL of pre-coating solution. Wait 5 min to allow the solution to flow through the microgrooves.

1.4 Add 100 µL of pre-coating solution to the upper right well and allow it to flow through the main channel into the adjoining well. Fill the lower right well with 100 µL of pre-coating solution.

1.5 Aspirate the solution from each well. Aspirate away from the main channels to avoid removing liquid from the main channels (**Figure 2A**). Immediately add 150 µL of phosphate-buffered saline (PBS) to the upper left well. Wait 1.5 min.

CAUTION: Do not aspirate all liquid from the enclosed main channels.

1.6 Add 150 µL PBS to the lower left well. Wait 5 min to allow liquids to flow through the microgrooves. Add 150 µL PBS to the upper right well. Add 150 µL PBS to lower right well. Wait 10 min.

1.7 Repeat steps 1.5–1.6 for a second PBS wash.

1.8 Check the chip under a tissue culture microscope for bubbles in the main channels. If bubbles are present, perform the procedures below. If no bubbles are present, skip to step 1.9.

1.8.1 Aspirate PBS from the wells angling the pipet tip away from the channel opening (**Figure 2A**).

1.8.2 Dispense 100 µL of pre-coating solution into the upper well, angling the tip of the pipet near the channel opening (**Figure 2B**). The bubbles should move through the channel into the lower well. Wait 1.5 min.

1.8.3 Repeat steps 1.3-1.8.

1.9 Aspirate PBS from the wells angling the pipet tip away from the channel opening (**Figure 2A**).

1.10 Add 100 µL of 0.5 mg/mL poly d-lysine (PDL) to the upper left well of the chip. Wait 1.5 min. Fill the lower left well with 100 µL of PDL.

1.11 Add 100 µL of PDL to the upper right well of the chip. Wait 1.5 min. Add 100 µL to the lower right well.

1.12 Close and seal the dish or container using paraffin film to prevent evaporation and place the chip containing PDL in an incubator at 37 °C for 1 h.

1.13 Repeat steps 1.5–1.6 twice to remove excess PDL.

1.14 Wrap Petri dish in parafilm and store for >1 h at room temperature or at 4 °C overnight.

1.15 Aspirate the PBS from the device.

1.16 Immediately add 100 µL of cell culture media to the upper left well of the chip. Wait 1.5 min. Add media to the lower left well. Add media to the upper right well. Wait 1.5 min. Add 100 µL medium to the lower right well of the chip.

2. Seeding Neurons into the Multicompartment Chips

2.1. Prepare cell suspension of dissociated rat hippocampal neurons according to established protocols[21,22] to yield a density of ~12 × 10^6 cells/mL.

Note: Use of cell suspension densities between 3 and 12 × 10^6 cells/mL is possible. If a lower density is used, the volume of cell suspension to be added to the chip may be increased (see below). The procedure described below is applicable for murine dissociated cortical or hippocampal neurons. Optimal cell densities for other neuron types may vary.

2.2. Remove the majority of media in each well of the chip, leaving approximately 10 µL in each well. Aspirate away from the main channels to avoid removing liquid from the main channels (**Figure 2A**).

CAUTION: Do not aspirate all liquid from the enclosed main channels. Air bubbles may become trapped in the chip if fluid is aspirated from the main channels. In this case, refer to step 1.8 above.

2.3. Load 5 µL of cell suspension in the upper right well and another 5 µL of cell suspension in the lower right well (~120,000 cells total). Load the cells by dispensing close to the main channel (**Figure 2B**). Check under a microscope to ensure the neurons are in the main channel. Wait for 5 min to allow the cells to attach.

Note: Neurons can be loaded into either compartment. For explanation purposes, the somatic compartment is the main channel on the right side, but either compartment can be used as the somatic compartment. Use of lower cell densities down to 60,000 cells per chip is possible. Up to 10 µL of cell suspension may be added to each well of the somatic compartment in combination with a cell suspension with fewer cells than described above.

2.4. Add approximately 150 µL of neuronal culture media to each of the upper and lower right wells, and then add 150 µL of media to each of the upper and lower left wells. Place the chip into a 5% CO_2 37 °C incubator.

2.5. After 24 h, perform a media change by removing media from the wells. Make sure the main channel remains filled. Add 150 µL of media to each top well, and then fill the bottom wells.

2.6. Place the chip back in the incubator for the desired number of days.

Note: Monitor the media every couple of days to make sure it remains light pink. If the media is yellowish, replace 50% of it with fresh media. If the fluid level is low, make sure there is adequate humidity and appropriate secondary containment of the chips to prevent evaporation. Minimizing, or even eliminating, media changes is possible using secondary containment and/or covering the dish containing the chip with polytetrafluoroethylene (PTFE)-FEP film.

3. Retrograde Labeling of Neurons within the Chip

Note: Retrograde labeling can be performed using multiple techniques, including using modified cholera toxin and rabies virus. Below are instructions for labeling neurons using G-deleted Rabies-mCherry or –eGFP virus. Handle potentially infectious materials according to the local organization's guidelines. Additional training may be required.

3.1. Warm fresh neuronal culture media to 37 °C. Estimate ~400 µL of media per chip.

3.2. Dilute 100,000 viral units of modified rabies virus in a total of 50 µL using media taken from either well of the axonal compartment.

Note: Dispose of tips and tubes in contact with the virus according to the organization-approved protocol.

3.3. Gently pipet the remaining media from the wells of the axonal compartment and store in a centrifuge tube at 37 °C.

3.4. Add 150 µL of fresh warm media and the 50 µL of diluted virus to the axonal compartment. Incubate for 2 h at 37 °C incubator.

3.5. Remove media containing virus and dispose of it properly.

Note: Air bubbles may become trapped in the chip if fluid is aspirated from the main channels. In this case, refer to step 1.8 above.

3.6. Gently add 75 µL of fresh media to one axon well and allow it to flow to the other axon well.

3.7. Remove flow-through from the second axon well and dispose properly.

3.8. Repeat steps 3.6 and 3.7 once.

3.9. Add back stored media to the axonal compartment. Add approximately 50 µL fresh media, if necessary, to maintain adequate volume and return the cells to the incubator.

Note: Fluorescent protein expression is visible by 48 h and persists for up to 8 days. Neurons can be imaged for up to 30 min at room temperature in neuronal culture media. Culture media can also be replaced with warmed CO_2-independent hibernate E with B27 and imaged for longer. Neurons can also be imaged within a well-humidified environmental chamber at 37 °C and 5% CO_2. In this case, humidification is critical for minimizing evaporative losses within the chips, which is exacerbated by heating and can compromise neuron health.

4. Fluidic Isolation of the Axonal Compartment within the Chip

4.1. Remove 20 µL from the lower left well of the axonal compartment and place into the upper right well of the somatic compartment. Wait 2 min for flow within each channel to equilibrate.

4.2. Remove 50 µL of media from the axonal compartment. Add 0.3 µL of 1 mM Alexa Fluor 488 hydrazide to this media, mix via pipet and return back to the axonal compartment. The chip is ready for imaging.

Note: Other compounds of interest can be added. Adding a fluorescent dye with a similar molecular weight as the compound of interest is recommended in order to monitor fluidic isolation over time.

5. Performing Axotomy Within the Chip

5.1. Remove media from the axonal compartment keeping the pipet tip away from the entrance of the main channel (**Figure 2A**) and store it in a centrifuge tube.

5.2. Aspirate the axonal compartment completely, placing the aspiration pipet near either entrance of the main channel of the axonal compartment (**Figure 2B**). Continue aspiration for 1–2 min. Make sure that solution is completely removed from the compartment and the axons are severed.

Note: The vacuum pressure for aspiration must be at least 18 inch-Hg for the axotomy procedure to work properly.

5.3. Replace the axonal compartment with the stored media.

Note: If bubbles form in the axonal compartment when replacing the media, repeat steps 5.1–5.2.

5.4. Return the chip to the incubator.

6. Fluorescence Immunostaining within the Chip

6.1. Prepare 4% formaldehyde fixation solution in PBS (4% formaldehyde, 1 µM $MgCl_2$, 0.1 µm $CaCl_2$, 120 mM sucrose)

6.2. Remove most of the media in the wells of the chip (do not dry interior compartments).

6.3. Immediately add 100 µL of fixation solution to the top wells of the axonal and somatic compartments.

6.4. After 1 min, add 100 µL of fixation solution to the bottom wells. Fix for 30 min at room temperature.

6.5. Remove most of the solution from the wells of the chip (do not dry interior compartments). Immediately add 150 µL of PBS to each of the top wells of the axonal and somatic compartments. Wait 2 min for the PBS to flow into the bottom wells.

6.6. Repeat step 6.5 twice.

6.7. Remove most of the PBS from the wells of the chip. Immediately add 150 µL of PBS with 0.25% TritonX-100 to each of the top wells of the axonal and somatic compartments. Wait for 15 min.

6.8. Remove most of the liquid from the wells of the chip and immediately add 150 µL of blocking solution (10% normal goat serum in PBS) to each of the top wells of the axonal and somatic compartments. Wait for 15 min.

Note: Effective blocking solutions should be specific to the secondary antibody, *e.g.*, for a donkey anti-sheep secondary antibody, use donkey serum in the blocking solution.

6.9. Remove most of the liquid from the wells of the chip and immediately add 100 µL of primary antibody (or antibodies) in 1% normal goat serum in PBS to each of the top wells of the axonal and somatic compartments. Cover to minimize evaporation and wait for 1 h at room temperature or 4 °C overnight.

6.10. Remove most of the solution from the wells of the chip (do not dry interior compartments). Immediately add 150 µL of PBS to each of the top wells of the axonal and somatic compartments. Wait 5 min for the PBS to flow into the bottom wells.

6.11. Repeat step 6.10 twice.

6.12. Remove most of the liquid from the wells of the chip and immediately add 100 µL of secondary antibody (or antibodies) in PBS to each of the top wells of the axonal and somatic compartments. Cover to minimize evaporation and wait for 1 h at room temperature.

Note: Refer to the manufacturer's instructions for the recommended dilution of secondary antibodies.

6.13. Repeat steps 6.10–6.11.

6.14. If imaging within 1 day of immunostaining, keep the chip filled with PBS. If chip will be stored longer than 1 day before imaging, wrap the dish containing the chip in paraffin film to prevent evaporation and store at 4 °C until ready to image.

6.15. For longer term storage of samples, mounting media (*e.g.*, Fluoromount-G) can be used.

6.15.1. Remove most of the liquid from the wells of the chip. Use a 1 mL disposable plastic pipet to add 2 drops of mounting media to each of the top wells of the axonal and somatic compartments.

6.15.2. Tilt the chip to encourage the flow of the mounting media through the channels. After 5 min add 2 drops to the bottom wells. Wait for 1 h before imaging.

Note: After using mounting media it will not be possible to re-probe for other targets.

REPRESENTATIVE RESULTS:

After approximately 5–7 days of neuron growth within the chip, axonal growth is evident. The chips are compatible with phase-contrast imaging as demonstrated in **Figure 3**, which shows neuronal growth at 24 days. The chips are also compatible with fluorescence imaging (**Figures 4–7**). Three days after rabies virus infection via the axonal compartment, mCherry-positive neurons with axons extending into the axonal compartment were imaged in the chip (**Figure 4**). To demonstrate the ability to fluidically isolate the compartments, a low molecular weight fluorescent dye (Alexa Fluor 488 hydrazide) was added to the axonal compartment. These results are comparable to PDMS-based chamber[17] and demonstrate the suitability of the plastic chip for phase-contrast and fluorescence imaging.

To illustrate neuronal growth with the plastic chips and PDMS devices, we cultured neurons in both platforms and monitored neuronal growth over time. **Figure 5** shows neuronal growth from 3 to 22 days in culture; these results are representative of 3 independent experiments. Neuronal growth is comparable within the two platforms up to 15 days in culture, but at longer culture ages (>21 days) isolated axons within the plastic chip appear healthier with less beading (**Figure 5A**). To further visualize axons within the axonal compartments, we immunostained for β-tubulin III which shows healthy axonal growth within the plastic chips at 22 days in culture (**Figure 5B**).

Axon injury and regeneration studies are common using microfluidic compartmentalized devices. To demonstrate the suitability of these studies using the chips, retrograde labeled neurons were imaged before and 24 h after axotomy (**Figure 6**). A retraction bulb and regenerating axon are both evident following axotomy. These results are equivalent to published data using PDMS-based devices[14,17].

Immunocytochemistry is a common technique performed within multi-compartment devices to visualize protein localization. After 24 days in culture, neurons within the chips were fixed and stained for both excitatory and inhibitory synaptic markers, vGlut1 and vGat, respectively (**Figure 7**). Retrograde labeled mCherry neurons were also imaged (**Figure 7C**). Imaging was performed using spinning disk confocal with a 60× silicone oil immersion objective, demonstrating the ability to perform high-resolution imaging. Importantly, dendritic spines were evident within a magnified region, demonstrating that the neurons cultured within the chips were forming mature synapses.

FIGURE AND TABLE LEGENDS:

Figure 1: A pre-assembled, plastic two-compartment microfluidic chip for compartmentalizing neurons. (A) Schematic representation of the multicompartment chip showing the locations of the upper and lower wells. **(B)** An enlarged schematic of the chip showing the main channels (or compartments) and microgrooves which connect the compartments. The main channels are approximately 1.5 mm × 7 × 0.060 mm (W × L × H). The width and height of the microgrooves are approximately 0.01 mm × 0.005 mm, respectively. The length of the microgrooves varies depending on the configuration, 0.15 mm to 0.9 mm. **(C)** A photograph of a representative multicompartment chip containing food coloring dye in each main channel or compartment demonstrating the ability to fluidically isolate each channel.

Figure 2: Pipetting techniques needed when using plastic multicompartment chips. (A) When adding and aspirating media for washes, the pipet tip should be angled away from the entrance of the main channel as shown. **(B)** When loading neurons or performing axotomy, the pipet tip should be angled towards the entrance of the main channel.

Figure 3: A phase contrast micrograph showing typical neuronal growth within the chip at 24 days in culture. Embryonic hippocampal neurons were seeded into the right somatic compartment. Axon growth is visible in the axonal compartment beginning at 5–7 days.

Figure 4: Retrograde labeled neurons express mCherry fluorescent protein and extend axons into a fluidically isolated axonal compartment. (A) A merged fluorescence micrograph showing live retrograde labeled neurons infected via a modified mCherry rabies virus briefly applied to the axonal compartment. Neurons were imaged 3 days post-infection at 21 days in culture. Creating an isolated microenvironment within the axonal compartment is demonstrated by application of a low molecular weight dye, Alexa Fluor 488 hydrazide. **(B)** A merged image of (A) including a differential interference contrast (DIC) image to visualize the microgrooves region of

the chip. Images were acquired with laser scanning confocal microscope using a 30×/1.05 N.A. silicone oil (ne = 1.406) objective lens.

Figure 5: Side-by-side comparison of neuronal growth within multicompartment PDMS devices and plastic chips. (A) Phase contrast micrographs of both platforms taken at 3, 7, 15, and 22 days in culture. At the bottom, a higher magnification region taken from the images at 22 days is included to illustrate axonal growth at this age within both platforms. Axons within the chip are more continuous and appear healthier than in the PDMS device at this age. **(B)** An invert immunofluorescence micrograph of β-tubulin III stained axons within the axonal compartment of both the PDMS device and plastic chip at 22 days in culture. Images were acquired with a spinning disk confocal imaging system using a 20x/0.45 N.A. objective lens. All scale bars are 100 µm.

Figure 6: Axotomy and regeneration of hippocampal neurons within the plastic multicompartment chip. (Top) Neurons were retrograde labeled using a modified mCherry rabies virus and then imaged before axotomy at 24 days in culture. Images were pseudocolored using the 'Fire' color look-up table. **(Bottom)** The same neuron imaged in the top panel was imaged 24 h post-axotomy. White dashed lines show the edges of the microgroove barrier. Axotomy occurred at the location of the left dashed line. The white arrowhead shows a retraction bulb. The white arrow indicates a regenerating axon. Images were acquired with a spinning disk confocal imaging system using a 20×/0.45 N.A. objective lens.

Figure 7: Synapses form between hippocampal neurons cultured within plastic multicompartment chips. Immunostaining was performed at 24 days in culture and imaged within the somatic compartment using a 60× silicone oil immersion lens. Neurons express **(A)** the excitatory synapse marker, vGlut1 (green) and **(B)** the inhibitory synapse marker, vGAT (blue). **(C)** Retrograde labeled mCherry neurons (red) were infected via modified rabies virus applied to the axonal compartment. **(D)** A merged fluorescence micrograph of vGlut1, vGat, and mCherry. **(E)** The magnified region in (D) indicated with a white box shows dendritic spines, the sites that receive synaptic input from other neurons. Images were acquired with a spinning disk confocal imaging system using a 60×/1.3 N.A. silicone oil (ne = 1.406) objective lens.

Table 1: Comparison of plastic and PDMS multi-compartment platforms for culturing neurons.

DISCUSSION:

Plastic multicompartment chips provide an easy-to-use option for compartmentalizing neurons, providing long-term neuronal cultures (>3 weeks). This protocol details how to culture cortical and hippocampal murine neurons within these chips. The creation of soluble microenvironments and how to retrograde label neurons, perform axotomy, and perform immunocytochemistry were also discussed. Importantly, these chips are compatible with high-resolution, fluorescence, and live imaging.

Plastic multicompartment chips provide many of the same functions as the PDMS-based compartmentalized devices, but have advantages, disadvantages, and some distinguishing

features. **Table 1** provides a feature comparison of both plastic chip and PDMS-based devices. First and foremost, the chips are pre-assembled and made permanently hydrophilic, which facilitates wetting, making them easier to use. The plastic is not gas permeable, unlike PDMS, so if bubbles unexpectedly form within the channels, they do not readily escape and must be removed. A pre-coating solution containing mainly ethanol and some other proprietary agents eliminates bubble formation.

Live imaging of projections following transduction of fluorescent proteins was performed within the chips (**Figure 4**) and there was no detectable autofluorescence of the plastic. A caveat is that immersion oils used with the chip for high numerical aperture objectives must be silicone oil based, and not mineral oil based. Mineral oil can cause an adverse reaction with the cyclic olefin copolymer. For brightfield imaging, it is important to note that the microgrooves in the chip are rounded at the ends and there is a gradual tapering of the z-direction of the main channels towards the microgroove barrier causing some light refraction at either end of the microgrooves during brightfield imaging (**Figure 3**). Because the chip is pre-assembled, antibody penetration into the micron-sized microgrooves may be uneven (as with permanently bonded PDMS-based devices); thus, quantitative analysis following immunostaining should be performed in the channels/compartments. Immunostaining of neuronal projections within the microgrooves can be improved by creating a volume difference between the compartments to aid the flow of antibodies and fluorophores into the microgrooves.

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DISCLOSURES:

A.M.T. is an inventor of the microfluidic chamber/chip (US 7419822 B2) and is a member of Xona Microfluidics, LLC. V.P. is an employee of Xona Microfluidics, LLC. J.H. is a member of Xona Microfluidics, LLC. T.N. declares no competing financial interests.

REFERENCES:

1 Neto, E. *et al.* Compartmentalized Microfluidic Platforms: The Unrivaled Breakthrough of *In Vitro* Tools for Neurobiological Research. *The Journal of Neuroscience.* **36** (46), 11573-11584, doi:10.1523/jneurosci.1748-16.2016, (2016).

2 Virlogeux, A. *et al.* Reconstituting Corticostriatal Network on-a-Chip Reveals the Contribution of the Presynaptic Compartment to Huntington's Disease. *Cell Reports.* **22** (1), 110-122, doi:https://doi.org/10.1016/j.celrep.2017.12.013, (2018).

3 Batista, A. F. R., Martínez, J. C. & Hengst, U. Intra-axonal Synthesis of SNAP25 Is Required for the Formation of Presynaptic Terminals. *Cell Reports.* **20** (13), 3085-3098, doi:https://doi.org/10.1016/j.celrep.2017.08.097, (2017).
4 Yang, Y. *et al.* Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. *Neuron.* **61** (6), 880-894, doi:10.1016/j.neuron.2009.02.010, (2009).
5 Sutton, M. A., Taylor, A. M., Ito, H. T., Pham, A. & Schuman, E. M. Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. *Neuron.* **55** (4), 648-661, doi:10.1016/j.neuron.2007.07.030, (2007).
6 Hengst, U., Deglincerti, A., Kim, H. J., Jeon, N. L. & Jaffrey, S. R. Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. *Nature Cell Biology.* **11** (8), 1024-1030 (2009).
7 Sharma, N. *et al.* Long-distance control of synapse assembly by target-derived NGF. *Neuron.* **67** (3), 422-434, doi:10.1016/j.neuron.2010.07.018, (2010).
8 Harrington, A. W. *et al.* Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. *Cell.* **146** (3), 421-434, doi:10.1016/j.cell.2011.07.008, (2011).
9 Zhang, Y. *et al.* Assembly and maintenance of nodes of ranvier rely on distinct sources of proteins and targeting mechanisms. *Neuron.* **73** (1), 92-107, doi:10.1016/j.neuron.2011.10.016, (2012).
10 Taylor, A. M., Wu, J., Tai, H. C. & Schuman, E. M. Axonal translation of beta-catenin regulates synaptic vesicle dynamics. *The Journal of Neuroscience.* **33** (13), 5584-5589, doi:10.1523/JNEUROSCI.2944-12.2013, (2013).
11 Tran, H. T. *et al.* Alpha-synuclein immunotherapy blocks uptake and templated propagation of misfolded alpha-synuclein and neurodegeneration. *Cell Reports.* **7** (6), 2054-2065, doi:10.1016/j.celrep.2014.05.033, (2014).
12 Calafate, S. *et al.* Synaptic Contacts Enhance Cell-to-Cell Tau Pathology Propagation. *Cell Reports.* **11** (8), 1176-1183, doi:10.1016/j.celrep.2015.04.043, (2015).
13 Cosker, K. E., Fenstermacher, S. J., Pazyra-Murphy, M. F., Elliott, H. L. & Segal, R. A. The RNA-binding protein SFPQ orchestrates an RNA regulon to promote axon viability. *Nature Neuroscience.* **19** (5), 690-696, doi:10.1038/nn.4280, (2016).
14 Taylor, A. M. *et al.* Axonal mRNA in uninjured and regenerating cortical mammalian axons. *The Journal of Neuroscience.* **29** (15), 4697-4707 (2009).
15 Pinto, M. J. *et al.* The proteasome controls presynaptic differentiation through modulation of an on-site pool of polyubiquitinated conjugates. *The Journal of Cell Biology.* **212** (7), 789-801, doi:10.1083/jcb.201509039, (2016).
16 Bigler, R. L., Kamande, J. W., Dumitru, R., Niedringhaus, M. & Taylor, A. M. Messenger RNAs localized to distal projections of human stem cell derived neurons. *Scientific Reports.* **7** (1), 611, doi:10.1038/s41598-017-00676-w, (2017).
17 Nagendran, T. *et al.* Distal axotomy enhances retrograde presynaptic excitability onto injured pyramidal neurons via trans-synaptic signaling. *Nature Communications.* **8** (1), 625, doi:10.1038/s41467-017-00652-y, (2017).
18 Taylor, A. M. *et al.* A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nature Methods.* **2** (8), 599-605, doi:10.1038/nmeth777, (2005).

19 Taylor, A. M., Dieterich, D. C., Ito, H. T., Kim, S. A. & Schuman, E. M. Microfluidic local perfusion chambers for the visualization and manipulation of synapses. *Neuron.* **66** (1), 57-68, doi:10.1016/j.neuron.2010.03.022, (2010).
20 Mukhopadhyay, R. When PDMS isn't the best. What are its weaknesses, and which other polymers can researchers add to their toolboxes? *Analytical Chemistry.* **79** (9), 3248-3253 (2007).
21 Banker, G. A. & Cowan, W. M. Rat hippocampal neurons in dispersed cell culture. *Brain research.* **126** (3), 397-342 (1977).
22 Banker, G. & Goslin, K. *Culturing Nerve Cells*. Second edition, (MIT Press, 1998).

Figure 1

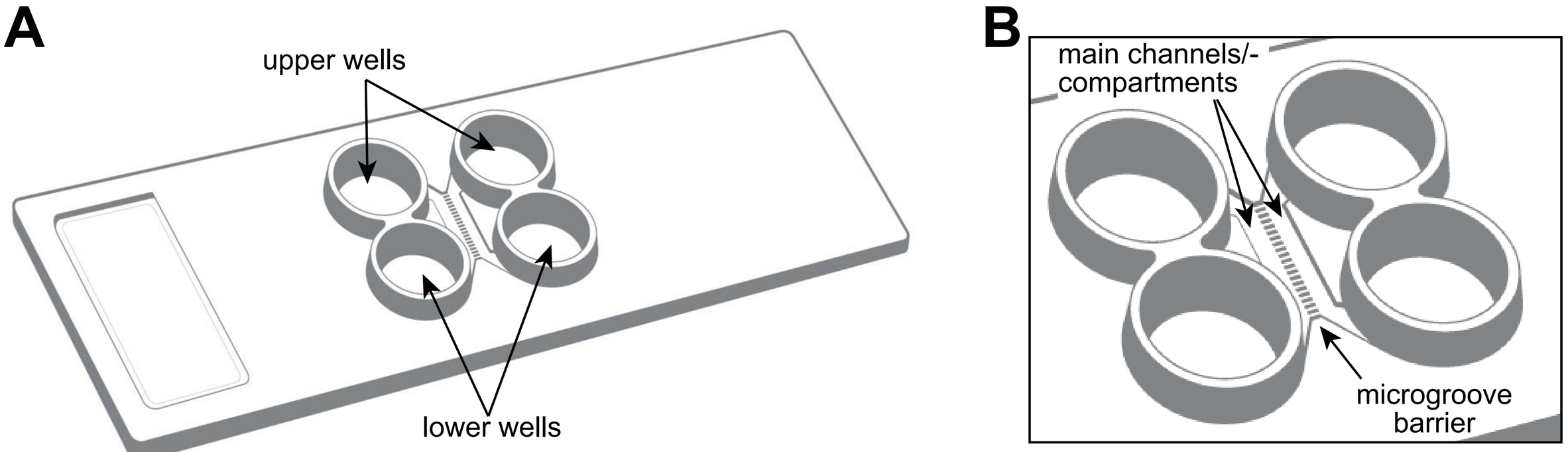

Figure 2

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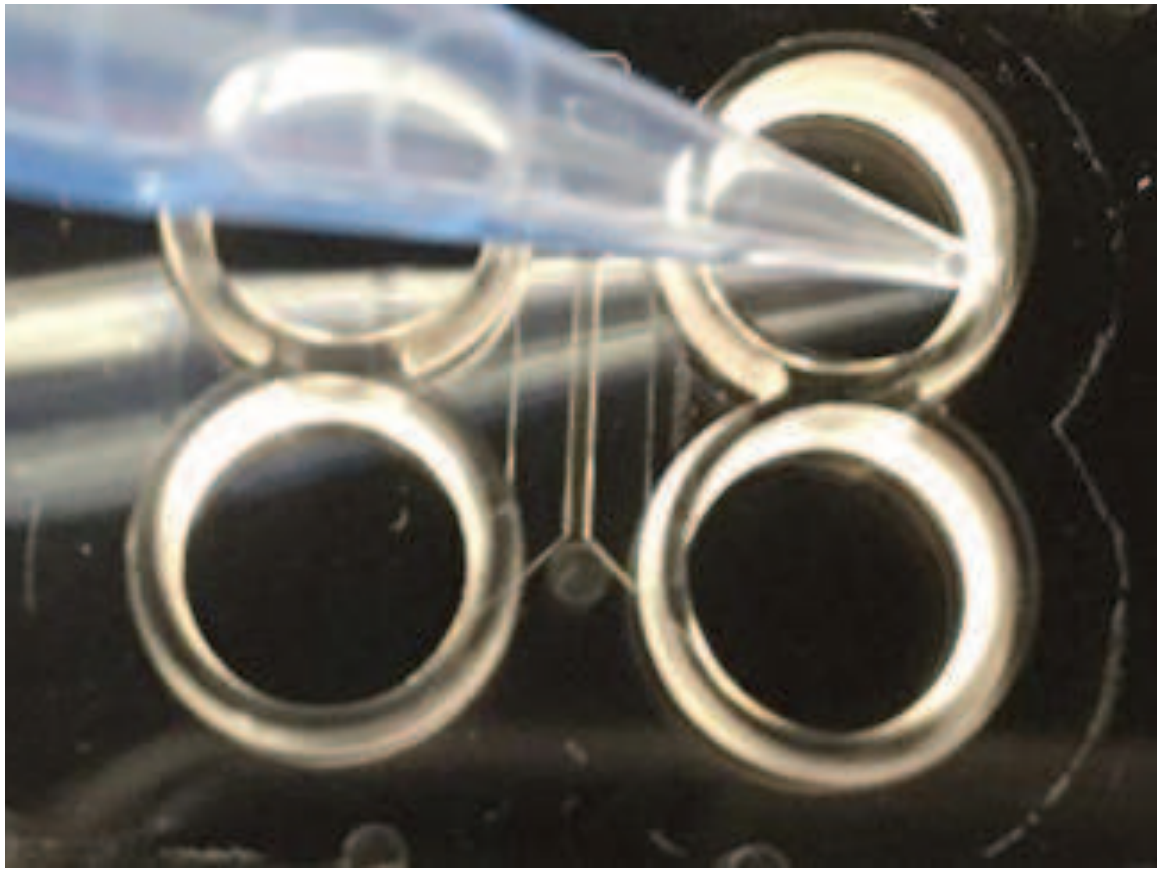
A



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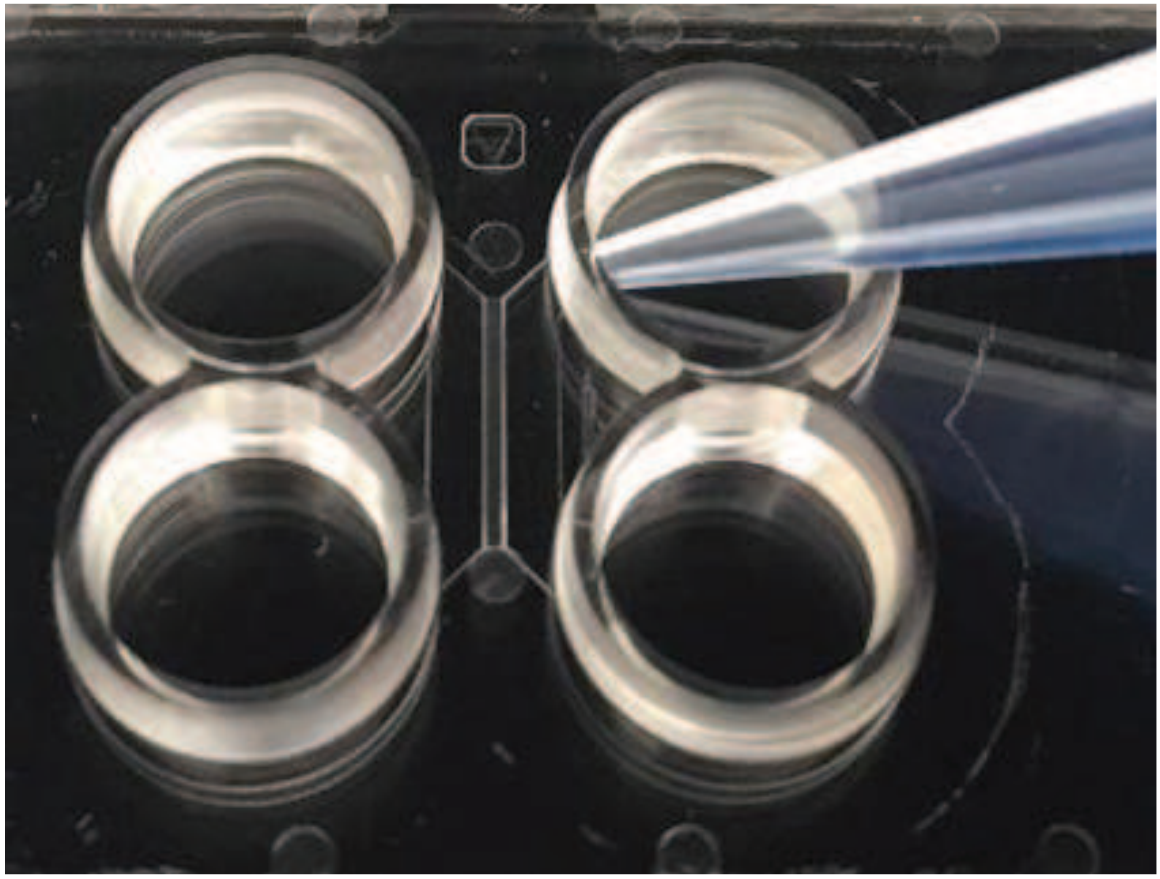

Figure 3

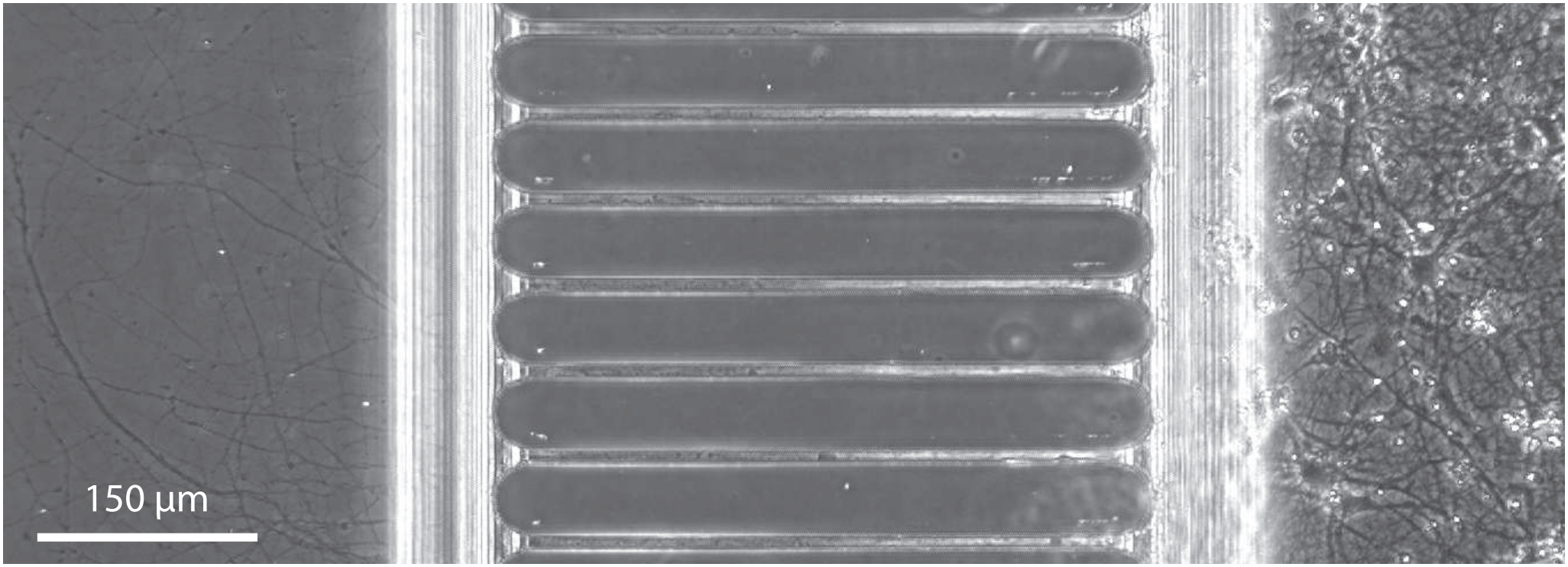

Figure 4

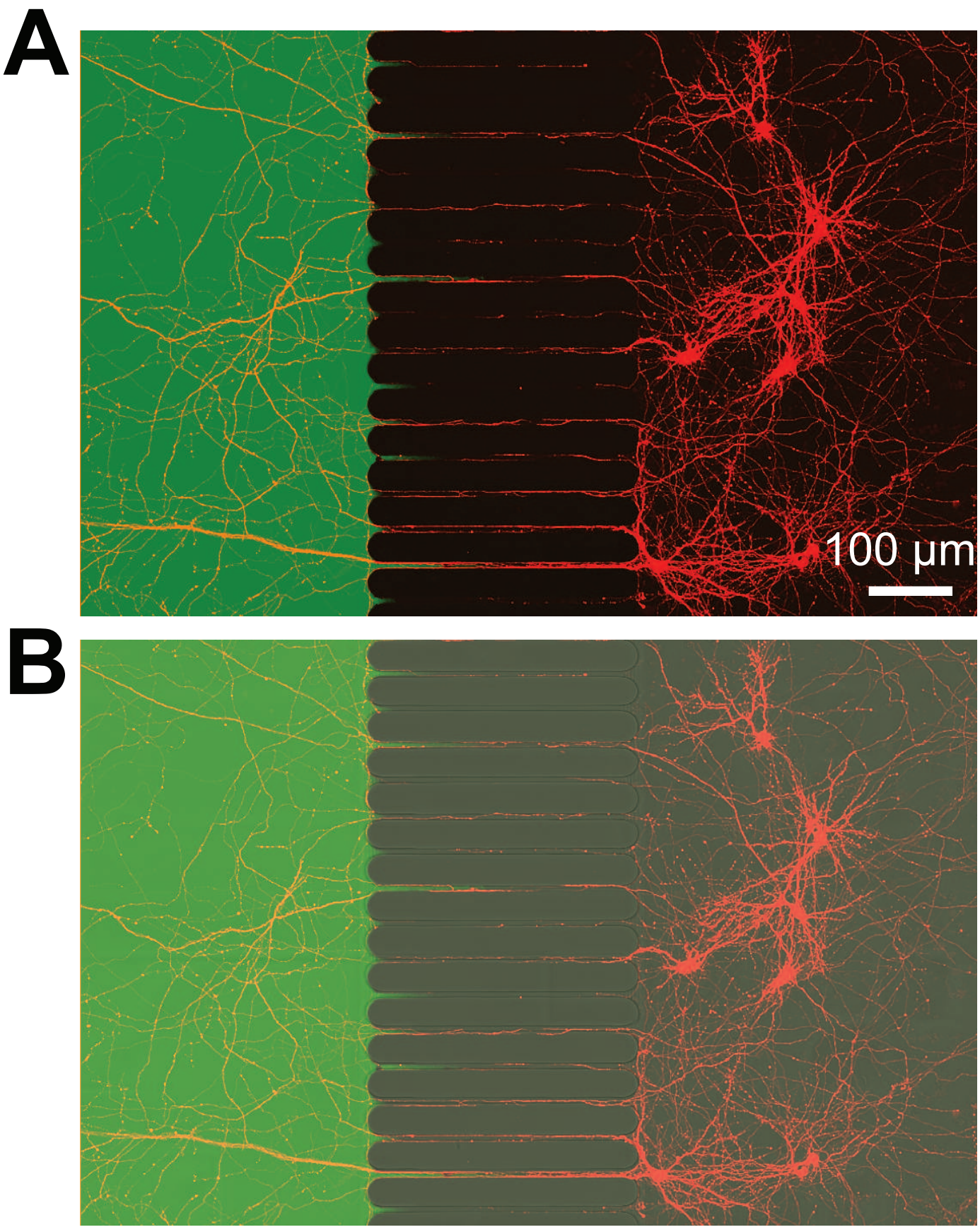

Figure 5

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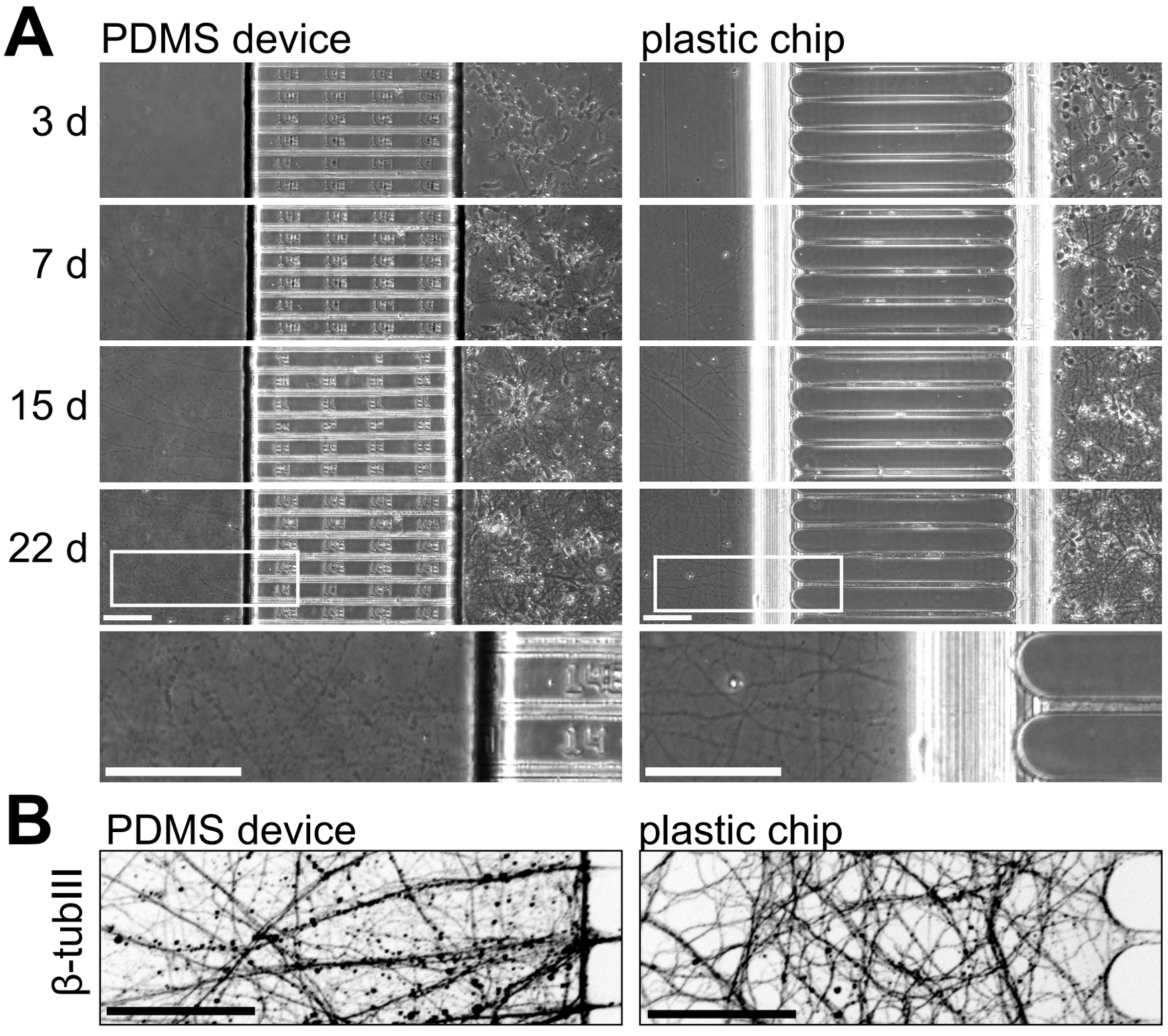

Figure 6

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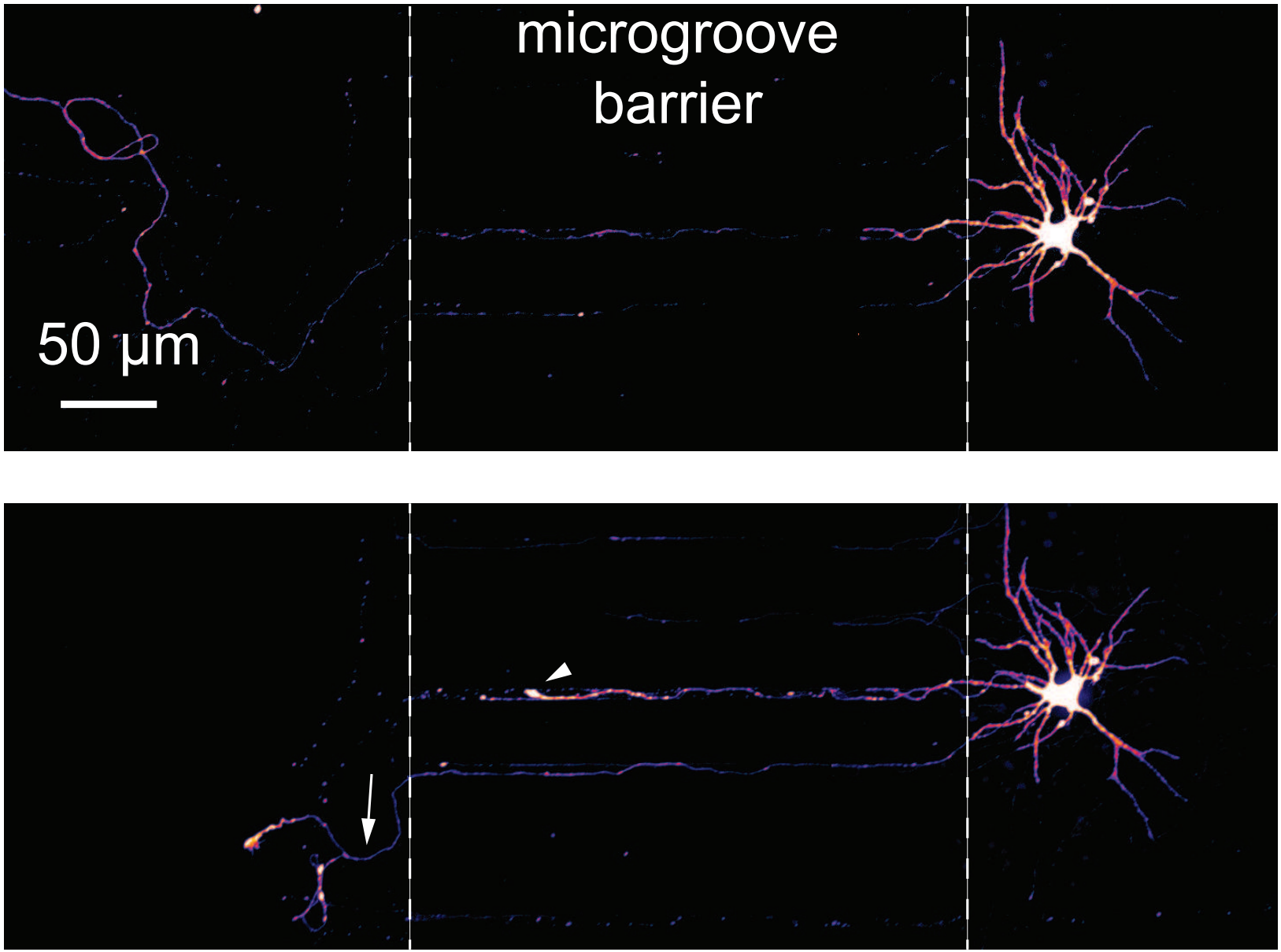

Figure 7

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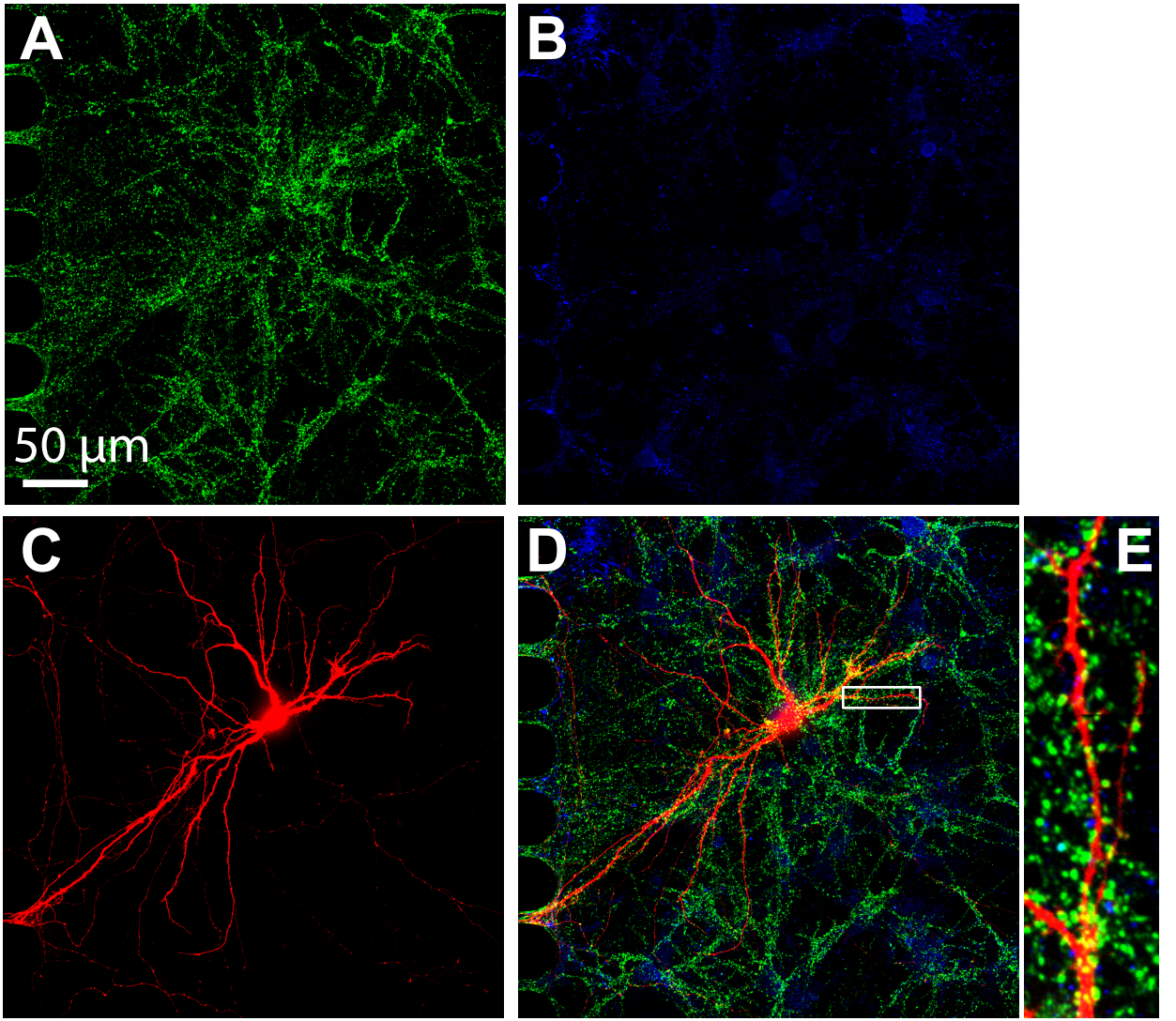

Table 1

Click here to download Table table 1- chips vs PDMS chambers.xlsx

Plastic multi-compartment chips	PDMS multi-compartment devices
isolate axons	isolate axons
establish microenvironments	establish microenvironments
axotomize neurons	axotomize neurons
optically transparent	optically transparent
compatible with high resolution imaging	compatible with high resolution imaging
compatible with fluorescence microscopy	compatible with fluorescence microscopy
fully assembled	assembly to substrate required
healthy axons >21 days	healthy axons >14 days
hydrophilic culturing surface	hydrophobic
gas impermeable	gas permeable
rounded microgrooves and channels	straight microgrooves
fewer preparation steps	top is removable for staining within microgrooves
not compatible with laser ablation	absorption of small molecules & organic solvents
not compatible with mineral oil-based immersion oils (silicone-based oils are fine)	

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
XonaChip	Xona Microfluidics, LLC	XC150	150 µm length microgroove barrier
	Xona Microfluidics, LLC	XC450	450 µm length microgroove barrier
	Xona Microfluidics, LLC	XC900	900 µm length microgroove barrier
XC pre-coat	Xona Microfluidics, LLC	XC Pre-Coat	included with XonaChips
XonaPDL	Xona Microfluidics, LLC	XonaPDL	
E17/E18 timed pregnant Sprague Dawley rats	Charles River	24100564	
neuronal culture media:			
- Neurobasal medium	ThermoFisher Scientific	21103049	
-B-27 Plus Supplement (50x)	ThermoFisher Scientific	A3582801	
-GlutaMAX Supplement	ThermoFisher Scientific	35050061	
-Antibiotic-Antimycotic (100x)	ThermoFisher Scientific	15240112	
fluorinated ethylene propylene film	American Durafilm	50A	0.5 mil thickness
modified rabies virus	Salk Institute for Biological Studies	G-deleted Rabies-eGFP	Material Transfer Agreement required
	Salk Institute for Biological Studies	G-deleted Rabies-mCherry	Material Transfer Agreement required
Alexa Fluor hydrazide 488	ThermoFisher Scientific	A10436	
Fluoromount G	ThermoFisher Scientific	00-4958-02	
Glass Pasteur pipettes	Sigma-Aldrich	CLS7095D5X SIGMA	5.75 in length
hibernate-E Medium	ThermoFisher Scientific	A1247601	
Pierce 16% formaldehyde	ThermoFisher Scientific	28906	
PBS (10x)	ThermoFisher Scientific	QVC0508	

normal goat serum	ThermoFisher Scientific	16210064	
triton X-100	ThermoFisher Scientific	28314	
anti-vGlut1 antibody	NeuroMab	75-066	clone N28/9, 1:100
anti-vGAT antibody	Synaptic Systems	131 003	1:1000
anti_beta-tubulin III	Aves	TUJ	1:1000
Alexa Fluor secondary antibodies	ThermoFisher Scientific		1:1000
Incubator, 5% CO_2 37 °C			
Epifluorescence imaging system	EVOS Fluorescence imaging system	AMF4300	10x objective
Spinning disk confocal imaging system	Andor Technology	CSU-X1, iXon X3 EMCCD	60x silicone oil & 20x objectives
Laser scanning confocal imaging system	Olympus	FV3000RS	30x silicone oil objective

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Author(s): NAGENDRAN, POOLE, HARRIS, TAYLOR

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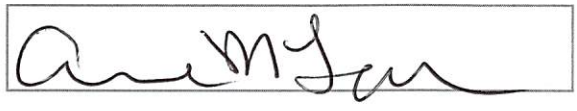
Signature:  Date: 5/4/2018

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Nagendran et al., **"**Use of pre-assembled plastic microfluidic chips for compartmentalizing primary murine neurons"

Point-by-point response to editorial and reviewer comments

Note: The authors' response is italicized and indented under each comment.

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

Thank you for taking the time to thoroughly review our manuscript. The outcome is much stronger than our original submission. We have proofread our manuscript and feel confident in the revision.

2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.

The email from Dr. Dsouza requests vector image files. EPS files have been uploaded.

3. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

Done.

4. Please remain neutral in tone when discussing commercial products. The accompanying video cannot become an advertisement.

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We removed these commercial symbols. We revised the manuscript and replace most of the commercial language with generic terms.

6. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of " XonaChip" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Done.

7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Done.

8. Please adjust the numbering of the Protocol. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Done.

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

Done.

10. 1 and 2.01: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We added more details to section 1 and added references to section 2.01 (now section 2.1).

11. 6.14: Please write the text in the imperative tense.

Done.

12. Please include single-line spaces between all paragraphs, headings, steps, etc.

Done.

13. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We believe we are within the 2.75 page limit. If not, many of the wash steps can be simplified to reduce the recording time. We can also eliminate the axotomy section, if necessary, and refer readers to the written protocol.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences).

Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Done.

15. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We added a section on needed compatible immersion oils for imaging using the COC material. We also added a table (Table 1) based on a suggestion from the reviewers to highlight the differences between the plastic and PDMS-based platforms.

16. Please follow the book citation example below to reformat book references:
Kioh, L.G. et al. Physical Treatment in Psychiatry. Blackwell Scientific Pubs. Boston (1988).

Done. See reference 22.

17. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Done. See revised table.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript provides a well-detailed protocol for the use of microfluidic chambers. Protocols for commonly used techniques like immunocytochemistry and retrograde labelling are well-adapted here for use in these devices. Some extra clarification is required at some points. These comments are listed below. The authors repeatedly contrast these new injection-molded plastic chambers to more conventional PDMS ones, highlighting the differences between them throughout the protocol when appropriate. The plastic devices address many issues that plagued PDMS-based chambers (toxicity, variability in bond, hydrophobicity), but are also disadvantageous in other ways. The authors address these shortcomings in the discussion section.

Major Concerns:
None.

Minor Concerns:
In section 2: Seeding neurons in the XonaChip, the authors state that a concentration of 12 million cells/mL is required. This concentration assumes that only 5uL of cell suspension will be added to the chambers. In our experience, adding larger volumes of cell mix (10uL on each side) yields a more even distribution of cells along the main channel, most likely due to the increased

volume differential created by using a larger initial loading volume. The protocol should explicitly mention that different concentrations of cell suspension can be used to accommodate this, up to a limit. For example, "a total of 120,000 cells are required in the main channel, with a maximum loaded volume of 20uL possible."

> *We thank the reviewer for this helpful comment and important clarification. As a result we added the following note below step 2.3:*
> *"NOTE: Use of lower cell densities down to 60,000 cells per chip is possible. Up to 10 µl of cell suspension may be added to each well of the somatic compartment in combination a lower cell density."*

120,000 cells in the main channel is 2X the amount we use in our lab. We have also found that the viability at given concentrations differs between cell types. For example, basal forebrain cholinergic neurons do not extend axons across microgrooves if <50,000 cells are present in the main channel. This is not true for cortical neurons, where lower cell counts do not have a significant impact on cell viability or morphology. This cell-type-dependent sensitivity to cell density should be stated.

> *Added the following note below step 2.1:*
> *"Note: The procedure described below is applicable for murine dissociated cortical or hippocampal neurons. Optimal cell densities for other neuron types may vary."*

In section 3: Retrograde labelling neurons within the chip, the authors state that neurons can be visualized for 30 minutes at room temperature, or longer if using Hibernate E. Another viable alternative would be using a microscope with an environmental chamber able to mimic incubator conditions. This would allow for microscopy to take place over an indefinite time. This would also mitigate the impact of temperature change on other assays like axonal transport.

> *We added the following note under step 3.9:*
> *"Note: Neurons can also be imaged within a well-humidified environmental chamber at 37 °C and 5% CO2. In this case, humidification is critical for minimizing evaporative losses within the chips, which is exacerbated by heating and can compromise neuron health."*

In section 6, the authors recommend using Fluoromount G for long term storage of fluorescently labelled samples. While effective, our lab has found that the solution blocks the main channels of the device over time, making it impossible to re-probe cells for another target. This should be stated.

> *We added the following sentence to the note under step 6.14: "Note that after using Fluoromount G it will not be possible to re-probe for other targets."*

A table highlighting the differences between PDMS chambers and the XonaChips would be a nice inclusion.

We added a table describing the differences as Table 1.

Because the XonaChip is not gas permeable, bubbles entering the main channel are an issue with these devices. This is mentioned in the manuscript, but a method of removing bubbles is absent. If reliable methods for removing bubbles have been tested by the authors, they should be included in the manuscript.

This has now been included in the revised manuscript under step 1.8.

Reviewer #2:

Manuscript Summary:

The manuscript by Nagendran et al describes a protocol for culturing and compartmentalizing primary rat hippocampal neurons into distinct soma and axon chambers, using a commercial microfluidic plastic chip manufactured and distributed by Xona Microfluidics. This chip replaces the original PDMS-based devices and claims improved cell viability and ease of use. The manuscript describes protocols for neuron loading and culture, retrograde labeling, fluidic isolation, axotomy and immunocytochemistry. The use of compartmentalized cultures in neuroscience have been popular since the original Campenot chambers and is relevant due to the different environments seen by the soma and the axons in vivo. Therefore, a detailed protocol with videos will be very helpful for neuroscience labs which might not have expertise in the finer details and complexities behind handling and using microfluidic devices. I would recommend publication of this protocol, after some minor concerns are addressed.

Major Concerns:

No major concerns.

Minor Concerns:

The following are some minor concerns that need to be addressed before publication:

1) Some more references need to be added:

- Page 1, Line 70: "The drawback of PDMS ..."
- Page 1, Line 71: "PDMS can be made hydrophilic temporarily ..."

We have now added a reference that summarizes many of the challenges in working with PDMS. Mukhopadhyay, R., 2007. When PDMS isn't the best. What are its weaknesses, and which other polymers can researchers add to their toolboxes? Anal Chem 79, 3248-3253.

2) In the long abstract (Line 39), the authors claim that the plastic device has improved cell health, but there is no reference in the Introduction or Discussion sections (Page 6, Line 297) that show that. There has to be some more discussion on cell viability and toxicity, since ease of use and viability are the main advantages that are being claimed in the manuscript.

We thank the reviewer for bringing up these issues. We conducted side-by-side comparisons to examine neuronal health within the chips and PDMS devices. We found that both devices resulted in healthy >14 day cultures. In the chips we found isolated axons were healthy for 21 days or more with no signs of degeneration, generally more

than in PDMS-based chambers. Representative results are now shown in new Figure 5 and were consistent over 3 independent cultures.

We have revised the text to reflect this new information. We also eliminate the toxicity wording related to PDMS. While others have reported toxicity, we have not observed consistent toxicity and PDMS devices have been used extensively for culturing neurons in hundreds of labs world-wide.

3) Please add dimensions of a standard microscope slide (Line 95)

Done

4) In Line 103 (Step 2.2), it would be good to have an approximate volume that needs to be removed.

Added the text, "leaving approximately 10 µl in each well".

5) Since removal of bubbles in a plastic chip is a greater problem than gas permeable PDMS chips, the authors should add to the cautionary note in Step 2.2, and say what to do if bubbles get introduced into the main channel.

We now have a cautionary note and refer to step 1.8 which now includes a troubleshooting guide for removing trapped air (bubbles).

6) In Step 2.4, the step of adding 150 uL of media in all the wells should be said in 1 line, avoiding repetition.

Done

7) In Line 120, the authors should elaborate what "monitoring the media" actually means; what should the users look out for.

We added the following note under step 2.6: "Note: Monitor the media every couple of days to make sure it remains light pink. If the media is yellowish, replace 50% of it with fresh media. If the fluid level is low, make sure there is adequate humidity and appropriate secondary containment of the chips to prevent evaporation."

8) In Step 3.5, it perhaps bears repeating that the fluid should be carefully removed from the wells, without introducing bubbles.

We added the following note under step 3.5: "Note: Air bubbles may become trapped in the chip if fluid is aspirated from the main channels. In this case, refer to step 1.8 above."

9) In Step 3.7, it is not clear what flow-through means - should the users remove media from both the wells?

We added "…flow-through from the second axon well …".

10) In Step 3.8, authors should clearly mention how many times should the user repeat the steps?

Added, "…. 3.7 once."

11) In Figure 5, A and B are not labeled and "white dashed lines" are not seen. In Line 229, the retraction bulb and the regenerating axon should be labeled in the figure, since it is mentioned in the text.

Done