**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.

*Done.*

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

*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*