Instituto de Medicina Molecular

João Lobo Antunes

Dear Editor,

We would like to thank the editor and the referees for the thorough review of our submitted manuscript "Isolation and Expansion of Neurospheres from Postnatal (P1-3) Mouse Neurogenic Niches".

Regarding their suggestions we believe that we have improved the paper and we are convinced that, in its present form, the manuscript addresses the main criticisms raised by the reviewers.

We are now submitting the revised version of the manuscript with changes highlighted in grey.

We hope that you will find that this new version, together with the specific point-by-point replies made below to the editor and referees, are adequate for the publication of this study in JoVE.

Best regards,

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Response to Editorial comments:

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.

The manuscript was thoroughly reviewed, taking into account the mentioned issues.

2. Please revise lines 43-45, 74-77, 357-365, 418-420 to avoid textual overlap with previously published work.

We have rephrased the abovementioned sentences (changes highlighted in grey in lines 43-45, 76-79, 425-433 and 437-439).

3. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

We have included an ethic statement at the beginning of the protocol (lines 90-94).

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

As suggested by the editor, the numbering of the protocol was changed to follow the JoVE Instructions for Authors. The changes are highlighted in grey. Due to the requested changes in the numbering, we have deleted sub-steps 2.1. and 2.2. identified as "SVZ Microdissection" and "DG Microdissection" respectively, moving these procedures to step 2 (lines 147-173). Moreover, we have also created a new step (step 13) which includes the different cell biology assays (line 340).

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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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: GlutaMAX, NeuroCult, Milli-Q, etc.

We have removed all commercial language from the manuscript. GlutaMAX was replaced by L-glumamine (line 100) and it was mentioned in the Table of Materials. We now only refer NeuroCult in the Table of Materials. We have Milli-Q water replaced by high purity water (lines 258, 259, 261, 262, 273, 282, 418, 426 and 427).

6. Please add more details 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. 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. See examples below.

7. Lines 91, 97: Please list an approximate volume of medium to prepare.

Regarding the Editor's concern about the appropriate volume of growth medium and dissection medium to be prepared at the day of the culture, it is difficult to define this in the protocol since it depends on the number of pups, and consequently the amount of SVZ and DG cells to be obtained from the dissection. However, we have now mentioned a range of volumes to be prepared (lines 104-106 and 110).

8. Line 105: How large is the petri dish?

The diameter of the petri dish is 60 mm and the height is 15 mm. Moreover, the actual inside growth surface diameter is 51.4 mm and the approximate growth surface area is 21 cm^2. This information was added in line 117.

9. Line 107: Please specify the euthanasia method.

The Editor raised an important question about the euthanasia method. In this protocol, we use the decapitation method as described in step 2.2..

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10. Line 130: Please specify the pore size of the tissue paper.

Regarding the pore size of the tissue paper, it is 11 μm. This information was added in line 143.

11. Lines 134, 142, etc.: Please specify the surgical tools used here.

We have now specified in the manuscript the surgical tools needed for this protocol (lines 147, 151, and161).

12. Line 173: How to obtain the pellet? Is centrifugation used here?

When we mention the word "pellet" we are referring to the tissue obtained from the SVZ or DG dissection, obtained in the previous steps (step 3.1. and 3.2.). Therefore, to be more understandable, we changed the word "pellet" to "digested tissue" (line 185).

13. Line 227: Please specify incubation time.

Regarding the incubation time needed to obtain secondary neurospheres, it is similar to that required to form primary neurospheres, mentioned in line 216. As suggested, we now specify that information in the lines 240 and 241.

14. Line 236: Neurospheres from which step? Please specify.

Concerning the storage of neurospheres, this procedure can be applied in primary or passage neurospheres. Therefore, the neurospheres that are collected can be obtained after performing steps 5.3. or 6.7.. This information is now specified (line 249).

15. Line 237: What happens after centrifugation, discard the supernatant? Please specify.

In fact, it is not clear which procedure should be performed after the centrifugation. Indeed, the supernatant which corresponds to the growth medium has to be removed after the centrifugation to keep just the pellet (neurospheres). To be clearer, this information is now specified in line 251.

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16. Lines 276, 282, 288, 323: At what temperature? Please specify.

PI (line 276 of the original manuscript) and BrdU are incubated (line 282 and 288 of the original manuscript) at 37°C. These procedures are performed in cell cultures and that are maintained in culture after the exposure with PI or BrdU. Therefore, all the incubations are performed at 37 °C with 5% CO2 and 95% atmospheric air. The temperature was added in the sentences of the mentioned lines.

Concerning the primary antibodies, they are incubated at 4°C, as is now mentioned in line 396 of the revised manuscript.

17. Line 289: Please specify the medium used here.

Since, we will evaluate the differentiation capacity of the cells, the medium is the SFM devoid of growth factors. This is now mentioned in line 359.

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

As suggested by the editor, all the protocol steps were reviewed to have 2-3 action and maximum of 4 sentences per step.

Steps 7.4. and 7.5. were combined into one step (lines 253 and 254).

Steps 10.1.2, 10.1.3. and 10.1.4 in the original manuscript were combined into one step which now is the step 14.1.2. (line 370).

The first two steps of the protocol of the immunostaining of neurosphere culture of day 2 (10.4.1. and 10.4.2.) were also combined into the step 14.4.3 (line 400).

Step 10.4.6. of the original manuscript was deleted because this information is repeated in step10.4.7.

19. Please include single line spacing between each numbered step or note in the protocol.

We have adjusted the spacing.

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20. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Concerning the steps to be featured in the video, we guarantee that we have them highlighted in yellow.

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Regarding the highlighted steps to be included in the video, we ensure that the guidelines stated by the editor were followed, including the cohesive narrative with a logical flow between the steps and the presence of at least one action written in the imperative voice per step.

22. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

Concerning the details of the steps to be featured in the video, we ensure that all the details are included in the highlighted text.

23. Table of Materials: Please remove any ™/®/© symbols.

We have removed these symbols from Table of Materials.

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Response to Reviewer 1:

The protocol described by the authors doesn't provided new information about how to culture and work with neurosphere cultures. It has been published by several groups how to make neurospheres cultures and there are several protocol manuscripts, even on Nature protocols describing this procedure in much detail. Also, one of the main weaknesses of this submission is the lack of a video showing the procedure, something that might be useful for most researchers interested in this particular type of cell culture.

Although there are several publications regarding the protocol of neurospheres, in our humble opinion our protocol brings new detailed information since we describe not only the neurosphere culture but also its applications including assays to evaluate the NSC properties: self-renewal, proliferation and multipotency as well as cell viability in different contexts. Unlike other published papers with this methodology, our protocol also integrates the test of different substrates for neurosphere adhesion and migration of the cells to form a pseudomonolayer (Supplementary figure 1).

Regarding the video, we have highlighted in yellow the steps (from the harvesting of postnatal mouse brain and SVZ/DG Microdissections to the Passaging of the neurospheres) which will be filmed after the final approval of the written manuscript.

Response to Reviewer 2:

Manuscript Summary:
The authors describe a protocol for isolating and propagating SVZ and DG progenitor cells.

Major Concerns:
None. I assume that the micro-dissections will be filmed by the Jove team since illustrations about the microdissections are missing in the Figures.

This part of the protocol was selected by us to be filmed by the JoVE team since this procedure involves many details that should be followed to guarantee an efficient isolation of the SVZ- and DG-derived NSPCs. In the text we have highlighted in yellow the steps (from the harvesting of postnatal mouse brain and SVZ/DG Microdissections to the Passaging of the neurospheres) which will be filmed after the final approval of the written manuscript.

Minor Concerns:
Some minor orthographic errors such as line 519 should read overcome instead of overcame.

The word "overcame" was changed to "overcome" (line 557).

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Response to Reviewer 3:

Manuscript Summary:
This could be a useful protocol in the list of existing protocols for the neurosphere assay, but careful editing and addition of missing information is necessary:

Major Concerns:
1) On page 3, line 165, for tissue dissociation, authors mention that they use a final concentration of 5%-10% of Trypsin-EDTA 0.05% to incubate for approximately 15 min at 37 °C. However, the concentration of trypsin EDTA is very important to decide how long to incubate. Please explain the use of different concentrations of Trypsin-EDTA for dissociation.

We have including the range of 5-10% of Trypsin because the final concentration of Trypsin-EDTA to dissociate the SVZ and DG tissue depends on the number of pups and therefore the amount of tissue that is obtained.

2) On page 4, line 202-203, authors describe " Incubate SVZ and DG cells for 6-8 days and 10-12 days, respectively to form primary 203 neurospheres, at 37°C with 5% CO2. " Were additional EGF and bFGF added to the dish and were the dishes rotating while incubating? Without enough growth factors and keeping the dish still in the incubator during the culture it is difficult to keep the neurospheres floating.

Regarding to the reviewer's question about the presence of additional EGF and bFGF in the 60mm-petri dishes, no additional growth factors are added in the dishes during neurosphere formation. When the cells are diluted in the SFM in the step 5.2., the medium already contains the proper concentration of growth factors needed to promote the proliferation of SVZ- and DG-derived NSPC for 6-8 days and 10-12 days, respectively. Moreover, during incubation, SVZ and DG petri dishes are not rotating in the incubator because this could induce an aggregation of the neurospheres precluding the formation of single neurospheres. Furthermore, neurospheres have already a spontaneous locomotion[1].

With these conditions, it is possible to obtain floating neurospheres as observed in Figure 1 of the Results section. Beyond the proliferative capacity of these cells, it is also not used any coating subtract in the petri dishes, preventing any cell adhesion and subsequently promoting the floating of the cells. This protocol (without adding extra growth factors or rotation) is well-established and used by different labs.

3) On page #5, line #235, the authors describe a method to freeze and store neurospheres in -20C. However, it is not clear if the neurosphere pellets were stored as such or in freezing media. Also, it is not clear how to recover those frozen neurospheres for further proliferation and differentiation. A protocol for recovering frozen neurospheres is missing.

The Reviewer raised an important question regarding the storage of the neurospheres. As mentioned in the last step of the section 7, what is stored is just the pellet of cells and we don't add any freezing media, and therefore there is

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no cell viability preventing the recovery of neurospheres. The stored cells are intended for DNA, RNA and protein extraction. With these procedures, different markers of proliferation and differentiation could be evaluated. For that, the cells have to be slowly thawed on ice.

4) On page 5, line 246-249, authors described two dissociation methods. One to dissociate the pellet of neurospheres in 1 mL of dissociation PBS. Authors gave the detailed components of dissociated PBS, but didn't describe the dissociation procedure. I assume it is mechanical dissociation which they should describe.

Concerning the dissociation procedure included in the protocol to evaluate the neuritogenesis, the dissociation is mechanical. To be clear, we included this information in the revised manuscript (line 312).

5) Authors mention on page 9, line 421, that SVZ neurospheres are larger compared to neurospheres from DG. However, readers may not appreciate such a difference as presented in figure 1 because of different scale bar sizes. I suggest the authors keep a) scale bars at the same size in both images; and b) zoom settings the same for SVZ and DG so that their point will be clear.

Regarding the scale bars of the Figure 1, we agree with the Reviewer about the difficulty to compare the size of SVZ and DG neurospheres having different scale bars. Therefore, we have changed the scale bar in the new images A1 and A3 of Figure 1. Regarding images A2 and A4 also of the Figure 1, we have replaced them by new images because they were acquired with different number of tiles, being impossible to have the same scale bar.

6) on page 9 line 422-424, authors make the conclusion that "Importantly, in differentiative conditions, SVZ- and DG-derived NSPCs migrate out of the neurosphere forming a pseudomonolayer of cells composed of different cell types (Figure 1B). From the figure 1B, I can't draw that conclusion. The rationale for coming up with their conclusion should be provided.

The Reviewers concern is valid because in the figure 1B it is just visible the migration of the cells out of the neurosphere and not specifically the presence of different cell types in the periphery. Therefore, we have re-phrased this sentence in the revised manuscript by deleting the last part (lines 440-442).

The presence of different cell types in the pseudomonolayer can be observed in figure 6 of the revised manuscript, where we show a pseudomonolayer of cells formed by different cell types including NSCs, oligodendrocyte precursor cell, myelinating oligodendrocytes, immature and mature neurons as well as astrocytes.

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Minor Concerns:
Page #1, line #61: Epidermal growth factor and not 'ependymal'
Page #1, line #62: Fibroblast growth factor and not 'Fibroblastic'
Page #2, line #131: Suggest authors provide Tissue Chopper information

We have corrected the name of the growth factors (line 63).

We have added the information regarding the Tissue Chopper (line 143 and table of materials).

Response to Reviewer 4:

Manuscript Summary:
Here the authors presented well described protocols for isolation and culturing of neural stem cells. The title is informative and contains important search words. Manuscript is well written and all procedures are clearly explained.

Major Concerns:
no

Minor Concerns:
Since this is methods manuscript in the Abstract the important information about key methods should be added, like basic design, the age of mice, duration of incubation etc.

Regarding the Abstract, the information asked by the Reviewer was added, namely the age of the mice (P1 to 3), the incubation period (which is 2-7 days, depending on the degree of differentiation that we want to study), as well as potential applications of the NSA (lines 49-56).

Please explain, why do you use bFGF without heparin, which protect bFGF from degradation at +37 (G. Chen et al., 2012. Stem Cell 30 (4):623-630)

Concerning the use of bFGF without heparin, this procedure was based in our previous studies[2, 3] and others[1, 4]. Although heparin is known to help the stabilization of bFGF we have successfully obtained neurospheres by using bFGF without heparine.

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In step 7, (Storage of Neurospheres) a question: why the neurospheres were frozen without DMSO, which routinely added as cryoprotectant, and why the authors recommend store the cells at -20 C? Did the authors check viability of the cells after re-freezing?

We store the neurosphere pellets, without adding any cryoprotectant because the stored cells are intended for molecular biology analysis (line 254). We have also addressed this question to Reviewer #3.

In the results concerning step 8: It will be good, but optional, add some pictures that will illustrate forming neurites.

As suggested, we have added pictures of the neurites at DIV 1, 2 and 3, in both neurogenic regions. To identify them we performed an immunocytochemistry against the protein βIII-Tubulin . The images are in the new Figure 4.

In step 9.3. to analyze differentiation fate, the authors offer use BrdU at DIV1 and then check expression of markers of mature cells at DIV7 in BrdU positive cells, but do not provide any illustration.

The Reviewer raised an important question about the results of the combination of BrdU with other mature markers regarding to the Cell Differentiation Assay. Therefore, we have now added in Figure 5 of the revised manuscript a graph (and representative images) showing the percentage of BrdU-positive/NeuN-positive cells divided by the total amount of NeuN-positive cells at DIV7. Importantly, with this result we can estimate the number of proliferating progenitors that differentiate into mature neurons. Moreover, in Figure 5, we have also added representative images of the graph A3 already present in the original manuscript (% of BrdU-positive cells at DIV1).

Response to Reviewer 5:

Manuscript Summary:
In this study the authors describe a protocol for the generation, expansion and differentiation of neural stem cells from the early postnatal subventricular zone (SVZ) and subgranular zone (SGZ), the two neurogenic niches described in the murine brain.

Major Concerns:
1. Neurosphere-forming assays have been widely used to identify stem cells based on their capacity to evaluate self-renewal and differentiation at the single cell level in vitro, mainly in the adult neurogenic niches. Thus authors should better clarify in the manuscript that all these experiments are performed in postnatal day 1-3 and not in adult NSCs. Also in the title.

We have reviewed all the sections of the document and we have now included in the revised manuscript the missing information in the title, introduction (line 84) and protocol (lines 115 and 210).

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2. Is still present in the field that the relationship between in vivo progenitors and neurosphere-initiating cells needs some refinement (Doetsch et al., 2002; Reynold sand Rietze, 2005; Pastrana et al., 2011). For example, it is shown that the neurosphere assay does not provide an accurate read-out of the number of stem cells in vivo, mainly because both transit amplifying progenitors and GFAP+ cells form neurospheres and can be serially passaged as multipotent stem cells. Moreover, the neurosphere assay likely does not detect quiescent stem cells as the purified population containing putative quiescent stem cells from the SVZ does not give rise to neurospheres (Pastrana et al., 2009). It is crucial to describe limitations of this assay and the critical points that researchers have to keep in mind before extrapolating results from the in vivo context. The authors should mention all these points in the introduction and discussion.

We are in agreement with the referee concerning the limitations of the neurosphere model. Therefore, while writing the manuscript we always mentioned NSPCs and not just NSCs since both GFAP+ and transit amplifying progenitor cells are able to generate neurospheres. In fact, unlike what happens *in vivo*, *in vitro* we cannot distinguish between type B and type C cells.

Regarding the fact that this method does not detect the presence of quiescence stem cells, since they do not proliferate under the presence of growth factors, we have added a new sentence in the revised manuscript (lines 551 and 552).

3. Sox2 is also expressed in differentiated cells thus in the cell-pair assay to study cell fate the immunocytochemistry against Sox2 in cell pairs should be combined with other markers such as Nestin or differentiated markers (DCX and NG2).

Concerning the question that Sox2-positive cells can be differentiated cells, we are in agreement with this observation. Therefore, we performed again the cell pair assay not only with Sox2 but also with Nestin and the differentiated marker DCX, as was suggested by the Reviewer. With this approach, we have observed symmetric pairs formed by two Nestin- and Sox2-positive cells which are negative for DCX (Figure 2A1 and B1), symmetric pairs composed by two DCX-positive cells, negative for Nestin and Sox2 (Figure 2A2 and B1) as well as asymmetric pairs with one Nestin- and Sox2-positive cell that is negative for DCX and the other cell positive for DCX and negative for Nestin and Sox2 (Figure 2A1 and B2), in both neurogenic niches. Therefore, we have changed the information related with the Figure 2 in the Results section (lines 445-449) as well as in the legend of the Figure (lines 500-503). Importantly, we have also added the Nestin and DCX markers to the protocol of the cell-pair assay in section 4 (lines 205-206).

4. For evaluation of neuritogenesis why the authors only incubate the cells for 1-3 days? Longer periods of cultures will favor neurite formation.

Regarding the neurite formation, we have differentiated cells from 1-3 DIV since with longer periods the network is so big making it almost impossible to

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distinguish different neurons. Moreover, with higher differentiation times the neurites are very long being difficult to follow them.

5. Cell survival assay can be improved combining the PI staining with a immunocytochemistry for activated Caspase3 or the staining with DAPI to also count apoptotic bodies.

Concerning the evaluation of cell death, we totally agree with the Reviewer regarding the combination of the PI staining with another method. Beyond activated Caspase3 and the staining with DAPI to access cell apoptosis, we can also do the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay[5]. We have added in the manuscript an extra note with these alternative approaches to access cell survival (lines 346-348).

Minor Concerns:
In 11.3, 11.4 and 11.5 authors show different coating procedures (PDL, PDL/Laminin and PLO/Laminin) however these three coatings are not explained or defined in the experiments.

Concerning the section related with the three different coatings, these were explained in the Supplementary information. However, we have now moved them to the main manuscript. The PDL coating plate proceduree is in sections 8, the PDL/laminin coating plate procedure is in section 9 and the Poly-L-Ornithine (PLO)/Laminin coating procedure is in section 10.

In Figure 1A, Nestin immunostaining is not clearly seen. In Figure 1B, please indicate the number of days of differentiation.

Regarding to the images of the Figures 1A and 1B, they were replaced to address the question 5 of the Reviewer 3. In the final images, we ensure that the Nestin immunostaining is clearly seen. These images correspond to neurospheres in proliferative conditions with 0 days of differentiation.

In Figure 5, markers in red (Nestin, NG2 and NeuN) are not clearly seen.

We have updated Figure 5 (now Figure 6) so that the staining for Nestin, NG2 and NeuN markers are clearly seen (new images A, F, G and I).

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