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Boston, July 14th 2019

To the editors of
JoVE

Dear Editors,

enclosed please find the revised manuscript **"Simultaneous flow cytometric characterization of multiple cell types retrieved from mouse brain/spinal cord through different homogenization methods"** by Molina Estevez, Mathews et al., submitted for consideration to *JoVE* as regular article.

We thank the reviewers for carefully reading our manuscript and for providing their feedback and suggestions. We have addressed reviewers' comments and edited the text and figures accordingly.

Please find below a point-by-point answer to editor's and reviewers' comments and questions.

Yours sincerely,
Marco Peviani

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.
   Answer: the manuscript has been proofread and edited to ensure there are no spelling or grammar issues.

2. Please revise lines 132-141 to avoid textual overlap with previously published work.
   Answer: lines 132-141 have been revised and sentences have been rephrased to avoid textual overlap with previously published work, as requested.

3. Please define acronyms/abbreviations upon first use in the main text.
   Answer: we checked the manuscript to ensure that acronyms and abbreviations are defined upon first use.

4. Please use the active/imperative voice and complete sentences throughout the protocol.
   Answer: we checked the manuscript to make sure the active/imperative voice and complete sentences are used throughout the protocol.

5. 2.1: Please specify the age, gender and type of mouse.
   Answer: we added the sentence "8w-old C57BL/6J mice, either sex, were used in the experiments" at line 127 of the revised manuscript.

6. 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.
   Answer: as per editor's request, we highlighted in yellow the protocol text to be featured in the video.

7. 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.

Answer: as per editor's request, we ensured that the highlighted text forms a cohesive narrative.

8. 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.
   Answer: as per editor's request, we ensured that all relevant details required to perform the steps in the highlighting are included.

9. Figure 1: Please define error bars in the figure legend.
   Answer: as per editor's request, we defined the error bars in the revised figure 1 and corresponding legend.

10. Table 1: Please abbreviate liters to L (mL) to avoid confusion.
    Answer: we abbreviated liters to L in the revised Table 1.

11. Table of Materials: Please sort the materials alphabetically by material name.
    Answer: as per editor's request, the list of materials have been sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Major Concerns:

1. Figure 1B showed that the yield of cells retrieved from the brain or the spinal cord upon tissue homogenization with DH is significantly higher that with the PD method (107 versus 105), this can be a limit, can you suppose which cell types are completely lost by the PD method?
   Answer: we currently don't have any data supporting the loss of specific cell types with the PD method. However, prompted by reviewer's observation, we carefully examined under the microscope the cells retrieved after the DH or PD method. As it can be observed from the microscope pictures shown in the revised Fig. 1C, the DH method determines the death of many cells both in the brain and in the spinal cord. Many of these cells form aggregates; this phenomenon could be due to the presence of highlighly interconnected cell networks (like the endothelial and glial cells lining the CNS vasculature) that cannot be disaggregated by the shearing force applied with the DH. These

aggregates of death cells will likely not be removed by the Percoll density gradient and end up in the final cell pellet used for cytofluorimetric analysis. On the contrary, the PD method is able to digest the extracellular matrix and cell-to-cell junctions efficiently, leading to a more uniform single cell suspension. Some of the cells that die during the mincing process could be further digested by the papain used with the PD method, leading to formation of cell debris that are more efficiently separated through the Percoll gradient, determining an overall lower yield of cells retrieved, as compared to the DH method. This comment has been added in the revised manuscript, in the paragraph that describes the results of Fig. 1 B, C.

We would like to highlight that during revision of this manuscript we realized that the graph in Fig.1B was displaying the total number of cells retrieved per sample irrespective of the normalization for the weight of tissue, contrary to what reported on the y axis. We apologize for this issue. We have rectified the graph in Fig. 1B of the revised manuscript to make sure that the number of cells normalized on 100 mg of tissue weight is displayed. Since we analyzed additional brain and spinal cord samples to take the pictures showed in the revised Fig. 1C, we decided to include the new results in the revised graph shown in Fig. 1B. Accordingly, the text describing this graph has been edited in the revised manuscript to reflect the results shown in Fig. 1B.

2. In fig 2b you showed that CD45+ cells are very low, so most of these cells can be not recoreved by the PD: author may discuss about that.

   <u>Answer:</u> the percentage of CD45+ cells shown in Fig. 2B is calculated as relative percentage on the total number of live cells retrieved in the sample. With the DH method, CD45+ cells represent about 30% of the few viable cells surviving in the sample. On the other end, with the PD method, a higher number of live cells is retrieved; CD45+ cells being only a small fraction of the total. We agree with reviewer's comment that the graph in Fig. 2B might wrongly induce to think that the recovery of CD45+ is lower with the PD method as compared to the DH method. Given that dead cells represent an important fraction of the total population retrieved with DH, we decided to revise Fig. 2B to show, within the same graph, both the live and dead fraction of CD45+ and CD45- cells. The revised graph helps to better appreciate how both methods allow retrieval of a similar fraction of live CD45+ cells; however, the PD method is able to yield a significantly higher fraction of viable CD45- cells.

3. Flow showed that the myeloid cell fraction CD45+/CD11b (FIG.2C) is extremely low in brain and spoinal after the PD method. P values would help in this analysis to better comprehend the comparison between the two methods, please add and discuss.

   Answer: following reviewer's suggestion, we added a table (Table nr. 2) showing the results of the statistical analysis comparing the yields of different cell populations retrieved with the two methods. Similarly to Fig. 2B, we decided to reshape the graphs in Fig. 2C (pie-charts instead of histograms) by displaying not only the different cell types retrieved within the viable cell fraction, but also the fraction of dead cells, to allow better understanding of the actual proportion of different cell types retrieved with the two methods.

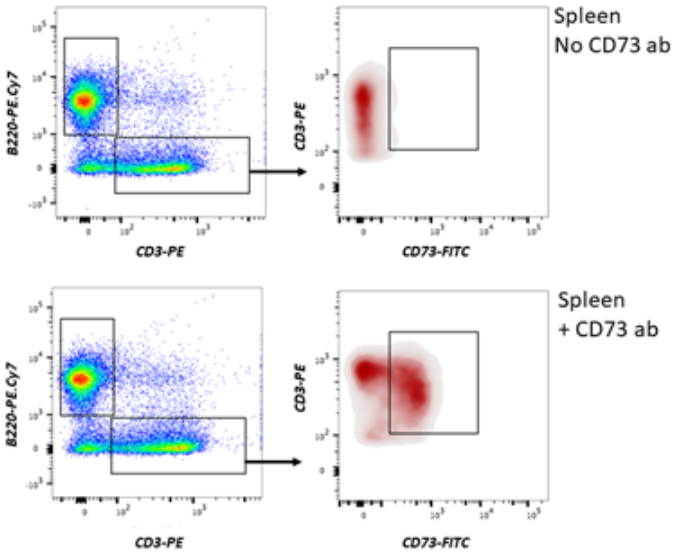
4. Fig 2c showed that almost the 50% of cells yelded with PD are "others", they are an important proportion: are you sure this method is good for the recovery of what you really need? Have you an idea of what "other cells" could be? This analysis could reveal the PD being more suitable for the recovery of different cell types, such as mesenchimal or stromal cells (being bigger and less sensitive to aggressive methods), you should control CD73 antibody in order to test this hypothesis.

   Answer: we thank the reviewer for this observation. The antibodies we used to identify different cell populations recognize well defined cell-specific antigens expressed by terminally differentiated cells. However, we are aware that the brain and spinal cord contain many other cell types, including progenitor cells at different stages of differentiation, such as Nestin+ neural stem cells, Nestin+Vimentin+ radial glia progenitors, Doublecortin+ neural progenitors, NG2+ oligodendrocyte precursor cells. As suggested by the reviewer, mesenchymal cells could also be part of the cell fraction resulting negative for all the markers used in our flow cytometric analysis.

   Following reviewer's suggestion, we processed the brain of 3 mice with PD method and analyzed it by applying the flow cytometric analysis described in our manuscript, adding to the antibody mix also a rat anti-mouse CD73-FITC conjugated antibody (clone TY/11.8, Mylteni Biotech, cat. #130-102-535). The spleen from one mouse was analyzed in parallel, as positive control for CD73 (Yamashita et al., EurJImm 1998). As shown in the figure below, the anti CD73-FITC antibody identifies a fraction of CD3+ cells in the spleen, as expected (Yamashita et al., EurJImm 1998). Interestingly, in the brain we detected about 14% of CD73+ cells within the "other cells" fraction retrieved through the PD method (see table below). Even though not all the "other cell types" could be accounted for, this analysis supports the existence of other CD45- cell types that could be further investigated upon isolation through the PD method.

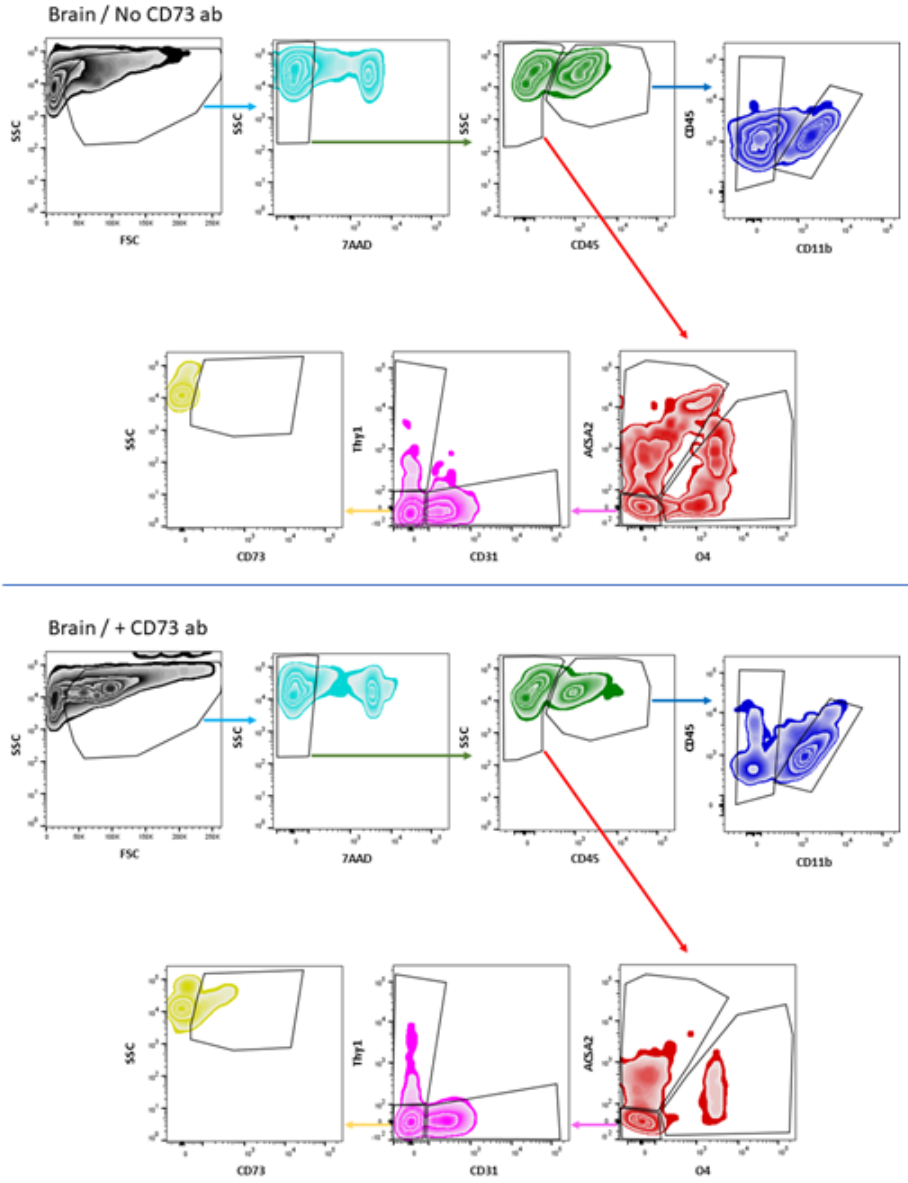
   These considerations have been added in the discussion section of the revised manuscript.

A



A. Flow cytometric analysis of CD73 in the spleen of a C57BL/6J male mouse. Single cell suspension from the spleen was stained with CD45, B220 (B cell) and CD3 (T cell) markers with or without anti-CD73 antibody. **B.** Flow cytometric analysis of CD73 in the brain of C57BL/6J male mice. Tissues were processed with PD method and stained with an antibody mix comprising anti-CD45, CD11b, O4, ACSA2, CD31, Thy1 antibodies with or without anti-CD73 antibody.

B



Mouse ID	*% CD73+ within "other cells"*
1	*10.6*
2	*15.6*
3	*16.3*
Average	***14.2***
SD	***3.1***

Reviewer #2:

Minor Concerns:

1. Regarding Method 5.5, It is unclear whether or how to remove the myelin-containing debris on the surface of the centrifuged solution.

   Answer: based on reviewer's comment, we rephrased the sentence describing removal of myelin-containing debris and we added a new figure (Fig. 4) showing a scheme with the critical steps of this protocol, including how to recognize the myelin-containing debris fraction and remove it properly.

2. This manuscript lacks readability because it has a lot of parenthetical phrases (using parentheses and commas) and slash-divided phrases. The authors had better reconstruct the structure of each sentence to gain better readability.

   Answer: we thank the reviewer for highlighting this issue with manuscript readability. Accordingly, we have edited the text and rephrased the sentences containing parentheses and slash-divided phrases to increase readability.