Submission of our revised manuscript JoVE 60588

Dear Alisha Dsouza,

In reference to your E-mail of September 06th 2019, please find enclosed our revised manuscript entitled « An easy and efficient high output method to isolate cerebral pericytes from mouse».

We thank the 4 reviewers for their productive criticism and helpful comments. We have made every attempt to address the concerns raised by the reviewers. New experiments have been performed and western blots have been added. Our responses to their suggested revisions are detailed in our point-by-point reply below. Please find the original remarks by the reviewers written in italic letters followed by our reply written in blue and upright letters.

We are convinced that our study is significantly improved by following the reviewers suggestions. We hope to have addressed all comments in a satisfactory manner to allow for publication of our revised manuscript in the JoVE Journal.

At last, please note that we also considered the editorial comments and have modified accordingly.

Yours sincerely,

Fabien Gosselet

Comments from Peer-Reviewers

Reviewers' comments:

Reviewer #1:

Major Concerns: The efficiency in terms of yield of pericytes as compared to other published alternative protocols has not been demonstrated. This would require a significant additional amount of work but it would most definitely increase the value and impact of the current study significantly.

We totally agree with the reviewer #1 that the comparison of efficiency in terms of yield of pericytes is only based on values and results claimed by authors in their original publications. The proposition of the reviewer to realize these 6 protocols (because 2 new protocols have been introduced in our discussion) in parallel to directly compare the different yields is an interesting suggestion but would require a huge amount of work as well as the use of several materials and equipment that we do not possess (FACs, etc). The objective of our study was to develop a cheap, fast and efficient method using traditional equipment easily found in all laboratories (centrifuge, etc). And we did it. Therefore we prefer do not follow this suggestion.

Minor Concerns:Language revision is warranted

Addressed. Manuscript has been deeply checked for language revision.

Reviewer #2:

Major Concerns: The authors have performed staining and immunoblotting (Figure 2) showing expression of NG2, PDGFRβ, and CD146 in isolated pericytes. These markers are mural cell markers but not 100% specific to the pericyte sub-population. A single-cell RNA seq analysis published last year showed that Pdgfrb and Cspg4 are expressed in both pericytes and smooth muscle cells (SMCs) but also in fibroblasts (FBs) and oligodendrocyte progenitor cells (OPCs) (Nature 2018; 554:475-480). Also, looking at Mcam gene (i.e., CD146 protein), the highest levels are found in SMCs and a little in oligodendrocytes. Even though the authors use filtration steps, it remains uncertain whether contamination from other cell types - namely, SMCs and FBs - may occur. Additionally, both the grey and white matters are taken into account which may increase contamination issues (e.g., myelin debris). An additional bulk RNAseq experiment could be performed and compared to the current Betsholtz database in order to reinforce the proposed protocol. No one will question the purity if this can be shown.

To address the question of brain pericytes purity raised by the reviewers #2, #3 and #4, we performed additional western blots using specific antibodies directed against CD31, GFAP and CD11b that specifically target endothelial cells, astrocytes and microglial cells, respectively. These new results have been included in the figure 3 and clearly demonstrate that there is no contamination by these different cell types in our final cultures. Therefore, new experiments have been done, a new figure has been added. All these results have been analyzed and discussed in the appropriate section of the manuscript.

- Authors should provide a picture of their initial preparation prior dissociation steps. This image should show vessels only and would be the first image of Figure 2A.

Dissociation steps often occur in our protocol, therefore we do not exactly understand which step is concerned by the reviewer's suggestion. However, as a JoVE protocol, our method will be translated into a movie and we are confident with the fact that all the mandatory steps will be filmed.

- Figure 2D: It is very surprising to see that PDGFRβ levels are equal in mouse brain vs pericyte samples (see Supplemental Figure 1a, Nat Neurosci. 2019; 22:1089-1098 and Figure 1, Neurosci Lett. 2015; 607:97-101)

We thank the reviewer for this comment. Indeed, as in the both studies cited above, we clearly demonstrate that brain pericytes express high level of PDGFR-β. In addition, these both studies claim that brain pericytes express higher levels of PDGFR-β than endothelial cells and vascular smooth muscle cells. In our study, we demonstrate that cerebral pericytes express equally PDGFR-β than total mouse brain. Interestingly, this receptor has also been reported highly expressed by neurons (J Cereb Blood Flow Metab. 2012, 32(2):353-367). Therefore, from our point of view, this is the explanation why we observe a high level of this receptor in total mouse brain extract that include the protein content from all the cell types (i.e. ECs, OPCs, vSMCs, neurons and pericytes, etc).

- Also, it is unclear why the authors decided to compare pericyte protein profiles with the total mouse brain. One would think that comparing pericytes to endothelial cells and/or commercially available pericytes from ScienCell (to name one) would be more informative and would definitely strengthen the manuscript in my opinion. Using the total mouse brain as comparison will only bring confusion to the readers.

The objective of our study was to demonstrate that our protocol and method are efficient to purify mouse brain pericytes from a total mouse brain. For this reason, we used the total mouse brain extract to compare the mRNA and protein expression levels with cultures of brain pericytes. Following reviewers'suggestions, we analyzed expression of specific markers of endothelial cells, microglial cells and astrocytes in our culture of brain pericytes and in total mouse brain fraction. We clearly demonstrate that the level of these markers decrease after extraction, purification and cultivation of these cells. In parallel, the expression of specific markers of brain pericytes is stable or increased (Figures 2B, 2C and 3). In addition, these cells show a specific morphology, as demonstrated by figure 2A.

Then, comparison of these cells with brain pericytes provided by ScienceCell is a very interesting suggestion, but we consider that there is an evident lack of evidence that these cells are really brain pericytes. There is no information on the extraction/purification protocol. In addition, these cells are not well characterized and only very few information are available on the website. It is mentioned that they express PDFGR-β and αSMA, but there is no evidence that they express other pericytes markers. Therefore, we consider that it would be not relevant to compare our cell culture with these cells.

Minor Concerns: It would be of interest to have a native English speaker checking the present manuscript before eventual resubmission.

Addressed. As suggested by the reviewer, the manuscript has been deeply checked for language revision.

Reviewer #3:

Major Concerns: The purity of the cell culture is not clear. The extracts will invariably contain brain endothelial cells and the authors need to demonstrate that pollution from at least these cells. There could also be pollution from macrophages and astrocytes. It would certainly be more convincing with double-labelings of hall-mark markers for endothelial cells, astrocytes, and microglia like CD31, GFAP and CD11b, and preferably in larger mags as this will convince the reader better about the purity of the pericyte cultures.

We thank the reviewer for this suggestion. Following this recommendation, we performed additional western blot experiments using specific antibodies against CD31, GFAP and CD11b that specifically target endothelial cells, astrocytes and microglial cells respectively. These new results have been included in the figure 3 and clearly demonstrate that our primary cultures are not contaminated by these different cell types. Results and discussion parts have been updated accordingly.

The yield may be high, but the authors are not quoting all papers that already set up protocol, e.g. disappointingly the authors fail to quote important papers (e.g. Thomsen et al, PMID: Thomsen MS et al. 27456748; Yamazaki Y et al. 26883501) in this context which also demonstrate high yield in their isolation of primary brain pericytes with virtually identical yield.

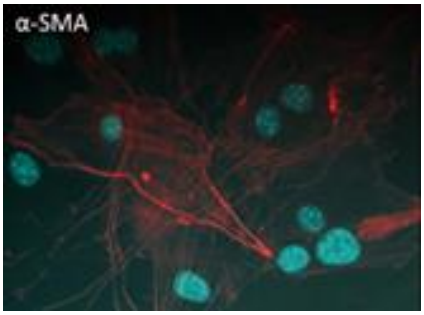
Following the reviewer's suggestion, we added and discussed these interesting references in the manuscript. However, we decided to do not include one of the reference of Thomsen et al., that describes brain pericytes purification using brain from pigs. The objective of our work being to compare the "mouse" protocols together, we decided that it would be not relevant to include data and methods obtained in porcine. Hope that the reviewer will understand our decision.

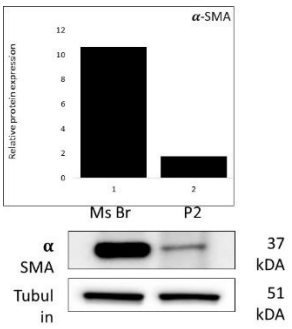
The functional characteristics of the isolated pericytes should be further explored. Did the pericytes enable the authors to co-culture the brain endothelial cells to improve the TEER and lower passive permeability ?

We thank the reviewer for this interesting suggestion and totally agree that functional characteristics of brain pericytes merit to be further investigated. For sure, influence of brain pericytes on brain endothelial cells physiology and monolayer integrity is an interesting point that we would like to investigate in our next studies. Indeed, objective of our study was to develop a fast and efficient method to obtain pure cultures of cerebral pericytes from mouse. But it was not for investigating influence of brain pericytes on blood-brain barrier physiology. However, following reviewer's suggestions, we included in the discussion part the fact that these primary cerebral pericytes might be used in such studies.

When cultured in 20 % FCS, will this change the phonotype of the pericytes ? Did the authors look for alpha-SMA expression?

When cultures in 20% FCS, morphology of brain pericytes is still normal with no visible change in phenotype. In culture conditions, they normally attain a tetrahedron like shape. To answer the second question, we checked for alpha-SMA expression, which can be stated as minimal expression when compared with mouse brain extract that is consistent with an undifferenciated state of brain pericytes as demonstrated previously (Tigges et al. and Dellavalle et al).





Left : IF of alpha-SMA in our cell culture. Right : Western blot showing low expression of alpha-SMA in total mouse brain compared to brain pericytes.

Minor Concerns: Abstact: …."we propose", use another verb. No indications of arrows in Fig.2

Addressed. This sentence has been changed. Arrows are now explained in the representative results section and in figures legend.

Reviewer #4: Manuscript Summary:

Major Concerns: One potential major concern of this protocol is that the authors did not address how to avoid the contamination of astrocytes. It would be highly recommended to check whether the authors' pericyte cultures would not be contaminated with astrocytes.

We thank the reviewer for this helpful comment. To demonstrate the absence of astrocytes contamination into our cell cultures, we tested the expression of the GFAP marker, specific of this cell type. Results are shown in Figure 3 and demonstrate that our protocol is efficient to decrease the expression of GFAP which is absent in our culture, but highly expressed in brain total fraction. In addition, we also investigated the contamination by microglial cells and endothelial cells. Again, our results show that there is no contamination.

Minor Concerns: Figure 2: the authors need to use the term "PDGF-R-beta" instead of "PDGF-R".

Addressed. We remplaced PDGF-R by PDGFR-β.