Dear Dr. DSouza,

Thank you very much for handling our submission! The listed below is the point-by-point response to editorial comments and reviewers’ comments.

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank the editor. We have corrected all the typos in the revised manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Thank the editor’s reminding. Here is the link to the website where the reusing policy is explicated stated, <http://www.rsc.org/journals-books-databases/journal-authors-reviewers/licences-copyright-permissions/#reuse-permission-requests>. The figures refer to the published reference has been cited correctly.

3. Please spell out each abbreviation the first time it is used.

We have added the full name for each abbreviation where they were used first time.

4. Please use centrifugal force (x g) for centrifuge speeds.

We have confirmed that all the description about centrifuge speed was in unit of centrifugal force. We used rpm as unit only at the step 5.8 for rotation speed of affinity purification incubation. We have added the description in this step.

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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ATCC, Triton X-100, etc.

Thank the editor for pointing this out. We have rewritten the related part with generic terms. Triton X-100 is a widely used non-ionic detergent. In order to make sure this is a generic term, we referred the published protocols by JOVE and found “Triton X-100” is directly used in the text.

6. Line 116: Please remove the weblink which contains commercial information.

We have removed the links.

7. 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:  
Approximate volumes for all buffers, gradients, and stock solutions to be set up should be given.

Thank the editor for reminding. We have added the details corresponding to the following listed points in the revised manuscript.

1.1: What container is used?  
1.2: Aspirate what medium, and from where? Please specify.  
1.4: What are the culturing conditions?  
2.2.2: Please describe how to remove the uterus and specify all surgical instruments used.  
2.2.3: What is used to cut?  
2.2.5: Please specify the volume of pre-chilled HBSS.

8. 2.2.6, 3.3: Please break up into two steps.

Thank the editor. We have separate step 2.2.6 into two steps. Step 3.3 describes to add Ac4ManNAz or DMSO into culture systems. We think it is not proper to separate it.

9. 2.2.9: Please spell out AM.

AM appeared for first time at step 2.1 and represents adherent culture medium.

10. 5.10: Please write the text in the imperative tense.

We have rewritten the text.

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

We have combined the short steps.

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

We have added spaces accordingly.

13. After you have made all the recommended changes to your protocol (listed above), 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.

We have highlighted the essential steps for video preparation.

14. 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. Please do not highlight any steps describing anesthetization and euthanasia.

Thank the editor for the instruction. We have highlighted the appropriate parts.

15. 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.

We have highlighted the appropriate parts in the revised manuscript.

16. Figure 1: Please define the error bars in panel C in the figure legend. Please include a space between numbers and their units in panel D (130 kD, 55 kD, 25 kD).

Thank the editor for pointing this out. We have defined error bar as SEM for panel C and add the space in panel D.

17. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

Thant the editor for the instruction. We have revised the discussion part according to the instruction listed above.  
  
**Reviewers' comments:**  
  
  
**Reviewer #1:**  
Minor Concerns:  
A couple of places the authors need to provide clarifications, which are labeled on the manuscript (Please see the attached file from Reviewer 1).

We are very grateful for the reviewer’s suggestions!

When seeding BEND3 cells into the transwell inserts for coculture, we chose to use the cells when the cell confluence in 10 cm dishes reachs 90% and then split the cells into the transwells at the ratio of 1:9. We found in this way the cell state of BEND3 and the cell density of plating is reproducible. We have added this step into the text.

According to the workflow shown by Fig. 1A, the labelling of culture began on D2 and ended on D6. The refeeding should be carried on D3 and D5. We have clarified the procedure in figure legend. Ac4ManNAz or DMSO are not needed to be supplied when refeeding and we have clarified it in the corresponding part of revised manuscript. The 55 kD band in old Fig1 D indicated nonspecific binding proteins. We have clarified this in the new figure legend.   
  
  
**Reviewer #2:**  
The authors present a useful protocol for the isolation of proteins from neural stem and progenitor cells. The protocol is detailed enough to enable reproducibility. However, the mass spec analysis was not discussed in detail. It is clear that for anyone using the present protocol and proceeding to mass spec analysis it will be necessary to obtain resources and protocols to go all the way to identify the neural stem cell proteome. Nevertheless, the labeling protocol described here is helpful but requires some improvement at certain places.

We thank the reviewer for the positive comments. We sincerely apologize for the typographical and grammatical errors. We have proof read the manuscript and made corrections accordingly. The details of mass spec analysis are not included in this manuscript but can be found in our previous publications. We have added references in the text.

1. A rigorous proof read is required to correct typographical and grammatical errors. I point out the most critical ones below but the entire text should be proof read.

Thank the reviewer for pointing this out. We apologize for the errors and corrected them in the revised manuscript.

2. In the abstract lines 40-42 the authors make a strong statement about cell transplantation and brain repair. Thus far this is not possible and the statement is too strong. It is better to say … may offer… rather than …offering…. This point should also be addressed in the introduction lines 62-64.

We completely agree with the reviewer. We have revised this part.

3. Lines 103-104 the authors should avoid negative statements about FACS with the intention to better highlight their technique. FACS has been used by leading labs (Kriegstein, Walsh, Huttner to name just a few) to isolate embryonic progenitor cells from many species and human. It is simply not true that FACS is 'labor-consuming'. In contrast, FACS is way more efficient than the method described here. Thus the authors are advised to remove such negative statement.

Thank the reviewer for the advice. We have removed this statement.

4. Line 114. Correct the typo: transwell not tranwell

We have corrected it.

5. Line 168. Correct the typo: 2x10… not 2\*10…

Thank the reviewer. We have correct it.

6. Line 202. Antibody dilution for anti-Nestin is 1:40, in the reagent list they state 1:20. Which dilution is the correct one?

Thank the reviewer for pointing this out. The dilution ratio for anti-Nestin antibody should be 1:20. We have corrected it in the revised text.

7. Line 203. Correct the typo: blocking buffer not block buffer

We have corrected it.

8.Line 230. Correct the sentence and remove After reaction,… It is obsolete.  
We have removed this part.  
  
**Reviewer #3:**  
Thank the reviewer for critical comments and helpful advices.   
it is essential that the authors include representative fluorescence images of both co-cultured and non-cocultured cells, so that the difference between them can be determined by the reader. FACS-mediated cell sorting could also probably assess the degree of differentiation in each culture type, but this would sacrifice the convenience of the FACS-free method described by the authors.

Using endothelial coculture system to expand NSPCs in vitro is first reported by our group in 2004. The secreted factors from endothelial cells stimulate NSPCs self-renewal. In the original paper, we have reported detailed comparison between the co-cultured and non-cocultured cells. In this revision, we have added a new panel in Figure 1 to show the neural clones formed by NSPCs in endothelial coculture have much more Nestin+ NSPCs and less Tuj1- differentiating neuronal cells than differentiated NSPCs culture. This is in line with what we have shown in original Fig. 1B and 1C.

The cytotoxic and morphological effects of the MOE substrate on cells is also vital for determining the applicability of this method. Although the authors claim that optimization was done to determine the 100µM optimal Ac4ManNAz concentration and that such a concentration had no effects on the cells' morphology, self-renewal or differentiation potential, only a "ratio of positive cells" for each treatment case was included. Such a ratio provides inadequate evidence that the substrate has no effect on the differentiation or proliferative capabilities of the cells, and provides no evidence whatsoever about effects on morphology. Cell viability data and morphology images showing the negligible cytotoxicity at this concentration are essential for demonstrating the utility and applicability of this method to other systems.

We appreciate the reviewer’s comments. We have added a new panel to show cell morphology and viability judged by nuclear morphology between Ac4ManNAz labeling and control. The images demonstrate that 100 μM Ac4ManNAz labeling does not affect cell morphology and viability significantly. In addition, we pointed out that the sensitivity and cytotoxicity of Ac4ManNAz to different cell types could vary, so the labeling concentration needs to be optimized individually.

Finally, based on the SDS gel in Figure 1d, no clear difference between protein purified from NPSC-expanded cultures and differentiated cultures can be observed. In addition, there appears to be unspecific binding to the streptavidin bead resulting, raising the question of how well the Ac4ManNAz-based purification protocol can identify labelled glycoproteins. MS data or other proteomic studies are essential to demonstrate that the culturing method described produces detectable amounts of NPSC glycoprotein enrichment, and that such enrichment yields specific detectable quantities of glycoproteins. It is also unclear from the acknowledgements section whether figures 1c and 1d actually correspond to the studies described in this manuscript.

The reviewer is absolutely right about the proteins revealed in gel imaging. The Coomassie blue staining of SDS PAGE gel in Fig. 1D does not show the difference between NSPCs with or without endothelial coculture. There could be two possible reasons. First, most abundant plasma membrane proteins are housekeeping proteins, like various transporters and channels. They are highly expressed by both NSPCs and differentiating cells. Because of their high abundance, they are very easy to be visualized after staining and corresponding to the major bands in gel. The cell type specific membrane proteins, however, are not as abundant as housekeeping membrane proteins. Second, purified membrane proteins are separated by SDS PAGE gel mainly based on their molecular weight. One protein band in gel could represent a bunch of membrane proteins with similar molecular weight. It may mask the cell type specific proteins if they have the similar size with housekeeping proteins. Therefore, the difference between the two groups is not easily discerned at this resolution. In our previous study, we show clearly mass-spec analysis could identify the difference in membrane proteins purified from NSPCs with or without endothelial coculture, providing quantitative evidence that our strategy can identify the membrane proteins which are specific or highly expressed in NSPCs rather than differentiating neurons.

Fig. 1C and 1D correspond to the study described here and we have rewritten this claim to make it clear.