Oct20, 2018

Dear Dr. Vineeta Bajaj

Please find the attached revised manuscript entitled “Neurogenesis using P19 Embryonal Carcinoma Cells”, which we would like you to consider for publishing in Journal of Visualized Experiments. We have substantially revised the manuscript to address all concerns and suggestions of the editor and the three reviewers and submitted the revised version for your kind perusal and consideration. We hope that the current version will now meet publication requirements in Journal of Visualized Experiments. We have confirmed that the revised manuscript fulfills the criteria suggested by you.

We thank you for extending the deadline for resubmission and should you have any queries, please feel free to ask me.

Best regards,

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**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 your constructive comment. We have checked the manuscript again.*

2. Figure 2: Please include a space between the number and the units of the scale bar.

*Thank you for your constructive comment. We have modified Figure 2 as suggested.*

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

*Thank you for your constructive comment. We have modified the summary as suggested.*

4. Please revise the Introduction to include all of the following:  
a) A clear statement of the overall goal of this method  
b) The rationale behind the development and/or use of this technique  
c) The advantages over alternative techniques with applicable references to previous studies  
d) A description of the context of the technique in the wider body of literature  
e) Information to help readers to determine whether the method is appropriate for their application

*Thank you for your constructive comment. We have newly added the information regarding e) Information to help readers to determine whether the method is appropriate for their application. We have checked the Introduction again and confirmed that our manuscript fulfills the criteria.*

5. Please define all abbreviations before use (PBS, etc.).

*Thank you for your constructive comment. We have defined all the abbreviations.*

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*Thank you for your constructive comment. We have modified the descriptions as suggested.*

7. 1.2: Please describe how cell passage is done.

*Thank you for your constructive comment. We have described the process in the revised manuscript as suggested.*

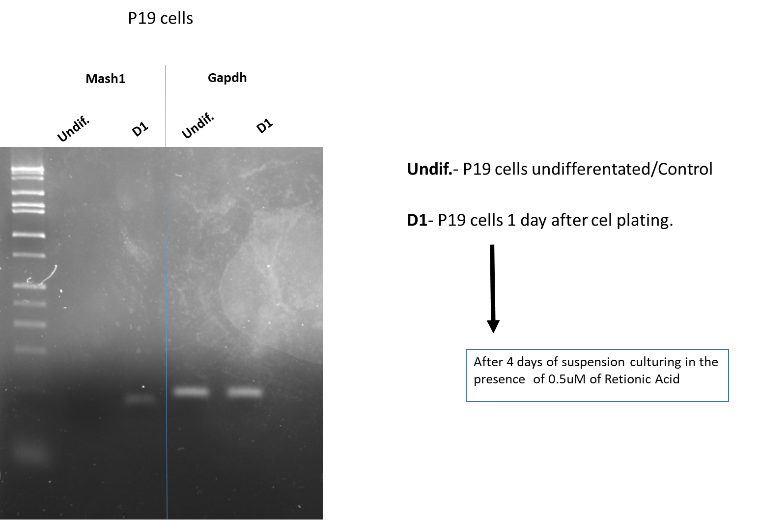
8. 2.3: Please specify the incubation temperature.

*Thank you for your constructive comment. We have modified the description as suggested.*

9. References: Please do not abbreviate journal titles.

*Thank you for your constructive comment. We have modified the journal titles as suggested.*

**Reviewers' comments:**  
  
**Reviewer #1:**  
JOVE58225: A simple method for inducing neurogenesis in P19 embryonal carcinoma cells  
This manuscript by Pawel Leszczynski et al, focuses on the description of a protocol for inducing neurogenesis from P19 embryonal carcinoma cells.  
  
protocol concerns:  
\*In our experience, culturing P19 cells in 10% FCS might provide a fraction of neuronal autodifferentiated cells due to the presence of traces of retinoid analogs, reason why 5%FCS + 5%FCS delipidated are used for decreasing such autodifferentiation potential. Do the authors observed potential autodifferentiation events during their culture maintenance? In Figure 2 we can observe some filamentous-like structures which on our experience could suggest the presence of an autodifferentiation potential. Furthermore in Figure 1 authors evaluates the level of expression of a certain number of markers to illustrate the gain in neuronal markers as well as the loss of stemness, or early responding markers during neurogenesis (e.g. Ascl1, Tal2 as illustrated by Kobayashi et al; SciRep. 2014) are missing, mainly to evaluate the presence of potential autodifferentiation.

*Thank you for your constructive comment. During P19 cell culture maintenance we did not observe autodifferentiation of P19 cells. We also verified the expression of Mash1 (Ascl1) in undifferentiated P19 cells (Undiff.) and D1 (1 day after cell plating-5 days after induction of neurogenesis during cell aggregation with the retinoic acid). Since we did not observe the expression of Mash1 in undifferentiated P19 cells whereas the expression was found in D1, it is concluded that P19 cells are not autodifferentiated in our culture system.*

\*While the protocol seems to generate neurons, the performance over other methods is not evaluated. Notably there are two major aspects that is not discussed/evaluated in this protocol: (i) the performance of cell aggregates versus the monolayer strategy described by Monzo et al (J. Neurosci Methods; 2012); (ii) the use of RA concentrations higher than previously described. This last point is of major relevance in the context of other strategies, namely those aiming to avoid the use of retinoic acid during neuro-ectodermal inuction for avoiding induction of Hox-related patterning. Overall, while I do agree that the procedure described on this manuscript allows to induce neurogenesis, none of the provided figures allows to judge about the yield of the procedure (fraction of neuronal cells induced after 2 +4 days of culture), neither to evaluate its efficacy relative to other well described methods.

*Thank you for your constructive comment. From what we have tested regarding the points you raised, we think making aggregates in the suspension culture is one of the most important points in our protocol. On the other hand, Monzo et al. has clearly demonstrated that neurogenesis can be introduced by the monolayer strategy. We appreciate their technique to introduce neurogenesis. However, their technique requires a matrigel coating plate for their neuronal differentiation. Moreover, it takes 14Days after 1uM RA treatment to observe neuronal-like cells in large numbers. On the other hand, our method allows us to observe similar cell morphology 8Days after 0.5uM RA treatment. Therefore, it is concluded that our method can still be useful in the field of neuroscience. More importantly, we use similar concentration of RA as previously reported studies including Monzo et al. Nevertheless, further studies are required for an optimization of RA concentration to avoid its side effect, and for comparison of our protocol and other methods using functional assays . To clarify this point, we have added the following sentence in the Discussion. “Monzo et al. have clearly demonstrated that neurogenesis is introduced by the monolayer culture method using P19 cells. Although their method is quite convenient as we can eliminate suspension culture process, further studies are required to compare their method with other well-described methods.”. Thank you very much for your very important suggestions.*  
  
**Reviewer #2:**  
Manuscript Summary:  
The manuscript entitled "A simple method for inducing neurogenesis in P19 embryonal carcinoma cells" presents a simple, easy and cheap method to differentiate P19 cell line into neurons.  
  
Major Concerns:  
Some improvement is necessary to clarify the described methods. Please consider the following comments and suggestions:  
1) In the Abstract and Introduction please change P19 cells for P19 cell line, to clearly state it is a cell line;

*Thank you for your constructive comment. The description of P19 cells in the manuscript was changed from “P19 cells” to “P19 cell line (P19 cells)” and the same modifications were performed wherever appropriate.*

2) A scheme containing timings for each procedure/step should be included for easily and rapidly assessing the whole procedure

*Thank you for constructive comment. We have newly added a scheme containing timings for each procedure/step*

3) Is it necessary to have a final step of neuronal enrichment with cell culture using anti-mitotic drugs? Discuss it

*Thank you for your constructive comment. During first days after cell plating mostly indicated cells display neuronal markers. Our protocol was designed to finish cell culture after 4 days of cell culture where number on neuron-like cells is high. We agree that anti-mitotic agent should be added to cell culture but only in situation when researcher is aiming to prolong his studies. Therefore, we have added the following sentence in the Discussion.*

*“ It is also recommended to perform neuronal enrichment with cell culture using anti-mitotic drugs (e.g. Cytosine arabinoside) for long term culture to inhibit extensive proliferation of glial cells..”*

4) Which is the final neuronal production yield? It should be included an average number of neuronal production whenever starting the process with 1x106 cells, for instance. Is the final yield an advantage?

*We assume that the final production yield can be evaluated by Map2 staining (neuronal marker) and therefore we have newly added Figure 4 to clarify this point. Our staining result shows that almost all the cells possess neuronal character.*

5) In figure 1 clarify whether the days are counted during aggregate-based differentiation process

*Thank you for your constructive comment. In the revised version of the manuscript we added the missing information. In our protocol we started counting days from the time P19 cell were replated on culture plates (after 4 days of RA treatment). We have added the following sentences to clarify this point. “Neurogenesis is induced by culturing P19 cells in 100 mm of non-treated culture dish with 5% of FBS and 0.5 µM RA (The cells form aggregates). After 4 days, the cell aggregates are dissociated with trypsin and seeded on adherent cell culture plate for following next 4 days.”*

6) In Figure 4B: does "day4" mean 4 days after plating being 8 days after starting neuronal differentiation process? Clearly state it

*Thank you for your constructive comment. In the revised version of manuscript we clarified that Day 4 represents 8 days after the induction of neurogenesis (RA treatment).*

Minor Concerns:  
General comments about the protocol:  
1.2 - about 90% of confluency?

*Thank you for your constructive comment. We have added the description (70-90% of confluency) in the manuscript.*

3.1 - please state temperature of RA storage

*Thank you for your constructive comment. We stored RA at -20 and we have added the description in the manuscript.*

3.2 - Clearly mention that differentiation medium is with RA. Better define what is dish dedicated to suspension culture. Please define the surface area for this dish.

*Thank you for your constructive comment. We have clearly mentioned that differentiation medium is with RA and described catalog number of suspension culture dish in the materials section.*

3.3 it is mentioned dish and in 3.4 it is mention flask; please maintain the same name for avoiding misunderstandings

*Thank you for your constructive comment. We have changed from “flask” to “plate” in 3.4 of the manuscript.*

5.1 - 3 mL per well - clearly state

*Thank you for your constructive comment. We have added “3 ml per well’’ to 5.1 of the manuscript.*

**Reviewer #3:**  
The manuscript entitled: "A simple method for inducing neurogénesis in P19 embryonal carcinoma cells." Introduce a simple method to differentiate the P19 cells into neuronal cells, employing accessible reagents and describing the process step by step to successfully fulfill the protocol. I believe that the protocol described will benefit the scientific community, however some steps should be further clarified to make it more clear and accessible for researchers not used to work with P19 cells.  
  
Minor and Major Concerns:  
-In the introduction, authors state that "DMEM with 10% FBS for maintaining P19 cells as well as for neuronal development can be utilized", however in the protocol it is described Differentiation medium, (DMEM with 5% FBS) and subsequent Retinoic acid is required for the differentiation into neurons. This is somehow confusing and should be clarified.

*Thank you for your constructive comments and we are sorry that our description was confusing. We meant that we only use FBS and RA for our neurogenesis which is relatively convenient compared with the method in which we should use FCS and FBS. Therefore, we have changed the description as follows: “In this study, we demonstrated that DMEM with FBS can be utilized for maintaining P19 cells (DMEM+10%FBS) as well as for neuronal development (DMEM+5%FBS+RA)”*

PROTOCOL:  
Step 1.2- How is the cell passage performed? In which kind of plate are the cells maintained?, flask? Which size? Is there any coating needed?

*Thank you for your constructive comments and we have described catalog number of our culture flask in the material section. We have also added the detailed method how we maintain the cells in sections 1-2.*

Step 2.9- and 4.12- It is possible to add an alternative method for cell counting?

*Thank you for this comment. We have changed this description to ”cell counter” instead of hemocytometer.*

Step 3.3 and 5.2- It is any specific coating required for seeding the cells? Are the aggregates free-floating or they are attached to the plate surface? Please specify

*Thank you for this comment. We do not need specific coating for plating the cells and the aggregates are floating in the suspension culture plate.*

Step 3.6 and 4.5- Is this step performed at RT or 37ºC?

*Thank you for this comment. We have added the missing information about the temperature. We have added “room temperature” in our revised manuscript.*

Step 5.3- After this step it is to be assumed that the cells are already neurons ? Could the authors specify this on the protocol? If so, should step 5.3 correspond then to the Day 1 depicted in figure 1?

*Thank you for this comment. It normally takes 4-6days after plating the cells to a 6 well plate and we have clarified this in the result section.*

Figure 2B would strongly benefit with an additional picture of an immunocytochemistry staining with neuronal markers, to support the statements of neuronal differentiation and to discard a potential heterogeneity in the cell culture (comprising both differentiated and undifferentiated cells)

*Thank you for this comment. We have performed an* *immunofluorescent staining for Map2 (neuronal cell marker)(shown in Figure 4).*