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March 0, 2012

Attn: Mr. Patrick Lansing

Re: Revision of Manuscript JOVE-4188, *Journal of Visual Experimentation*.

Dear Mr. Lansing,

We are delighted that our manuscript was of high interest and appreciate the reviewers time and expertise in identifying areas of the submission that previously were not clear. We have responded to the reviewer's comments and provide a detailed listing of comments outlining the changes made in the manuscript. We look forward to hearing from you soon.

Sincerely,

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Reviewer 1 Comments and Responses:

1. The manufacturer of Accumax should be referenced in the text, as this is a key component of the protocol.

We have added the manufacturer's name to the product information table. This item is manufactured by Innovative Cell Technologies, but distributed by Sigma. So while we purchased it from Sigma, we've included the manufacturer's name for reference.

2. Is the DNAse I stock solution the same used for Quench and Quench 1.5?

We've added additional text to the protocol to clarify this aspect on step 3.4

3. It would be helpful to suggest a FACS collection buffer for viable cells that will be cultured, as opposed to TRIzol, since this is referred to later in the text.

Step 5.5 has been added to the protocol indicating that the cells can be indicating that cells can be sorted directly into 6 well plates for culture, with references to multiple published manuscripts that describe in detail the media that is used and proper preparation of such plates.

Reviewer 2 Comments and Responses:

1. The authors suggest that their method is more suitable to get better yield and survival as indicated in their abstract "Prior efforts to isolate NC-derived populations relied upon digestion with a cocktail of collagenase and trypsin to obtain cell suspensions for flow cytometry. In our hands these methods produced cell suspensions from the LUT with relatively lower viability." It would be helpful for scientist to know what was the survival rate of cells achieved by Buehler et al using the other methods relative to their particular protocol. So that one can judge how significantly does cell survival and viability increase. For example, using collagenase they found 60% cell death, trypsin 65% cell death while using their protocol with Accumax only 30% cell death was seen in the E15 lower urogenital tract.

We have provided details of percent survival for enteric and LUT progenitors isolated using three separate conditions: trypsin+collagenase, dispase, accumax. In the past most studies published isolation of enteric neuronal progenitors performed with a blend of enzyme (trypsin/collagenase, not each one independently) and we have added this additional detail to the discussion with citations. We have also incorporated a table summarizing the survival of LUT neuronal progenitors as compared to enteric progenitors in all three conditions. We described in detail in the discussion explaining how the flow sort gates are set so that it is clear that the percent survivals we provide are from the "parent" population which has already been narrowed to exclude debris and clumps of cells by forward, side, and voltage pulse geometry gates.

2. Omit "expressing distinct neurotransmitter phenotypes" from the abstract as this is not shown by the authors.
This has been corrected in the text of the abstract.

3. Wording regarding appearance of TH+ progenitors is confusing, re-word to read as "Enteric neuronal progenitors begin to express TH during their migration in the fetal intestine[1] and TH is also present in a subset of adult pelvic ganglia neurons[2-4] . The first appearance of this lineage and the distribution of these neurons in other aspects of the LUT, and their isolation has not been described."

We have changed the wording in the abstract as recommended.

4. Requested clarification on fetal staging to - add in text if morning of the plug is considered as day 1 or day 0.

We have added a phrase to the longer abstract to clarify that morning of plug is considered day 0.5

5. Requested change wording of "In our hands these methods produced cell suspensions from the LUT with relatively lower viability." From lower to low.

We have changed this wording in the abstract.

6. Requested clarification on use of solution in Step 1.1: At what stage in the protocol is the solution made in step 1.1 being used ? Is this solution the same as " Dissociation solution" ? Unclear.

We have modified the text wording in Step 1.1 to indicate that this solution is used in Step 3.4 to prepare the quenching solutions Quench and Quench 1:5.

7. Spacing between numbers and text needs to be systematical throughout the manuscript.

We have corrected the text so that there is consistently a space after numbers

8. Step 1.3: Replace "um" with "µm".

This has been corrected in the text.

9. Step 2.1: Insert space after 100
This has been corrected in the text.

10. Step 2.4: Replace the word “element” with “tissue” instead.
This has been corrected in the text.

11. Step 2.4: Keep space between 15 and ml.
This has been corrected in the text.

12. Step 2.4: Keep stop sign after closed bracket symbol and not before.
This has been corrected in the text.

13. Step 3.3: Insert time necessary to digest 14dpc LUT and 15 dpc LUT as this is shown in Figure 4.
We have provided typical dissociation times for LUT tissue.

14. Step 3.4: Is “dissociation solution” the same as Accumax solution or is it the solution made in step 1.1. Unclear.
This has been clarified in the text of Step 3.4.

15. Step 3.4:Replace “ul” to “µl”. Done twice.
This has been corrected in the text.

16. Step 4.1: Insert space after 15
This has been corrected in the text.

17. Step 4.3: Insert space after 1
This has been corrected in the text.

18. Step 4.4: Insert space after 5 and 15
This has been corrected in the text.

19. Step 5.1: “transfer 1/10 to 1/20th of the sample volume to a new tube”. Is this a 5 ml or 15 ml tube ? since both are mentioned in the reagents list
This has been changed to indicate that that volume is tranfered to a 5ml polystyrene tube.

20. Step 5.2 “Fill all tubes with Quench 1:5” Is this a 5 ml or 15 ml tube ? since both are mentioned in the reagents list
These are the 5 ml tubes that the cell suspension was filtered into during step 4.4, Step 5.2 has been modified to indicate that those 5 ml tubes are what is being filled.

21. Step 5.2: Replace text “4oC” to read as “4°C”
This has been corrected in the text.

22. Step 5.2: Insert space after 200. Replace “ul” to “µl”.
This has been corrected in the text.

23. Step 5.3: Replace text in chart “7AAD” to read as “7-AAD”. Is this the final volume to be added ?
The text in Chart has been updated to indicate that the Volume of 7-AAD is what is added to the tube. The amount added is indicated. The final amount will vary by an amount ranging from 50-100 ul since the aspiration of media is an approximate process and performed to avoid the pellet. Final volumes are NOT measured so as to minimize handling of cells in solution. WE’ve added a note beneath the chart to clarify this aspect.

24. Step 5.4: Insert space after 0.75
This has been corrected in the text.

25. Step 6.2: Insert space after 100.
This has been corrected in the text.

26. Step 6.2: Replace “um” to “µm”
This has been corrected in the text.

27. Step 6.2: Flow rate of 1.0 Mention units used here
This is a software setting on BD Aria instruments that does not have a unit associated with it. We have replaced this value with the number of events per second which can be applied to different flow sorters to indicate that we sort at a threshold of 3,000 events per second.

28. Step 6.3: Replace text in chart “7AAD” to read as “7-AAD”.
This has been corrected in the text.

29. Figure 1 Panel a: There is an “a” labeled within the tissue (Top right). Indicate in figure legend what “a” stands for
30. Figure 1 Panel a: Include in figure legend what t, k, gt stand for.
The figure legend has be updated to indicate that the labeling of anatomical structure is as follows: adrenals (a), kidneys (k), testis (t), bladder (bla), and genital tubercle (gt).

31. Figure 1 Panel b: Include labels in panel b.
Labels have been added to the figure.

32. Reviewer requested revision of figure legend to read:
Brightfield images of 15dpc fetal LUT **(a)** and intestine **(e)**, respectively, imaged halfway through the dissociation incubation period, at the end of the dissociation incubation before disruption (b, f), after manual disruption (c, g), and in a sample that has been overly dissociated (d, h).
This wording has been changed in the figure legend.

33. Figure 4 Panel a: Y axis is written as “14 dpc” but figure legend is written as “14.5 dpc” Keep consistent between figure and legend.
The text in the figure has been corrected to read 14.5 dpc.

34. Figure 4 Panel b: Y axis is written as “15 dpc” but figure legend is written as “15.5 dpc” Keep consistent between figure and legend.
The text in the figure has been corrected to read 15.5 dpc.

Reviewer 3 Comments and Responses:

1. The reviewer wanted additional information about Accumax “this product in the abstract and the dissociation of subdissected tissues section. This is a proprietary reagent but some general information about it would be helpful to readers that are unfamiliar with this product and how it differs from other chemicals used for cell dissociation.”
We have added the manufacturer’s name to the product information table and have clarified in the discussion that Accumax is a proprietary blend of protease, collagenolytic activity and DNAse of unknown composition. Because the ingredients and proportions of reagents within this product are not provided in detail we are not able to compare the composition to other enzymatic treatments that are often used for cell dissociation.

2. In section 4) Filtering cell suspension, the authors describe the use of a “square of nylon mesh”. It would be very useful for the authors to provide a more detailed description of this mesh in this section rather than just including it in the table of reagents towards the end of the manuscript.

The text of section 4 has been modified to include additional detail.

3. In section 5) Preparing samples for FACS, the authors describe collection of cells for RNA in 5.4. The authors might want to detail the methods used to collect cells that are going to be used for other experiments rather than RNA isolation since often the FACS isolated cells may be used for in vitro culturing.

We have additional information on this aspect and refer the reader to multiple publications where the components of the media have been previously described. In addition, we provide a URL to the laboratory of Dr. Sean Morrison where a PDF listing detail about the media composition can be downloaded.