

Date: July 31, 2018

To

Science Editor,

Journal of Visualized Experiments

1 Alewife Center, Suite 200,

Cambridge, MA 02140

Subject: Revised manuscript # JoVE58690\_R0

Dear Dr. Ronald Myers,

We are glad to receive positive comments from the reviewers. I would like to submit revised version of our manuscript to JoVE. We have addressed all editorial and reviewer’s comments. Please find an updated manuscript file, and a point-by-point rebuttal.

Sincerely,



Anirban Paul.

Senior Research Associate,

Cold Spring Harbor Laboratory,

NY 11724.

**Editorial comments:**

Changes to be made by the Author(s) regarding the manuscript:

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.

All changes tracked

2. Please note that Open Access is checked in the uploaded ALA, while in the Questionnaire Responses Standard Access is selected. Please be consistent.

We have chosen Standard Access, and have modified our Author License Agreement accordingly.

3. 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].”

Cell editorial policy says “You do not need to ask our permission to use images or information that you have previously published in a Cell Press journal; we ask only that you cite the original publication.” Link https://www.cell.com/cell/authors

4. Figure 3: Please provide the unit for x-axis in panel A.

x-axis unit is arbitrary fluorescence units [FU], explanation added in legend for Figure 3

5. Figure 4F: Please remove the citation in the figure; instead cite the reference properly in the figure legend.

Citation moved to legend

6. Please do not number the Table of Materials in the article.

Table of Materials not numbered in article

7. Table 2: Please used SI abbreviations for all units and include a space between all numbers and their corresponding units. Please remove all commercial language (Tocris, Sigma, etc.).

SI abbreviations used, added space, commercial language removed

8. Please revise the title to be more concise if possible.

Title reworded

9. Please provide an email address for each author.

Email ID of all authors added in affiliations

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

First time abbreviations spelled out

11. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

SI abbreviations used

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

Spaces inserted

13. 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. For example: Fluidigm C1, Sutter, Airgas, Sylgard, Qiagen, Illumina, AMPure, Nanodrop, etc.

Commercial language removed as much as practicable

14. 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. For example:

1.1: Please specify the source materials for the microcapllaries and the length of microcapiilaries.

Specified and also mentioned in material table

1.2: Please describe how to oxygenate. Please mention how oxygen is supplied to the liquid.

Described

1.6: Please specify the age, gender and strain of mouse. Please specify all surgical instruments used. Please do not highlight euthanasia.

specified

1.7: What container is used in this step?

specified

1.8: Is the brain frozen before sectioning?

No and clarified as such in text

1.9: What is used to hold the fresh slices? How many slices are needed? How to keep bubbling oxygen?

Specified and added to text

1.10: what containers are used? Are the slices placed in the same container? What volume of the solution is needed?

Specified and added to text

1.12: What is used to cut cortical areas?

Specified and added to text

1.13: How many pieces are placed in one tube?

Specified and added to text

1.16: How to observe GFP/RFP signal?

Specified and added to text

2.2.4: What is incubation temperature?

Specified and added to text

2.3.5: Please specify the type of wash tube.

Specified and added to text

2.3.9/2.5.8: Please provide composition of wash buffer.

Wash buffer comp not specified by vendor of IVT MessageAmp-II (Ambion) but supplied as part of Cat#AM1751

7.1: Please add more details here. What wavelengths are measured?

Specified and added to text

7.2, 8.1 and 8.2: Please describe how these steps are actually done.

Will vary depending on sequencing facility requirement. Generic amounts mentioned.

15. Please specify centrifugation parameters throughout.

Specified and added to text

16. Please revise the protocol (2.1.2, 2.1.3, 3.1.1, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Text moved to discussion where needed

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

Combined where possible

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

Single space used throughout

19. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There are a 10 page limit for the Protocol, and a 2.75 page limit for filmable content. 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.

Authors understand the 10 page limit. Protocol itself is 9 pages. However this protocol is three methods combined; manual sorting, aRNA amplification and cDNA library generation. Reduction of protocol length is not possible without impacting content and clarity.

20. 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.

Euthanasia step removed

21. 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.

Steps highlighted

22. Line 436: Should Figure 2B&C be Figure 3B&C instead?

Corrected to Figure 3B&C

23. As we are a methods journal, 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

Additional text added to Discussion

Table 2 modified to correct errors

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

In this manuscript, Paul and Huang report a detailed protocol of the method to prepare cDNAs for single-cell RNA sequencing, in which manual sorting of single neurons is combined with two rounds of in vitro amplification protocol, called Double IN-Vitro transcription with Absolute counts Sequencing (DIVA-Seq). This is a fairly useful approach to dissecting gene expression profiles of single neurons especially when a large number of input cells with the significant fraction of fluorescently labeled neurons are not available and thus FACS is unusable. As standardized protocols for this method have been missing, publication of this paper should be of great interest to scientists in many fields. However, the authors need to modify/correct several parts in the text and figures for clarification and improvement before publication.

Major Concerns:

-The authors claim that manual sorting is faster and gentler than FACS. This should be more specifically discussed based on real numbers and references. How long does it take for FACS on average?

We thank the reviewer for raising this important issue. The actual times needed for FACS can be vary considerably depending on the sorting demands. Typically even a routine 2 color sort session requires additional steps beyond extraction of the brain, slicing the cortex, neurotransmitter blocking, mild protease digestion and trituration. These steps are now mentioned in the text lines 42-70 and later in lines 518-532. Overall by eliminating these steps in manual sorting, cells can be captured in a relatively shorter duration. However a major drawback of manual sorting is it requires some skill and practice to gather single cells in microcapillary pipettes. Also the number of cells captured is far lower than that possible using FACS. We have also mentioned this specific limitation of manual sorting in our discussion lines 1078-1080.

What kind of and what percentage of genes are misregulated by FACS?

This is an important issue and we thank the reviewer for this question. We added the following paragraph in Discussion in lines 1059-1066. “We did not do any comparative study with the same input sample from litter mates subjected to FACS and manual sorting using DIVA-Seq. Hence we do not claim that any particular gene category will be misregulated in FACS and not in manual sorting. Possibly both FACS and manual sorting will likely introduce some gene expression artifacts. For differential gene expression situations, any such effect in theory should cancel each other as it will be manifested in both control and sample groups. Recently a cocktail of transcription inhibitors have been used to prevent the activation of immediate early gene expression, and such steps can also be incorporated to this protocol [Hrvatin et. al. 2018].”

-The authors provide specific numbers of unique reads and genes per cell (average unique reads: 1.4x10^5; genes: on average 10000) obtained by the method combining manual sorting and DIVA-seq. Providing corresponding numbers in other experimental systems (e.g. FACS+other cDNA preparation method) should be helpful for readers to understand the superiority of authors' method.

We thank the reviewer and have added a sentence under Results, line 986-989. “This number compares favorably against published mouse brain derived single neuron data such as 1,865 – 4,760 genes in Zeisel et. al. 2015, 7,250 genes in Tasic et. al. 2016 and 8,000 genes in Okaty et. al. 2015. Readers are directed to Poulin et. al. 2016 review for details.”

-Manual sorting enables researchers to analyze sparsely labeled neuronal populations. What is the minimal number of labeled cells in brain tissues that is required to find labeled cells in a dish during single cell pick-up? Given that loss of labeled cells occurs during cell dissociation, this should be useful information for readers.

We thank reviewer for seeking clarification. We have been routinely successful in manually picking cells across slices containing only ~10-50 labeled cells total. This is now mentioned in Step 1.13

-Trituration is a crucial step for cell dissociation and not trivial. How many times should tissues be triturated using a pipette (line 91)?

We thank reviewer for seeking clarification. This is now clearly mentioned in step 1.15

-Single cell collection is one of major parts in this method. Thus, a detailed description for a glass pipette preparation is necessary. What are parameters for capillary puller (line 69)? What kind of tubing is connected to a patch pipette (line 96)? How do authors suck single cells? Any tips?

We thank reviewer for seeking clarification. Details are now mentioned in Steps 1.2, 1.3, 1.18. Pipette puller settings are not absolute and will likely vary from one machine to another, depending on the type of filament used and humidity, etc.

-The authors describe little about principles of single cell RNA sequencing. Due to lack of explanations, it is very difficult to follow what each step does and what final products the protocol aims to get. Concise explanations for key steps should be added. In relation to this, a legend in Fig.2 should be elaborated more. The role of each segment in primers should be explained (e.g. RAS, UMI, SBC, and RA3).

We thank the reviewer for asking us to elaborate on the principle of this biochemical process. Principle of single cell RNA sequencing using IVT is explained in original research articles cited Eberwine et. al. 1992 and Hashimshony et. al. 2012 referenced in lines 79-80 in the Introduction. To keep main text length within limits we did not elaborate any further. We have however modified the headings in steps 2.1, 2.2, 2.3, 2.4, 4.1, 3.2, 3.4, 3.5 to indicate the purpose of the steps without adding to the length of the manuscript considerably.

Figure 2 legend now explains the RA5, RA3, UMI and SBC primers and their usage.

Minor Concerns:

The names of kits must be described in the text (cDNA purification, cDNA syhthesis, aRNA production, and aRNA purification; from 2.1 to 3.5 in PROTOCOL).

Names not mentioned in the kit due to publication house policy of not mentioning commercial language; however, they are mentioned in Table of Materials with catalogue numbers and other details.

line 25-26; The sentence makes no sense.

Sentence reworded and we apologize for the mistake.

line 71; How long should ACSF be oxygenated?

Now specified in step 1.3

line 72-74; What is the volume that is necessary for experiments?

Volumes specified

line 75-76; No need of perfusion with ACSF?

Yes, and we did not detect any transcripts from blood cells as contaminants.

line 80; What kind of container is useful to keep sections?

Mentioned in step 1.9

line 85-86/89-90/93-94/96-97; What is the temperature?

Temperature specified

line 93-94; In what cases dishes should be coated? What percentage of agar or Sylgard should be used?

Agarose percentage specified on step 1.16, sylgard 1:10 added

line 101; What is the volume of sample collection buffer?

Specified

line 109; There should be "on ice" after the first heating.

Mistake corrected

line 110; It is hard to understand what this line means.

Line removed

line 118; It should be "1 ul/tube".

mistake corrected

line 181/277; It should be "50-55 ˚C".

mistake corrected

line 256; It should be "2nd round".

Corrected

line 366/368; Which one is correct, -20 or -80?

mistake corrected

line 377; It should be "SuperscriptIII".

mistake corrected

line 424; It should be "Figure 3".

mistake corrected

line 436; It should be "Figure 3B&C"

mistake corrected

.

line 443; It says R^2=0.96 but Figure 4C says 0.94. Which one is correct?

Mistake corrected to R^2=0.94

Figure 1; Microdissceted brain appears to contain a lot of GFP cells. Is this a representative one? A picture containing sparser GFP cells should be used.

Picture used was from a Pv-ires-Cre; H2B animal. There are significant variations between different Cre driver mouse lines.

Figure 1: Pics for phase/phase+GFP do not have a good resolution. It is also good to show high manification images in insets.

These images were acquired using a webcam attached to a microscope so inherently the resolution is not good. The manuscript uploaded had JPG high compressed quality. The final version TIF images are better resolution.

Figure 1 (pic showing manual sorting); A GFP cell of interest should be indicated. A glass pipette should also be indicated.

GFP cell and glass pipette indicated

Figure 1 (cartoon showing "aRNA library"); It should be UUUU....

Mistake corrected

Figure 2 (right column); Both ends of DNA strands are indicated as 5'.

Mistake corrected

Figure 4C; In the text, it says R^2=0.96. Which one is correct?

Mistake corrected in text to R^2=0.94

**Reviewer #2:**

Manuscript Summary:

In the manuscript titled "A single-cell RNA sequencing method using manual sorting and DIVA-Seq for fluorescently labeled neurons in mouse", the authors describe a procedure of manual isolation of neurons from mouse brain followed by in vitro mRNA amplification and library construction for RNA sequencing at the single cell level. Current single-cell RNA-seq methodology is generally more suitable for analysis on mixed or FACS-sorted cell populations which is not optimal for rare cell types in the whole cell population. Using an engineered fluorescence labeled mice, the authors demonstrate a powerful method to sort out interneurons from freshly diassociated mouse brain slices. Due to the bypass of the FACS, the samples can be maintained in oxygen enriched optimal solutions during the isolation procedure, therefore decreasing the stress for the cells. With several minor revisions, I believe, publication of this method will greatly benefit future scRNA-seq analysis for other neurons or rare cell types.

Minor Concerns:

1) Line 68, this section describes the method to sort mouse neurons. Overall it is very clear. But could the authors provide some numbers regarding how many slices were used and how many cells were obtained, so that the readers may have a better picture of the application of this method.

Authors thank this review for seeking clarification. We have now indicated approximate neurons needed in steps 1.9, 1.13

2) Line 110, not sure what was meant by these questions here.

Mistake corrected, line removed

3) In some places, the information of kits or reagents are not provided

As per publisher’s guidelines, we have referred to the reagents by their generic names and not used trademarked or copyrighted commercial names. However, a full table of materials used and corresponding catalogue numbers have been updated.