Point-by-Point Responses to Reviewer Comments

We sincerely thank the editor and reviewers for their helpful comments and suggestions to our previously submitted manuscript. Here, we respond to the editorial and reviewer comments with revisions. As displayed below, editorial and reviewer comments are presented in black followed by detailed replies to each comment in blue. We also tracked the major changes in the manuscript. For the paragraphs that we have changed significantly, we have labeled them with a pair of red round brackets. For the sentences that we have improved, we have underlined and italicized them.

We believe our revisions to the manuscript have significantly improved the paper. We hope these improvements will be favorably considered for publication in JoVE.

Editorial Comments

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: We thank the editor for the suggestion. We have went through the manuscript and ensured that there are no spelling or grammatical errors.

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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. Examples:
1) 1.1., 1.2: Please mention all details of the surgical steps including how sterility is maintained, tools used, incision etc.
2) 1.3: How is the dissection performed?

Response: We apologies for not including enough details in some protocol steps. We have went through the whole protocol and included as many details as we can in each step. For example, we have included more details on the mouse dissection at step 1 (line 100-101, 108-109, 113-114) and sequencing data analysis at step 5.11 and 6.1.7 (line 365, 391).

Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations.

Response: We thank the editor for the suggestion. We have adjusted the numbering and format accordingly.

Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your

protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Response: We thank the editor for the suggestions. We have highlighted about 2.5 pages for filmable content and made sure the highlighting include all relevant details and form a cohesive narrative.

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We thank the editor for the critical comment. We have revised our discussion part to focus on the listed points (Line 547-570).

Figures
1) Fig 2: Axis ticks are too small to read.

Response: Thanks. We have updated it.

Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are 10x Genomics Inc., Drop-seq, InDrop, MULTI-seq and Cell Hashing, falcon, Flowmi Cell Strainers, SD100 Cell Counting Chamber, Template Switch Oligo, NexcelomCellometer Auto 2000, TruStain FcX™ PLUS (FcX, BioLegend), Chromium Single cell 3' v3, Chromium Chip B, Dynabeads MyOne SILANE, Dynabeads, Agilent, Agilent TapeStation, SPRIselect, TruSeq RPIX , KAPA HiFi HotStart, CellRanger, Seurat V2 or V3, Co-Anchoretc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
3) Please check Table 1 as well.

Response: We thank the editor for the comments and suggestions. We have replaced all the commercial sounding names with generic names and removed the registered trademark symbols. However, for the term multi-seq, cell hashing, seurat v3, and co-anchor, we don't think they are commercial names. Although we have updated these names as generic names, we think it will be better to keep these names as they were to avoid confusing the readers. Please let us know if the editor agrees about that. If so, we can change those names back to their original name. If not, we can use these names as they currently are.

Table of Materials: Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as anitbodies with concentrations and RRIDs,

Response: We thank the editor for the comment. We have updated the table with detailed name/company/catalog number information and included a concentration/RRID information for the antibodies. In addition, the table has been saved as "table of materials".

If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: We thank the editor for the important comments. Considering part of the figure 1A and figure 4 (Figure 4A, 4B, 4C, 4D) in our previous submission were derived from one of our published paper, we have updated all of them with unpublished figures. We have also updated the texts by removing the citations.

Comments from Peer-Reviewers

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
Here, Feng et al present a protocol for performing 10x scRNA-seq on embryonic heart. The protocol is well described, the heart dissociation protocol can be useful for scientist working with this model. Rest of the protocol is relevant to any scientists using 10x genomics. Although as it is, the protocol does not bring anything new compared to what is already available from 10X and a few references (correctly cited), but it is not a JOVE requirement. It would be helpful for the scientific communities if the Multi-seq anchor / barcode synthesis / experimental design and limitation (cell type dependence) could be discussed more.

Response: We thank the reviewer for noting our protocol is well described. We also greatly appreciate the reviewer for pointing out the weaknesses of the manuscript.

Major Concerns:
- Line 114 (and other places): please report precisely the Flowmi strainers used (size + ref)

Response: We thank the reviewer for the comment. The cell strainer we used is 40µm. We have updated it in the manuscript and table of materials (line 127).

- MULTI-seq procedure: line 126
Here the authors only refers to the already published paper. If I am correct, the synthesis of the anchor, co-anchor is not trivial and not accessible to everyone. Maybe the authors could comment more on how they performed the synthesis?

Response: We thank the reviewer for the comment. We have added a paragraph to comment on the synthesis of anchor and co-anchor (Line 146-148).

- Line 133 (and 154). The authors do not comment on the number of cells that can be used at this step.

Response: We thank the reviewer for the important comment. We have updated the cell number information for both Multi-seq and Cell hashing procedures (line 141,167).

- Line 142. 1% BSA has been reported to inhibit 10X reaction. So cells have to be diluted. Can the authors comment on the maximum final %BSA the cells are just before loading on the 10x chip. This might be relevant to scientists working on the same cell / tissue model as presented here.

Response: We thank the reviewer for the comment. Since the cells will need to dilute with nuclease-free water to a specific cell concentration before loading into the Chromium, the BSA concentration after dilution will range from 0.1%-0.3%, which will depend on the targeted recovery cell number.

In addition, according to the information from 10X Genomics website (https://kb.10xgenomics.com/hc/en-us/articles/218170703-What-is-the-highest-BSA-concentration-that-can-be-used-in-the-cell-buffer-), the BSA concentrations ranging from 0.1-2% have no adverse effects on the workflow or sequencing data.

- line 277-278: mentioned step 6.1. But is this not 5.1?

Response: We thank the reviewer for the comment and apologies for the error. We have corrected it in our updated protocol (line 329).

Minor Concerns:
- writing ScRNA-seq is unusual (e.g. line 61). scRNA-seq seems more appropriate
Response: Thanks. We have updated all ScRNA-seq as scRNA-Seq.

Reviewer #2:

In this manuscript Li and collaborators describe how to perform single cell RNA-seq library preparation from individual heart cells from E18.5 embryos using the 10X genomics platform. They also describe 2 options for multiplexing multiple samples in the same reaction, thus reducing the batch effect and the cost of each experiment.

Response: We thank the reviewer for summarizing our study.

Major Concerns:
The manuscript needs a moderate revision before being ready for publication. While the protocol is well described, the authors should better explain the general workflow of the protocol at the very beginning (maybe with a clearer figure) and explain the technical differences between the 2 options they are describing. They should state at the beginning advantages and disadvantages of each of them and help the reader in the decision.

Response: We thank the reviewer for the great suggestions. We have updated figure 1 by including more details to show the workflow differences between Cell hashing and Multi-seq. In addition, we have included a few sentences in the introduction to explain the advantages/disadvantages of the two methods (Line 77-83).

They should also indicate clearly how many samples and how many cells they can multiplex in the same experiment. As the ultimate goal of multiplexing is the cost reduction (on top of the batch effect removal), a sentence about the magnitude of the cost reduction is also needed.

Response: We thank the reviewer for the important suggestions. We have included a few sentences in the introduction to talk about the number of samples/cells that can multiplex in the same experiment and a sentence for the magnitude of the cost reduction (line 82-89).

The whole sentence about FACS in the discussion is misleading/wrong. FACS and micromanipulation are not used to isolate single cell, but to isolate "specific" single cells with a specific surface molecule pattern or in a specific position. FACS especially is it still is the only option available for specific purpose. In the protocol presented there is no possibility to isolate specific subpopulations of cells, but only the way to obtain a single cell suspension from multiple samples.

Response: We thank the reviewer for the comment and apologies for the misleading. We have removed all the sentences about FACS in the discussion.

As different people will use different tissues or different embryonic stages, a general sentence about the total number of cells required as a starting point is needed.

Response: We thank the reviewer for the suggestion. We have added a sentence to talk about the total number of cells required as a starting point (line 113-114).

Minor Concerns:

The manuscript would benefit from a revision from a native English speaker. Some sentences are truncated (line 63) and some words are wrong (unbiased/unbiasedly)

Response: We thank the reviewer for the suggestion and apologies for the truncating sentences and grammar errors. The revised manuscript has been carefully read through by a native English speaker.

The word “recently” in the abstract must be explained, as everything is very recent in this field.

Response: Thanks. We have replaced the “recently” with “in the last two to three years”.

The sentence “significant cell variation” must be explained (line 44), what does significant mean?

Response: We thank the reviewer for the comment and apologies for the confusion. We have updated the sentence as “The transcriptional profile of each single cell varies among cell populations during embryonic development” (line 43).

The words DNA or cDNA must be used appropriately.

Response: Thanks. We have went through the manuscript to make sure the two words were used appropriately.

Some abbreviations need to be spelled out (EP tubes?).

Response: We thank the reviewer for the suggestion. We have updated the protocol by replacing some abbreviations including EP tubes with their full names.

A sentence describing the need of both a pre- and a post-amplification areas in the lab is needed.

Response: Thanks. We have added two sentences to emphasize this point in the protocol (line 135-137).

I think that if the authors would add a sentence about how long each main step takes and if there is any safe stopping point, the reader will have a better grip on the protocol.

Response: We thank the reviewer for the important suggestion. We have added an estimated time for each main step and marked that step if it is safe to stop.