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Ronald Myers, PhD.
Senior Science Editor
JoVE
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Dear Dr. Myers,

We would like to thank you and the reviewers for their favorable revision of our work and for the constructive feedback. We feel this has helped improve the manuscript substantially. Additionally, we are thankful for the opportunity to share this study with the scientific community.

We have made all of the changes suggested by the editor. We have also incorporated all the suggestions in the manuscript and we address the reviewers' comments point-by-point as follows:

Reviewer #1:

1) In the introduction, the authors make the statement "while gene expression analysis of mixed cell populations has been improved with single-cell RNA-seq technologies, the fragility and variability in size of repopulating hepatocytes leads to dropout of lowly-abundant transcripts and inefficient capture of the target cells." Certainly, the amplification used in sc-RNA seq can result in the dropout of abundant transcripts, but I do not feel that there is inefficient capture of target cells. Multiple papers have been published on single cell sequencing of hepatocytes with capture of their target population of cells. Nature. (2017) 542: 352-356. Nature (2017) 546: 533-538. Even very rare populations of cells have been resolved with sc-RNA seq such as the pulmonary ionocyte. It would be helpful for the authors to explain more how this method will fit into this landscape and what advantages/disadvantages it may have.

We thank reviewer 1 for pointing out the discrepancies between the text and others' studies and made several changes to the manuscript. In the revised manuscript, we mention our difficulties in capturing live cells in the *Fah*$^{-/-}$ injury and repopulation model. We found that in this model, capturing live cells after liver perfusion and hepatocyte dissociation was highly inefficient due to cell fragility. We discuss scRNA-seq and explore its potential in future studies of liver repopulation. Should novel methods to efficiently isolate hepatocytes be established, scRNA-seq would certainly aid in understanding the role of all cell types during liver repopulation and potentially identify novel transitional cell types during the regenerative process. All discussions regarding the limitation and potential of scRNA-seq can be found in the second-to-last paragraph of the discussion.

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2) Figure 3b: This is not really a control experiment. Comparing high RIN and Low RIN sequencing data seems unnecessary as it is commonly known that low RIN RNA is degraded and should not be sequenced.

We thank reviewer 1 for this comment and have modified the text and figure legend to emphasize the necessity to assess RNA degradation, especially for downstream RNA-seq analysis. While using a Bioanalyzer to assess RIN is a recommended step in our protocol, we feel that a demonstration of how a low-quality RNA sample could lead to poor sequencing results would be useful for the readers. We have also added a representative results section that low RIN should not be sequenced at all since it indicates RNA degradation.

3) No technique is perfect, the authors should comment briefly about the limitations of TRAP so that others can decide which approach to take.

We thank reviewer 1 for the constructive suggestion and have included the limitations of TRAP in the third-to-last paragraph in the discussion. Mainly, (1) TRAP-seq is a measurement of the 'translatome' and not 'transcriptome', (2) TRAP-seq is not an assessment of ribosome footprinting, (3) TRAP isolation still requires a certain number of cells (at least 1:100 in our experience) to express GFP:RPL10A, and (4) genetic elements are required to ectopically express the GFP:RPL10A transgene in the cell-type-of-interest and could therefore limit its use in cells types that do not yet have an efficient expression or delivery system.

Reviewer #2:

1) Can the authors provide some criteria for evaluating RNA quality? How are the assignments of "high-quality" and "low-quality" RNA made in figure 3B?

We thank reviewer 2 for the constructive feedback and have expanded the description regarding the evaluation of RNA quality for RNA-seq purposes in the final paragraph of the representative results section. We suggest analysis of RNA quality with a Bioanalyzer assay of RNA quality. If there is evidence of RNA degradation, the samples should be excluded. However, as different RNA-seq library preparation kits use different chemistries, it is difficult to make a clear cut-off for RIN. For instance, poly(A) selection-based kits generally require RIN values above 7, while ribosomal RNA removal kits sometimes allow RIN to be as low as 5. We have also edited the figure legend and removed the usage of 'high-quality' and 'low-quality', and instead suggest referring to the kit to decide whether or not to include an RNA sample.

2) What is the minimum amount of tissue or cell number needed for TRAP-seq to work?

We have included an estimation of the lowest amount of cells for a successful TRAP-seq experiment in the manuscript; this can be found in the 4th paragraph of the discussion. We have used 200 mg of the repopulating liver in which only 1-2% of hepatocytes were expressing the GFP:RPL10A transgene. We calculate that ~150 ng of RNA can be purified from roughly 2x105 hepatocytes using TRAP. In the original TRAP paper, the authors were able to isolate as

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little as 50 ng of RNA from 30 mg of mouse striatum that includes ~7x105 neurons expressing the transgene.

Regards,

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