Rebuttal for JoVE62680 "Polysome Profiling Without Gradient Makers or Fractionation Systems."

Our replies to the editor's comments are shown in (blue).

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. Please define all abbreviations at first use.

We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues and have defined all abbreviations.

2. Increase the word count of the abstract to be 150-300 words.

The word count of the abstract has been increased to 163.

3. Please provide an email address for each author.

Email addresses haven now been provided for both authors.

4. Please revise the following lines to avoid overlap with previously published work: 31, 100-101, 186-188, 256-257, 259-260, 261-262, 263-264

These lines have been revised/rewritten to avoid overlap.

5. Please add only one space after punctuation.

There is now only one space after punctuation.

6. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the Representative Results in the manuscript text.

The embedded Table has been removed. A new table has been generated using Excel and a Table Legend has been added to the revised manuscript.

7. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All instances of “could be,” “should be,” and “would be” have been removed unless mentioned in a “Note”. The protocol has been edited to conform to the imperative tense.

9. 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 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The details of each step in the described in this manuscript has been checked to ensure viewers can replicate the protocol.

10. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

One line space is between each protocol step.

11. In figures, e.g., Fig 3, please ensure there is a space between the number and units, e.g., 200 mL not 200mL.

The figures have been edited to ensure that there is a space between numbers and units.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ital.). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.

References have been corrected. All references were generated using EndNote and the 2020 JoVE style.

13. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table is now in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Polysome profiling by sucrose density gradients is a key method to analyze cellular ribosomes and their association with mRNAs and translation factors. Most commonly, to achieve high

quality and reproducibility, polysome profiling requires expensive automated gradient makers and fractionation systems. The manuscript of Sobhany and Stanley describes a simplified protocol aimed to obtain polysome profiles using sucrose density gradients without the need of the costly instrumentation. The approach is rather simple: the gradient is formed by layering five different stocks of sucrose solutions and, following ultracentrifugation, the gradients are manually fractionated by pipetting small aliquots from top to bottom to then analyze the obtained fractions. I agree with the authors that the approach could be easily implemented in any lab. In addition, the protocol is well-written and, as far as I can tell, it would be easily followed by any lab member with basic lab skills. However, I find critical issues that may discourage the readers to use the described approach.

Major Concerns:
1. Fractionation seems to be very time consuming. I'd estimate that withdrawing 200 µL aliquots of a ~10 mL gradient would take approximately 12-14 min (~15 sec to pipette the aliquot from the tube to the 96-well plate). This time would be for a single gradient and would be doubled (30 min!) if to take 100 µL aliquots. Readers need to be made aware of this point, moreover taking into account that labor is very expensive.

The reviewer is correct that manual fractionation can be time consuming, however with our experience manual fractionation takes roughly the same amount of time (or can actually be faster) than using a commercial fractionation system. We have considerable experience using the Brandel BR-188 Density Gradient Fractionation System. It takes five minutes and thirty seconds for the chase solution to entirely push a ~10mL gradient through the detector flow cell and into the fraction collector. After reversing the syringe pump, it takes another five minutes to remove the chase solution from the polypropylene tube and reset for the next tube. When you factor in placement and removal of the polypropylene tube into the tube piercing system, and replacing centrifuge tubes in the fraction collector, it takes roughly 15 minutes per gradient to generate a profile. For an experienced user it takes less than ten minutes to manually load 100 µL aliquots into 96-well plates.

2. Resolution is very low, moreover that of the polysome fractions. Besides a peak corresponding to disomes, no distinct peaks for groups of more than two ribosomes can be distinguished. The low resolution issue will make it really difficult to reach any solid conclusions about mRNAs or proteins associated with a particular group of translating ribosomes.

The reviewer is correct that the resolution of the peak is lower for manual versus automated systems, however the relative peak height is distinguishable between samples and can indicate differences in ribosomal profiles. This protocol is not meant to be a replacement for automated fractionation but rather an option for those who do not have access to automated instruments.

3. Reproducibility of the results seems low. It is worrisome that the representative profiles shown in the manuscript reveal inconsistencies. For example, even though more aliquots are collected in the profile shown in Figure 3C, the 60S peak is not defined. Also, the number of data points in the 80S peak is as few as three in panel B of the same figure and something like 10-12 in panel C. These inconsistencies reflect irreproducibility of the data.

While there is a significance difference in resolution of the chromatogram with manual versus automated fractionation in our experience, we have not had issues with data reproducibility with manual fractionation. There are seven data points for the 80S peak in panel B and fourteen in panel C. This is consistent with the aliquot volumes. Even in commercial fractionation systems, the 60S peak can be ill-defined.

An additional point is that authors could mention that a well-known alternative technique to fractionate sucrose gradients without the use of an expensive automated instrument is to simply carefully insert a needle/capillary tube to the bottom of the gradient tube, attach it to a peristaltic pump, properly adjust the flow and collect aliquots with either a fraction collector or manually. This approach is less labor intense and yields reproducible data with very acceptable resolution.

We thank the reviewer for this suggestion and have added this as a Note in the protocol.

Reviewer #2:

Manuscript Summary:

This manuscript introduces a new and easy method to profile polysome without commercial gradient makers. The method is simple and detailed in the manuscript. Therefore, for labs with limited resources, this manuscript will be helpful.

Major Concerns:

None.

We thank the reviewer for their support of our manuscript and valuable suggestions for refining the manuscript which are detailed below.

Minor Concerns:

(1) in the manuscript it states that overnight incubation is used to create linear gradient of sucrose. However, the authors didn't detail why overnight is sufficient, or how to judge whether a satisfied gradient has been built.

The reviewer is correct with this assessment. Additional details about length of incubation have been included. Also, we now suggest the use of a densitometer to assess the quality of a gradient.

(2) Just to confirm, Fig 3A is the results from a commercial fractionator, not the gradient maker.

The reviewer is correct. However, we realize that the Representative Results section of our manuscript lacked clarity on this issue, and therefore added several lines to eliminate any potential confusion.

(3) How the profiling results are compared with that from a commercial gradient maker? It will be better if the authors can provide or refer to some profiling results obtained from commercial gradient makers.

The reviewer is correct; comparison with a commercial gradient maker would make the manuscript better. We now refer to a paper that utilizes the Biocomp Gradient Maker.

Reviewer #3:

Manuscript Summary:

The manuscript by Sobhany and Stanley describe a method to generate polysome gradients without the need of expensive devices using only commonly available lab equipment. Although not novel, the beauty of this manuscript is the direct comparison between these 2 techniques (shown in figure 3) showing that for most of the common purposes (differentiate between monosomes and polysomes) the resolution obtained with this technique is enough. This also can be used as a way to perform preliminary experiments before deciding on the purchase of an expensive machine or the collaboration with other groups.

This report could also motivate the use of polysome profiles in laboratories that are not necessarily experts in the field and therefore expand the range of techniques and results produced by those labs.

I still have some criticisms/suggestions.

Major Concerns:

Please indicate in each possible point when the sample can be flash frozen and stored (during cell harvesting and sample preparation). This is a crucial piece of information in an experimental manuscript.

The reviewer is correct; this is crucial information and the text has been revised to include when samples and gradients can be flash frozen and stored.

Representative results: It is not clear if the profiles shown in figure 3 were generated using a gradient maker or the hand pouring/overnight incubation described in the manuscript. If not only the gradients harvesting but also their generation are different the authors should show the effect of each of these 2 steps play in the final gradient quality (for example by generating the gradients manually but harvest them with a automated fractionation system.

The reviewer is correct; we had not made this point clear. The manuscript has been revised to clearly state that all profiles in Figure 3 were created using gradients that had been prepared as described in the protocol.

Minor Concerns:

Line 36: a single mRNA molecule

This has been changed.

Labe 145: RNA, lysates or both could be frozen down?

This has been revised to “lysates”.

Lane 152-154: Not RNA but lysates are pipetted on top of the gradient

This has been corrected.

Lane 160: using which buffer?

This step of the protocol has been revised accordingly.

182: the word reach is missing: until you reach the bottom of the tube

"Reach" has been added.

Point 2.6: In our experience cooling the sample between shacking is better (by doing for example 5 rounds of 1min with 1min incubation on ice in between).

Thank you for this suggestion. We have not tested this variation of the protocol so we did not note this in the manuscript, but this is something to keep in mind for the future.

Reviewer #4:
Here the authors describe a layman's method of polysome fractionation. This reviewer appreciates the clarity of their presentation, along with an impressive figure showing the non-linear sucrose gradient using blue dyes. There is no concern except that this reviewer recommends to cite the latest work of high quality related to polysome profiling below.
Recommendation
This reviewer wants to highlight the recent work of polysome profiling (Chikashige et al., 2020) that is worth being cited here in the lines 38, 69 and 264.

Reference to be added
Chikashige, Y., Kato, H., Thornton, M., Pepper, W., Hilgers, M., Cecil, A., Asano, I., Yamada, H., Mori, C., Brunkow, C., et al. (2020). Gcn2 eIF2α kinase mediates combinatorial translational regulation through nucleotide motifs and uORFs in target mRNAs. Nucleic Acids Research 48, 8977-8992.

We thank the reviewer for their support of our manuscript. The recommended reference is now specifically described within the body of text in the Representative Results section and cited in the introduction and discussion as suggested.