**Answer to Editorial comment**

We thank you for consideration for the publication of our manuscript, “Orbital shaking culture of mammalian cells in O-shaped vessels for production of uniform aggregates”, in Journal of Visualized Experiments. According to your comments, we have revised the manuscript and indicated revised part in red color. We hope to confirm our revision and reconsider for the publication of our 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.*

**Response:** Thank you for remind. The manuscript has been already proofread by English-native editing service.

*2. Please use SI units, e.g. please use "µL" instead of "µl". Please leave a white space between the values and the units.*

**Response:** Thank you for reminding. We used SI units in the manuscript.

*3. Please define all abbreviations before use.*

**Response:** Thank you for reminding. We defined all abbreviations and removed the unnecessary abbreviation (H&E staining).

*4. 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.*

**Response:** The manuscript does not have commercial language.

*5. 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. 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.*

**Response:** According to the comment, we improved the protocol 5.1 and 5.4 in our manuscript.

*6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.*

**Response:** According to the comments, we confirmed that the steps contain lower than three actions in the manuscript.

*7. 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.*

**Response:** According to the comment, we added the information about cell counting on the protocol 2.3.

*8. Please leave a blank line between all protocol steps as well as Notes.*

**Response:** According to the comment, we confirmed that there was a blank line between all protocol steps.

*9. Protocol: 1.1: How much is the pH of the solution?*

**Response:** We clarified the pH of solution (6.8-7.6) in protocol 1.1.

*10. Protocol: 2.3: How do you count the cells? Please describe.*

**Response:** We counted cells by trypan blue staining and automatic cell counter. We added the information of automated cell counter in the material list.

*11. Protocol: 3.2, 4.3, 4.5: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*

**Response:** We converted centrifuge speeds to centrifugal force (x g) in the protocol 3.2, 4.3, and 4.5.

*12. Protocol: 4.2, 4.6: How much is the pH of the PBS?*

**Response:** We updated the information of the pH of the PBS on the protocol 4.2.

*13. Protocol: 5.1, 5.4: Please use the imperative tense for all steps in the protocol.*

**Response:** We improved the protocol 5.1 and 5.4.

*14. Figures: Please upload each Figure individually to your Editorial Manager account as a .png, .pdf, or a .tiff file. Please combine all panels of one figure into a single image file.*

**Response:** We have uploaded the Figure individually for submission.

*15. Figure 3: Please define the error bars.*

**Response:** According to the comment, we defined the error bars in the caption of Figure 4.

*16. If you are reusing 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 [AUTHOR] et al.[REFERENCE]".*

**Response:** All figure is the original and has not been published anywhere.

*17. 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 in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.*

**Answer to Reviewer #1**  
*Manuscript Summary:  
The manuscript presents a new culture system termed "o-shaped bag" for the cultivation of cell-aggregates in suspension. The main idea behind the platform is to prevent local accumulation of cells to the center-bottom of a vessel while cultured on an orbital shaker. In consequence, the technology aims at generating better controlled and more homogeneous cell aggregates e.g. compared to conventional culture dishes or Erlenmeyer flasks placed on an equivalent orbital shaker device.  
In principle, the manuscript is interesting and the technology deems straightforward. However, a number of key issues and questions remain.*

We thank you for careful reviewing and scientifically important comments. All your comments are so helpful to improve our manuscript. According to your comments, we revised our manuscripts and indicated the corrections in red color. We also answered your each comment below. We hope that the revised manuscript and answers make you satisfied.

*Major Concerns:  
1) Without any pictures, schematic and movies, which have not been provided by authors, it's difficult to understand and validate the features of the technology; these must be provided before a proper review can be performed. An additional schematic outlining the details of the experimental procedure e.g. culture inoculation, propagation etc. would be very helpful as well.*

**Response:** All figure is the original and has not been published anywhere.

*2) Technologies' advantages and limitations must be better compared and discussed to other approaches in the field e.g. the use of stirred tank bioreactors (STBRs) or wave motioned bags for cell aggregation. Authors mussed not focus on review article but also include some primary papers on this topic e.g. the following might be helpful: Zweigerdt R, Burg M, et al. Cytotherapy. 2003;5(5):399-413.; Niebruegge S, Nehring A, et al. Tissue Eng Part A. 2008 Oct;14(10):1591-601.; Singh H, Mok P, Stem Cell Res. 2010 May;4(3):165-79; Olmer R, Lange A, et al. Tissue Eng Part C Methods. 2012 Oct;18(10):772-84.;*

**Response:** We thank you for various helpful references and we added some of them into the reference (reference 7 and 14). According to the comment, we have added the discussion of the technical advantage and limitations of stirred suspension culture, orbital shaking culture and O-shape vessels (Page 5, line 13-28.)

*3) The results part misses important information, which makes it difficult to evaluate the value of the presented data.*

**Response:** We are sorry for poor explanation of the experiment and results. We answered the question below.

*Protocol:  
- For centrifugation the unit rpm is used but x g should be used instead to make it more comparable*

We used the x g as a unit instead of rpm for the condition of the centrifugation.

*- How was cell counting performed (trypan blue?); this must be mentioned especially if a special counting devices was used*

We used trypan blue staining and automated cell counter.

*- What is the total- and the min/max culture volume of the o-shaped bag?*

We fixed the culture volume at 20 mL.

*Results:  
- Details on the dimension of different culture systems used in the paper are missing; it's thus essentially impossible to evaluate the conclusions made. Different culture volumes and different dish sizes incubated at same shaking speeds will lead to different shear forces and therefore to different aggregate sizes; this has not been comprehensively considered in the manuscript.*

We agree that the difference of culture volume and vessel diameter affects the medium flow and the shear forces even at same shaking speeds. In the experiment, we used same culture volume at 20 mL and designed culture vessels with as a similar diameter to 90 mm culture dish as possible in order to minimize the difference of shear force (Figure 1).

*Minor Concerns:  
- P5 L14/15: Authors stated: "This result showed that enough oxygen was supplied to aggregates in culture bag through the gas permeable film." This statement is inadequate on the basis of the data presented in the manuscript. Using e.g. oxygen sensors (e.g. [www.presens.de/o2sensors](http://www.presens.de/o2sensors" \t "_blank)) for culturing hiPSC as suspension culture aggregates in conventional flasks or dishes, we found >80% of dissolved oxygen (DO) in the medium, even at high cell densities of up to 2 million cells / ml. This deems not surprising given the very high surface-to-liquid ratio in such systems on an orbital shaker. Actually, the data in the manuscript may suggest a more homogeneous size distribution of aggregates generated in o-shaped backs, which is likely the most important selling point of the system.  
But it's not convincingly shown in the paper draft, that cells/aggregates cultured on conventional dishes are less viable. However, depending on the culture conditions applied (e.g. cell density at inoculation, duration of culture etc.), aggregates may become >300µm in diameter which may result lack into the lack of other nutrients in the core of aggregates and thus limit cells viability.  
These issues must be more comprehensively discussed.*

**Response:** We appreciate your valuable comment and we agree that discussion of oxygenation is not appropriate. According to the comment, we emphasized the effect of homogeneous size distribution of aggregates and reduced the description of oxygenation in the discussion (page 5, line 30-39).

*- P5 L20/22: Authors stated: "According to our preliminary experiments, these O-shaped vessels can be used for suspension culture of human induced pluripotent stem cells, Vero cells, and HepG2 cells (data not shown)." This statement alone is insufficient. Please show exemplary data on human iPSC cultivation in you system to indicate that these more challenging cells can really be successfully cultivated in your setup, remain pluripotent etc.*

**Response:** According to the comment, we removed the mention about the results of other cell lines (page 6, line 1-4)

*- It fig1 and fig3: It remains entirely unclear how often have been repeated? It must be demonstrated that independent biological repeat using cells from at different passages has been performed.*

**Response:** We repeated the measurement twice for the histogram and three-times for the cell growth. We clarified the number of experiments in the caption of fig 3 (current fig 4). Although we did not show the number of experiments in the caption of fig 1 (current fig 2), the results have good reproducibility.

*- Demonstrate homogeneity of your cell suspension used for culture inoculation. It seems possible that some inhomogeneity in aggregates may result from an inhomogeneous cell dissociation i.e. not all cells where fully dissociated into single cells ahead of culture inoculation.*

**Response:** We totally agree with your opinion that incomplete dissociation may results in inhomogeneity of aggregate size. Therefore, to remove cell clamp causing inhomogeneity, we did filter cell suspension through a 40 µm cell strainer (protocol 2.2).

*- Provide data on the absolute cell density rather than showing "fold increase". This is important to understand and reproduce the protocol.*

**Response:** We thank you for comment. According to your comment, we used absolute cell density instead of fold increase in figure 4b.

**Answer to Reviewer #2**

*Manuscript Summary:  
The authors describe a new O shaped vessel for culturing cell aggregates. Here are the technical and data short comings*

We thank you for reviewing and comments. According to your comments, we revised our manuscripts and indicated the corrections in red color. We also answered your each comment below. We hope that the revised manuscript and answers make you satisfied.

*Major Concerns:  
There is no description of how media can be simply exchanged in the O-shaped vessels, even though this is claimed.*

**Response:** In HEK293 culture, we did not changed culture medium. If we culture cells requiring frequent medium change such as pluripotent stem cells, when medium change, we collected all suspension from O-shaped bag and separate cells and medium by centrifugation. We agree with you that it is effective to change medium in O-shaped bag directly, but it is still developing for next publication.

*There is no measure of oxygen permeability improvement. Where are the measurements vs. controls? What instrument used? What is the control O2 levels?*

**Response:** We agree that effect of oxygenation was just speculation. According to your comment, we reduced the mention of oxygenation in the discussion (page 5, line 30-39).

*There is no picture of the bag. There is no video of the bag in operation. No video of aggregates growing in cultures vs. controls.*

**Response:** According to your comment, we added the schematic images and pictures of vessels in figure 1. The video of the bag operation will be captured after publication for preparing video journal.

*There is only 1 set of data with HEK cells. What about the ones for hiPSC, Vero and HepG2?*

**Response:** We chose HEK293 cells as a model cells forming aggregates. For other cell lines, we performed just only preliminary experiments. According to the comments, we removed the mention about the results of other cell lines from discussion (page 6, line 1-4).

*No growth rate and growth curves.*

**Response:** We agree that the growth profile is the one of important part in the manuscript. According to the comment, we calculated and added the specific growth rates in the manuscript. We think that the specific growth rate is enough for evaluating growth in the manuscript. In order to count cells, we have to dissociate cell aggregates as a periodic destruction sampling. In the experiment in the manuscript, the culture volume is not enough for such periodic sampling to draw growth curves.

*No cell characterisations*

**Response:** The most important point of this manuscript is producing uniform aggregates form novel O-shaped vessel. Although characterization is also important, we think further characterization does not support this point and will be point of next publication.

*This paper therefore needs more, major revisions, data inclusion and videos to be convincing to the readers. Definitely a picture of the O bag should be shown or a schematic.*

**Response:** We appreciate your comment and we hope the revision satisfies you. According to your comment, we added picture and schematic image of O-shaped vessels in figure 1.