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Corresponding Author:	Ikki Horiguchi The university of tokyo Tokyo, JAPAN
Corresponding Author's Institution:	The university of tokyo
Corresponding Author E-Mail:	horiguchi@bio.eng.osaka-u.ac.jp
First Author:	Ikki Horiguchi
Other Authors:	Ikumi Suzuki
	Takashi Morimura
	Yasuyuki Sakai
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TITLE:

An Orbital Shaking Culture of Mammalian Cells in O-shaped Vessels for the Production of Uniform Aggregates

AUTHORS & AFFILIATIONS:

Ikki Horiguchi[1,2], Ikumi Suzuki[3], Takashi Morimura[3], Yasuyuki Sakai[1,4]

[1]Department of Chemical System Engineering, The University of Tokyo, Tokyo, Japan
[2]Department of Biotechnology, Osaka University, Osaka, Japan
[3]Biotech Business Unit, Fukoku Co. Ltd., Saitama, Japan
[4]Institute of Industrial Science, The University of Tokyo, Tokyo, Japan

Email Addresses of Co-Authors:

Takashi Morimura (t_morimura@fukoku-rubber.co.jp)
Yasuyuki Sakai (sakaiyas@iis.u-tokyo.ac.jp)

Corresponding authors:

Ikki Horiguchi (horiguchi@bio.eng.osaka-u.ac.jp)
Ikumi Suzuki (i_suzuki@fukoku-rubber.co.jp)

KEYWORDS:

Suspension culture, orbital shaking, mass production, mammalian cell, culture bag, aggregate

SHORT ABSTRACT:

Here we present a protocol for using O-shaped vessels, specialized for suspension cultures of cellular aggregates, with orbital shaking. The HEK293 cells grown in this bag form more homogeneous aggregates than those grown in conventional culture vessels.

LONG ABSTRACT:

Suspension cultures of mammalian cell aggregates are required for various applications in medical and biotechnological fields. The disposable bag-based method is one of the simplest techniques for the mass production of cellular aggregates, but it does not protect the cultures against over-aggregation, which occurs when they gather at the bottom center of the culture vessel. To solve this problem, we developed an O-shaped dish and an O-shaped bag, neither of which contains a central region. Aggregates grown in either O-shaped culture vessel were noticeably more uniform in size than aggregates grown in conventional vessels. Histological analyses showed that aggregates in conventional culture dishes contained necrotic cores most likely caused by a poor oxygen supply. In contrast, aggregates that were grown in the O-shaped bag, even those with similar diameters to aggregates in conventional culture dishes, did not show necrotic cores. These results suggest that the O-shaped bag provides sufficient oxygen to the aggregates due to the oxygen permeability of the bag material. We, therefore, propose that this novel gas-permeable O-shaped culture bag is suitable for the mass production of uniform aggregates that are necessary in various biotechnological fields.

INTRODUCTION:

Suspension cell culture plays an important role in the cell production for regenerative medicine[1] and recombinant protein production[2] because it is easy to scale up and achieve high cell densities, sometimes up to more than 10^7 cells/mL[5]. Chinese hamster ovary (CHO) cells[3] and human embryonic kidney cells 293 (HEK293)[4] have been grown in suspension culture, and human pluripotent stem cells (hPSCs) have recently been grown in suspension culture systems for regenerative therapies[1,6,7]. The use of suspension cultures is expected to increase in the future.

In suspension culture, some cell lines cannot grow as single cells and must, therefore, form aggregates. For example, hPSCs cannot survive in suspension conditions without forming aggregates. However, this requirement for aggregated growth presents difficulties for uniform suspension cultures. One difficulty is the formation of uniform aggregates in the early period of the culture, which determines the efficiency of the suspension culture[8]. Another difficulty is the mass transfer of nutrients into aggregates. In particular, the oxygen supply limits the maximum size of aggregates, and a poor oxygen supply causes necrosis in the center of aggregates[9]. Consequently, suspension cultures of cell aggregates are more difficult to obtain than conventional suspension cultures. Nevertheless, suspension cultures are crucial for biomedical and biotechnology applications.

Orbital shaking vessels are one of the simplest suspension culture systems that achieve culture medium mixtures without impeller agitation. Shear stress from the impeller and the dynamic medium flow is the major problem of suspension culture because it causes cell damage and differentiation. To achieve agitation with a lower shear stress, researchers and industries have developed a variety of orbital shaking vessel systems[2,10].

However, conventional culture vessels are not designed for orbital shaking cultures. In orbital shaking vessels, cells gravitate towards the center-bottom of the vessels by a type of medium flow known as the “Einstein's tea leaf paradox”[11], which causes the inhomogeneous aggregation of cells. The circular flow, caused by a centrifugal force and a friction between the culture medium and a vessel, sweeps cells into the center[11,12]. In addition, conventional culture bags for mass culture are square-shaped, which is not suitable for orbital shaking.

In this study, we developed a novel culture bag suitable for orbital shaking cultures. The novelty of this bag is its O-shape which does not have a center region, so cells are prevented from gathering at the bottom center region. We have also demonstrated the handling of these vessels with an HEK293 culture to demonstrate the possibility of these bags for biotechnological applications.

PROTOCOL:

1. Preparation of the Cells and Materials

1.1. Cultivate HEK293 cells in Dulbecco’s modified Eagle medium with a 10% fetal bovine serum (FBS) and 1% non-essential amino acids. Adjust the pH of the culture medium to 6.8 - 7.6.

1.2. After culturing for 5 to 7 d, dissociate the cells with a 0.25% trypsin-ethylenediaminetetraacetic acid solution (trypsin-EDTA) and reseed the cells at 5,000-10,000 cells/cm^2.

2. Seeding the Cells in an O-shaped Bag

2.1. Dissociate the cells as described in step 1.2 and resuspend them in 2 - 5 mL of culture medium.

2.2. Filter the cell suspension through a cell strainer (40 µm) to collect a single cell suspension.

2.3. Count the number of cells by trypan blue staining and automatic cell counter, and then prepare 20 mL of the cell suspension (2.0 x 10^5 cells/mL).

2.4. Connect the inlet of the O-shaped bag (**Figure 1a**) to a clamped 50 mL syringe without a plunger.

[Insert **Figure 1** here]

2.5. Pipette the prepared cell suspension into the O-shaped bag through the clamped syringe.

2.6. Replace the first clamped syringe with a clean syringe. Add 55 mL of clean air through the clean syringe to expand the bag completely.

2.7. Clamp the inlet tube and then close the inlet. Finally, remove the clamp from the tube.

2.8. Incubate the cells in the O-shaped bag by shaking them at 45 rpm in conditions of 37 °C and 5% CO_2.

3. Medium Change (optional)

3.1. Transfer the cell suspension into a 50 mL tube through the inlet.

3.2. Centrifuge it for 2 min at 200 x g at room temperature, and then aspirate the supernatant.

3.3. Add 20 mL of culture medium and resuspend the cells.

3.4. Add the resuspended cells to the O-shaped bag by following steps 2.4 - 2.7.

3.5. Incubate the cells in the O-shaped bag by shaking them at 45 rpm in conditions of 37 °C and 5% CO_2.

4. Collecting the Cells from the Bag

4.1. Transfer the cell suspension into a 50 mL tube through the inlet.

4.2. Wash the inside of the bag with 20 mL of calcium/magnesium-free phosphate buffered saline [PBS(-), pH = 7.4 - 7.6] and then drain the contents into a tube to collect the remaining cells from the bag.

4.3. Centrifuge the collected cell suspension for 3 min at 200 x g at room temperature, and then aspirate the supernatant.

4.4. Add 10 mL of PBS(-) and wash the aggregates.

4.5. Centrifuge them for 3 min at 200 x g at room temperature, and then aspirate the supernatant to collect the aggregates.

4.6. Add 4 mL of PBS and 1 mL of trypsin and incubate them with the aggregates for 10 min at 37 °C to dissociate the cells for counting (optional).

5. Preparation of an O-shaped Dish (optional)

Note: See **Figure 1b** for a schematic image of the O-shaped dish.

5.1. Cut out the bottom of a 60 mm or 35 mm dish with a hot knife and utilize it as an inner dish.

5.2. Put the 60 mm dish upside-down on the center of a 100 mm dish.

Note: A guide sheet can help to decide the position of the 60 mm dish.

5.3. If required, put viscosity-adjusted cyclohexanone on the commissure from the inside of 60 mm dish.

5.4. Dry the O-shaped dish for a few days and sterilize it by gamma ray or ethylene oxide gas.

REPRESENTATIVE RESULTS:

According to our measurements, aggregates grown in the conventional dish had varied diameters after 5 d of orbital shaking. In contrast, aggregates grown in O-shaped vessels for 5 d had much more uniform diameters. The conventional dish culture showed small aggregates (50 - 200 µm), whereas the cultures in the O-shaped vessels did not contain any small aggregates (**Figure 2a**). According to the image-based size measurement, the conventional dish culture showed two different peaks (**Figure 2b1**), indicating a wide deviation of aggregate size. This result implied that aggregates in the conventional dish were growing under two different conditions in the same culture, which could result in heterogeneous cell quality. On the other hand, aggregates in O-shaped vessels showed a single peak and less deviation in diameter than those in the conventional dish (**Figures 2b2** and **2b3**), suggesting that such aggregates may be of more uniform

quality.

[**Insert Figure 2 here**]

Hematoxylin and eosin staining of aggregate cross-sections showed that aggregates grown in the conventional dish had some denucleated cells and necrotic cores (**Figure 3a**). However, aggregates grown in O-shaped vessels did not show any necrotic cores (**Figures 3b** and **3c**). In particular, aggregates grown in the O-shaped bag were as large as those in the conventional dish yet did not have any necrotic cores (**Figure 3c**). These results suggested that the gas-permeable bag supplied enough oxygen to the culture.

[Insert **Figure 3** here]

Cell counts showed that more than 85% of the cells survived for 5 d of suspension culture in each vessel (**Figure 4a**). The final cell density was approximately 1.5 - 2.0 x 10^6 cells/mL. Although there was no significant difference, the growth ratio in the O-shaped bag was higher than those in other vessels (**Figure 4b**). The specific growth rates of the cells in the conventional dish, the O-shaped dish, and the O-shaped bag between day 2 and day 5 were 0.018, 0.025, and 0.020 h^{-1}, respectively.

[Insert **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic images and pictures of O-shaped vessels. (**a1**) This is a schematic image of an O-shaped bag. (**a2**) This is a picture of an O-shaped bag. (**b1**) This is a schematic image of an O-shaped dish. (**b2**) This is a picture of an O-shaped dish. The outer and inner diameter of the O-shaped bag are 90 mm and 20 mm, respectively.

Figure 2: Morphologies and diameters of aggregates grown in various vessels. These panels show (**a**) brightfield images and (**b**) histograms of aggregates grown in either (**a1** and **b1**) a conventional dish, (**a2** and **b2**) an O-shaped dish, or (**a3** and **b3**) an O-shaped bag.

Figure 3: Cross-sections of aggregates in various vessels. The cross-sections are 12 μm thick and were prepared from frozen samples. The sections were stained with hematoxylin and eosin to visualize the cells. (**a**) The cell aggregates grown in a conventional suspension culture showed necrotic cores. Cell aggregates grown in either (**b**) an O-shaped dish or (**c**) an O-shaped bag showed very little necrosis in their cores.

Figure 4: Cell viability and growth. These panels show the (**a**) cell viability and (**b**) cell density of HEK293 cells in various culture vessels after 2 d of culture (day 2) and 5 d of culture (day 5). The values shown represent the mean of the results from 3 independent experiments. The values for each experiment were calculated from trypan blue-stained cells counted with an automatic cell counter. The error bars indicate standard deviation.

Supplemental Figure 1: Bead distribution in 2 different dish formats in various shaking conditions. This panel shows the bead distribution during various shaking conditions (30, 40, and 45 rpm) in a conventional and O-shaped dish.

DISCUSSION:

In this study, we developed O-shaped vessels and performed an HEK293 suspension culture in them for a uniform aggregates formation and expansion. In the conventional culture dish, an orbital shaking culture produced two different diameters of aggregates, whereas we observed uniform aggregates in the O-shaped vessels (**Figure 1**). According to the observation of the distribution of stained beads in the orbital shaking conditions, beads gather in the center-bottom of a conventional culture dish. That gathering presumably caused the difference in cell density leading to various sizes of aggregates. Alternatively, beads were distributed in the O-shaped dish which does not have the center-bottom region (**Supplemental Figure 1**). This distribution probably causes the uniformly-sized aggregates in O-shaped vessels. Another—widely used—approach to producing uniform aggregates is the culturing in microwells, but this approach has some problems, such as in supplying a culture medium without aggregates dropping out of the microwells[13]. In the case of O-shaped vessels, uniform aggregates can be produced in a simple suspension culture.

The orbital shaking culture is a popular culture system utilized for mammalian cells. There are various culture methods for the mass production of mammalian cells, such as the stirred tank bioreactor[14], wave-motioned bags[15], and rotating bottles. Orbital shaking cultures do not include an inner impeller for stirring the medium, unlike the stirred tank bioreactor does. This feature is similar to the wave-motioned bags and rotating bottles. These impeller-free culture systems can avoid cellular damage from the shear force surrounding the impellers and realize low shear stress in suspension culture. Especially, orbital shaking culture systems are effective for the mass production of sensitive cells such as mammalian cells because of their high scalability and low shear stress.

O-shaped vessels can improve the remaining problem of orbital shaking culture in the formation of aggregates. In orbital shaking culture systems, floating cells migrate to the center and bottom of the vessel, which is known as the "Einstein's tea leaf paradox"**Error! Reference source not found.**. This migration causes the inhomogeneous aggregation and non-uniform aggregate production in conventional orbital shaking vessels. In this study, O-shaped vessels prevented the concentration of cells into the center-bottom of the vessels, which is speculated as the reason of uniform aggregation in orbital shaking O-shaped vessels.

Histological analyses showed that the aggregates in the conventional dish contained denucleated cells (**Figure 2a**). In contrast, denucleated cells did not appear in the aggregates from O-shaped vessels (**Figure 2b** and **2c**). It is possible that these denucleated cells were caused by a shortage of substrates such as glucose, glutamine, and oxygen[9]. According to the size measurement, aggregates in the O-shaped vessels had homogeneous diameters lower than 400 μm. In contrast, in a conventional dish, some aggregates had a diameter larger than 400 μm, and these aggregates

included denucleated cells. This result suggests that creating homogeneous-sized aggregates in O-shaped vessels is effective in controlling the quality of aggregates. In addition, it is also speculated that the oxygenation through the gas-permeable polyethylene film prevented the appearance of denucleated cells in the O-shaped bag.

These experiments showed the possibility of these O-shaped vessels as a simple system for producing uniform aggregates. Although other culture bags for suspension culture have been developed[15], those culture bags are square-shaped, which prevents the culture from getting effectively mixed in orbital shaking. The bag in this study has a novel round shape suitable for orbital shaking to produce aggregates with a homogeneous size. This characteristic of vessels is important for controlling the conditions and the high reproducibility of cells in mass production. The possible application of the O-shaped vessel is widespread. It can be used when producing recombinant proteins from cells and for regenerative medicine when dealing with stem cells.

In conclusion, we developed a novel O-shaped bag suitable for producing uniform cell aggregates with an orbital shaking culture. The bag shows possibilities for various biomedical applications such as in regenerative medicine.

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DISCLOSURES:
The authors have nothing to disclose.

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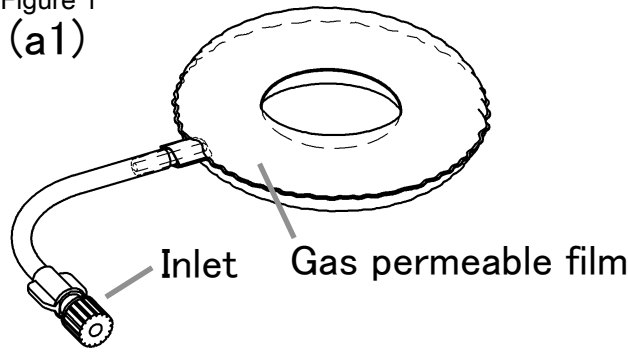
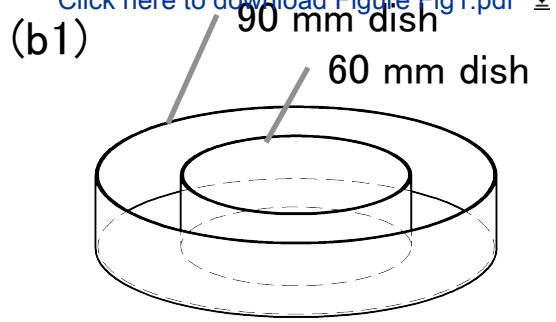
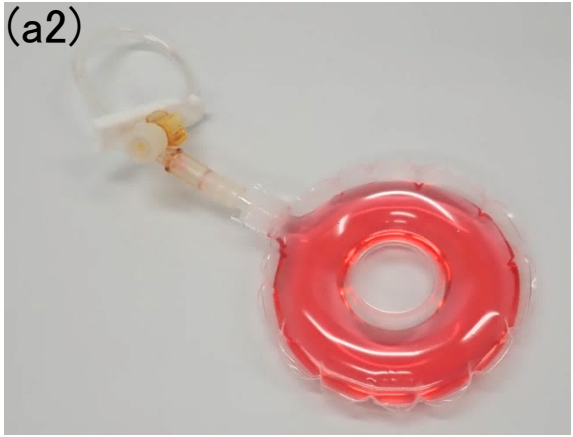
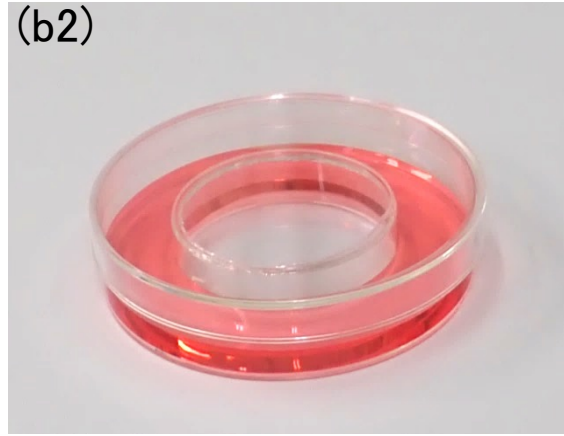
Figure 1

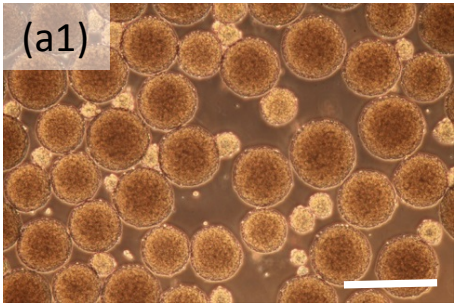


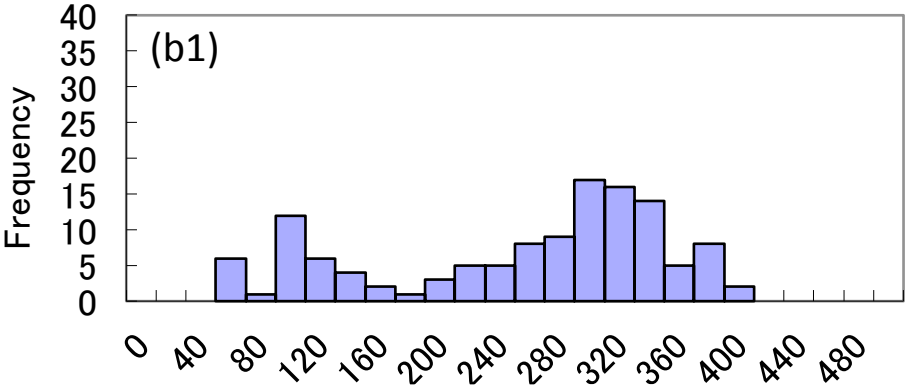




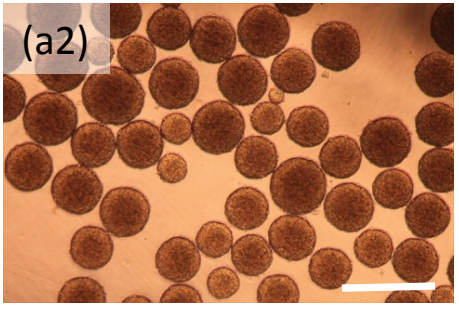


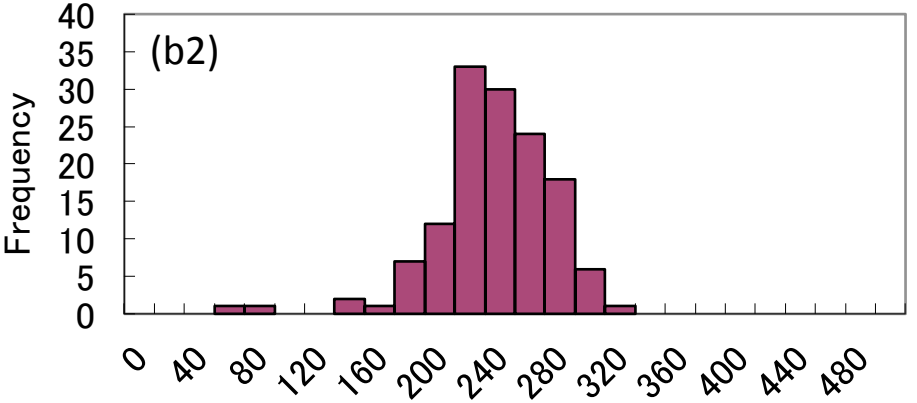
Conventional Dish



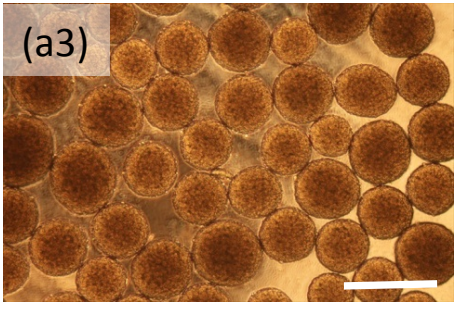


O-shape Dish

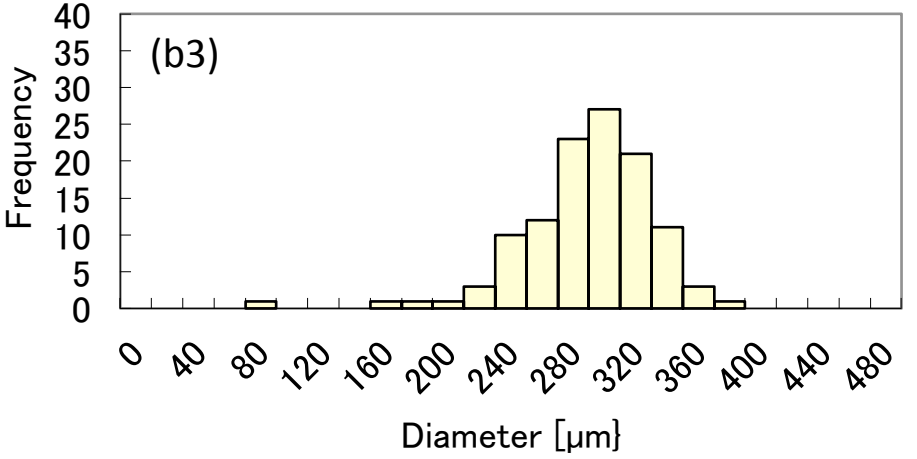




O-shape Bag



Bar = 500 µm



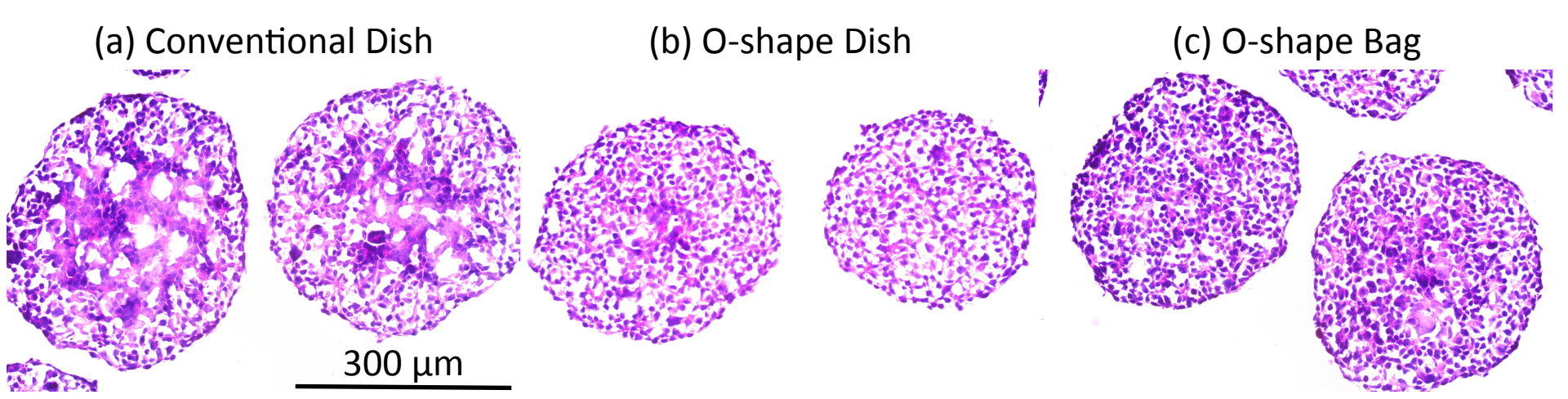
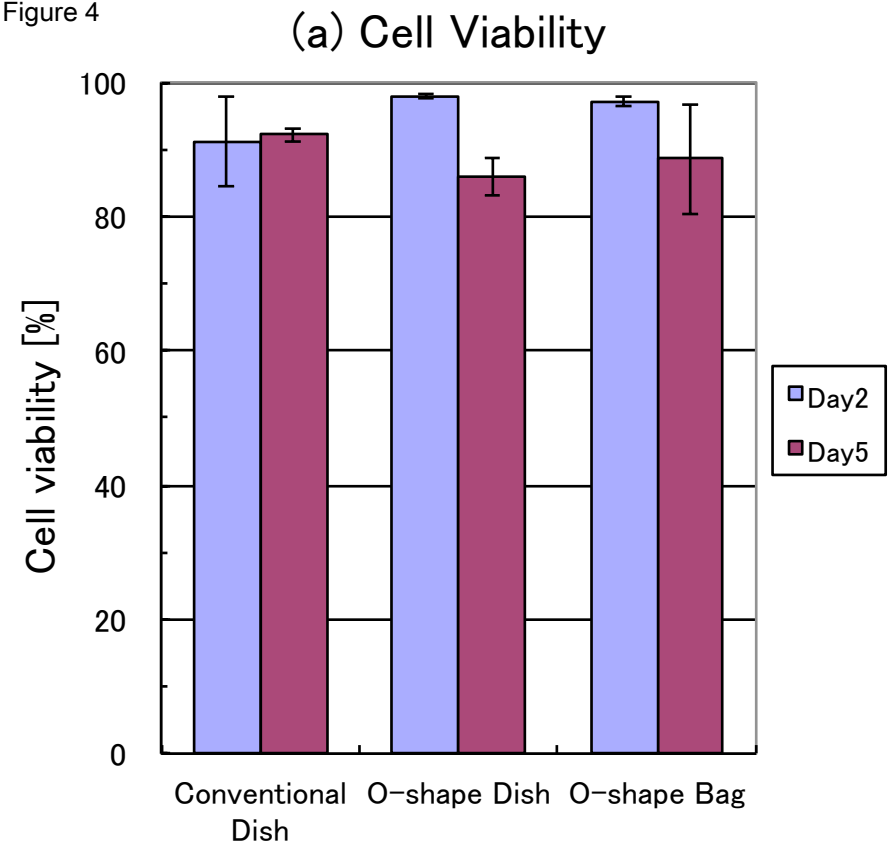
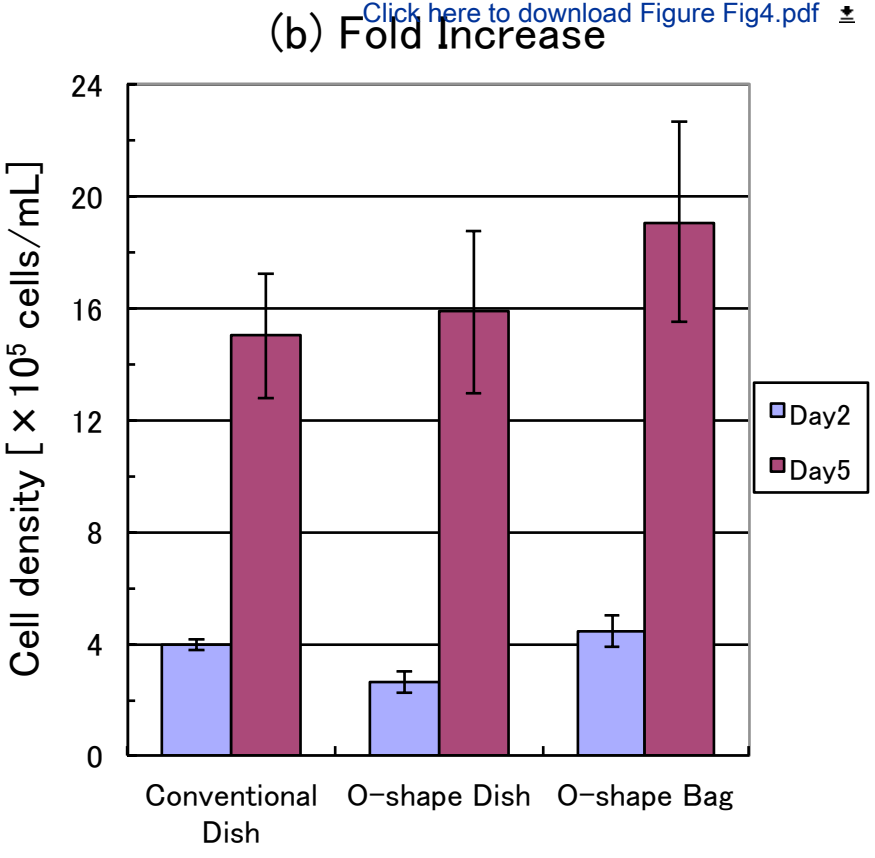

(a) Conventional Dish
(b) O-shape Dish
(c) O-shape Bag
300 µm

Figure 4





Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
HEK293	RIKEN Bio resorce centre	RCB1637	
DMEM, high glucose, pyruvate	GIBCO	11995040	
Fetal Bovine Serum, qualified, USDA-approved regions	GIBCO	10437-028	
MEM Non-Essential Amino Acids Solution, 100X	GIBCO	11140050	
Trypsin-EDTA (0.25%), phenol red	GIBCO	25200056	
Dulbecco's PBS （—）	Cell Science & Technology Institute	1102P05	
Cell Strainer 40µm	CORNING	352340	
50 mL Syringe	TERUMO	SS-50ESZ	
Shaker	AS ONE	2-1987-02	
Centrifuge Tube 50 mL	AS ONE	1-3500-02	
Automated cell counter	BioRad	TC20	

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Name:	Ikki Horiguchi
Department:	Department of Chemical System Engineering
Institution:	The University of Tokyo
Article Title:	Orbital shaking culture of mammalian cells in O-shaped vessels for production of uniform aggregates
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Name:	Ikki Horiguchi
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Institution:	The University of Tokyo
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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.

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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) 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.

We are sorry for late reply and now we revised the manuscript according to the editorial comments. We also answered your each comment below.

1. (Short Abstract) The title says vessel here it says bag. Please verify.

Response: Thank you for informing. We have changed the word "vessels" instead of bag in the short abstract

2. (Page 2, Line 74) Needs a citation here if this term is mentioned here. Also if adding a citation, please ensure that the references are changed accordingly thoughout for intext formatting and perform the necessary changes in the reference section as well.

Response: According to the comment, we added the citation (Page 2, Line 74).

3. (Protocol 2.4) Syringe of what size?

Response: We used 50 mL syringe. We added the information about the size of a syringe.

4. (Figure 2b1) How these measurements were performed?

Response: We used image-based size measurement from the picture. We added information of method in the representative results. (Page 4, Line 167)

5. (Discussion paragraph 1) We cannot have data not shown. This sentence is important, and it will be good if data is shown here.

Response: Thank you for an advice. According to the comment, we added the supplemental figure illustrating the different distribution of two different formats of dishes (Supplemental Figure 1).

TITLE:

Orbital Shaking Culture of Mammalian Cells in O-shaped Vessels for the Production of Uniform Aggregates

AUTHORS & AFFILIATIONS:

Ikki Horiguchi[1,2], Ikumi Suzuki[3], Takashi Morimura[3], Yasuyuki Sakai[1,4]

[1] Department of Chemical System Engineering, The University of Tokyo, Tokyo, Japan
[2]Department of Biotechnology, Osaka University, Osaka, Japan
[3] Biotech Business Unit, Fukoku Co. Ltd., Saitama, Japan
[4] Institute of Industrial Science, The University of Tokyo, Tokyo, Japan

Email Addresses of Co-Authors:

Ikki Horiguchi (horiguchi@bio.eng.osaka-u.ac.jp)
Ikumi Suzuki (i_suzuki@fukoku-rubber.co.jp)
Takashi Morimura (t_morimura@fukoku-rubber.co.jp)
Yasuyuki Sakai (sakaiyas@iis.u-tokyo.ac.jp)

Corresponding authors:

Ikki Horiguchi (horiguchi@bio.eng.osaka-u.ac.jp)
Ikumi Suzuki (i_suzuki@fukoku-rubber.co.jp)

KEYWORDS:

Suspension culture, orbital shaking, mass production, mammalian cell, culture bag, aggregate

SHORT ABSTRACT:

Here we present a protocol for using an O-shaped bag specialized for suspension culture of cellular aggregates with orbital shaking. HEK293 cells grown in this bag formed more homogeneous aggregates than those grown in conventional culture vessels.

LONG ABSTRACT:

Suspension culture of mammalian cell aggregates is required for various applications in medical and biotechnological fields. The disposable bag-based method is one of the simplest techniques for mass production of cellular aggregates, but it does not prevent over aggregation from gathering in the bottom center of the culture vessel. To solve this problem, we developed an O-shaped dish and an O-shaped bag, neither of which contained a central region. Aggregates grown in either O-shaped culture vessel were noticeably more uniform in size than aggregates grown in conventional vessels. Histological analyses showed that aggregates in conventional culture dishes contained necrotic cores most likely caused by poor oxygen supply. In contrast, aggregates grown in the O-shaped bag, even those with similar diameters to aggregates in conventional culture dishes, did not show necrotic cores. Our results suggest that the O-shaped bag provides sufficient oxygen to the aggregates due to the oxygen permeability of the bag material. We, therefore, propose that this novel gas-permeable O-shaped culture bag is suitable for mass production of uniform aggregates that are necessary in various biotechnological fields.

Commented [VB1]: The title says vessel here it says bag. Please verify.

INTRODUCTION:

Suspension cell culture plays an important role in cell production for regenerative medicine[1] and recombinant protein production[2] because it is easy to scale up and achieve high cell densities, sometimes up to more than 10^7 cells/mL[5]. Chinese hamster ovary (CHO) cells[3], human embryonic kidney cells 293 (HEK293)[4] have been grown in suspension culture, and human pluripotent stem cells (hPSCs) have recently been grown in suspension culture systems for regenerative therapies[1, 6, 7]. The use of suspension culture is expected to increase in the future.

In suspension culture, some cell lines cannot grow as single cells and must, therefore, form aggregates. For example, hPSCs cannot survive in suspension conditions without forming aggregates. However, this requirement for aggregated growth presents difficulties for uniform suspension culture. One difficulty is the formation of uniform aggregates in early period of culture, which determines the efficiency of suspension culture[8]. Another difficulty is the mass transfer of nutrients into aggregates. In particular, oxygen supply limits the maximum size of aggregates and poor oxygen supply causes necrosis in the center of aggregates[9]. Consequently, suspension culture of cell aggregates is more difficult to obtain than the conventional suspension culture. Nevertheless, suspension cultures are crucial for biomedical and biotechnology applications.

Orbital shaking vessels are one of the simplest suspension culture systems that achieve culture medium mixture without impeller agitation. Shear stress from impeller and dynamic medium flow is the major problem of suspension culture because it causes cell damage and differentiation. To achieve agitation with lower shear stress, researchers and industries have developed a variety of orbital shaking vessel systems[2,10].

However, conventional culture vessels are not designed for orbital shaking culture. In orbital shaking vessels, cells become localized to the center-bottom of vessels by a type of medium flow known as the "Einstein tea leaf paradox" which causes inhomogeneous aggregation of cells. The circular flow caused by a centrifugal force and a friction between culture medium and a vessel sweeps cells into the center [11, 12]. In addition, conventional culture bags for mass culture are square-shaped, which is not suitable for orbital shaking.

Commented [VB2]: Needs a citation here if this term is mentioned here. Also if adding a citation, please ensure that the references are changed accordingly thoughout for intext formatting and perform the necessary changes in the reference section as well.

In this study, we developed a novel culture bag suitable for orbital shaking culture. The bag had a novel O-shape, which did not have a center region to prevent cell gathering into the bottom center region. We have also demonstrated the handling of these vessels in HEK293 culture to demonstrate the possibility of these bags for biotechnological applications.

PROTOCOL:

1. Preparation of Cells and Materials

1.1. Cultivate HEK293 cells in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS) and 1% non-essential amino acids. Adjust the pH of the culture medium to 6.8-7.6.

1.2. After culturing for five to seven days, dissociate cells with 0.25% trypsin-ethylenediaminetetraacetic acid solution (trypsin-EDTA) and reseed cells at 5000-10000 cells/cm^2

2. Seeding Cells in O-shaped Bag

2.1. Dissociate cells as described in 1.2 and resuspend in 2-5 mL of culture medium.

2.2. Filter the cell suspension through a cell strainer (40 μm) to collect a single-cell suspension.

2.3. Count the number of cells by trypan blue staining and automatic cell counter, then prepare 20 mL of a cell suspension (2.0×10^5 cells/mL).

2.4. Connect the inlet of the O-shaped bag (**Figure 1a**) to a clamped syringe without a plunger.

Commented [VB3]: Syringe of what size?

[**Insert Figure 1 here**]

2.5. Pipette the prepared cell suspension into the O-shaped bag through the clamped syringe.

2.6. Replace the first clamped syringe with a clean syringe. Add 55 mL of clean air through the clean syringe to expand the bag completely.

2.7. Clamp the inlet tube and then close the inlet. Finally, remove the clamp on the tube.

2.8. Incubate cells in the O-shaped bag by shaking at 45 rpm in conditions of 37 °C and 5% CO_2.

3. Medium Change (optional)

3.1. Transfer the cell suspension into a 50 mL tube through the inlet.

3.2. Centrifuge for 2 min at 200 $\times g$ and room temperature, and then aspirate the supernatant.

3.3. Add 20 mL of culture medium and resuspend cells.

3.4. Add the resuspended cells by following steps 2.4 - 2.7.

3.5. Incubate cells in the O-shaped bag by shaking at 45 rpm in conditions of 37 °C and 5% CO_2.

4. Collecting cells from the bag

4.1. Transfer the cell suspension into a 50 mL tube through the inlet.

4.2. Wash the inside of the bag with 20 mL of calcium/magnesium-free phosphate buffered

saline (PBS(-), pH=7.4-7.6), then drain into a tube to collect remaining cells in the bag.

4.3. Centrifuge the collected cell suspension for 3 min at 200 ×*g* and room temperature, and then aspirate the supernatant.

4.4. Add 10 mL of PBS(-) and wash aggregates.

4.5. Centrifuge for 3 min at 200 ×*g* and room temperature, and then aspirate the supernatant to collect aggregates.

4.6. Add 4 mL of PBS and 1 mL of trypsin, incubate for 10 min at 37 ºC to dissociate cells for counting (optional).

5. Preparation and Use of an O-shaped Dish (Figure 1b, optional)

5.1. Cut out the bottom of a 60 mm or 35 mm dish with a hot knife and utilize it as an inner dish.

5.2. Put the 60 mm dish upside-down on the center of 100 mm dish. A guide sheet can help to decide the position of the 60 mm dish.

5.3. If required, put viscosity-adjusted cyclohexanone on the commissure from the inside of 60 mm dish.

5.4. Dry the O-shaped dish for a few days and sterilize it by gamma ray or ethylene oxide gas.

REPRESENTATIVE RESULTS:

According to our measurements, aggregates grown in the conventional dish had varied diameters after five days of orbital shaking culture. In contrast, aggregates grown in O-shaped vessels after five days culture had much more uniform diameters. The conventional dish culture showed small aggregates (50-200 μm), whereas the cultures in the O-shaped vessels did not contain any small aggregates (**Figure 2a**). According to the histogram, the conventional dish culture showed two different peaks (**Figure 2b1**), indicating a wide deviation of aggregate size. This result implied that aggregates in the conventional dish were growing under two different conditions in the same culture, which could result in heterogeneous cell quality. On the other hand, aggregates in O-shaped vessels showed a single peak and less deviation in diameter than those in the conventional dish (**Figure 2b2-b3**), suggesting that such aggregates may be of more uniform quality.

Commented [VB4]: Need to state how these measurements were performed?

[**Insert Figure 2 here**]

Hematoxylin and eosin staining of aggregate cross-sections showed that aggregates grown in the conventional dish had some denucleated cells and necrotic cores (**Figure 3a**). However, aggregates grown in O-shaped vessels did not show necrotic cores (**Figure 3b** and **3c**). In

particular, aggregates grown in the O-shaped bag were as large as those in the conventional dish yet did not have necrotic cores (**Figure 3c**). These results suggested that the gas-permeable bag supplied enough oxygen to the culture.

[**Insert Figure 3 here**]

Cell counts showed that more than 85% of cells survived for five days of suspension culture in each vessel (**Figure 4a**). The final cell density was approximately 1.5-2.0 $\times$ 10^6 cells/mL. Although there was no significant difference, the growth ratio in the O-shaped bag was higher than those in other vessels (**Figure 4b**). The specific growth rates of cells in conventional dish, O-shaped dish and O-shaped bag between day 2 and day 5 were 0.018, 0.025, 0.020 h^{-1} respectively.

[**Insert Figure 4 here**]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic images and pictures of O-shaped vessels. Schematic images (1) and pictures (2) of O-shaped bag (a) and dish (b). The outer and inner diameter of O-shaped bag was 90 mm and 20 mm respectively.

Figure 2: Morphologies and diameters of aggregates grown in various vessels. Brightfield images (a) and histograms (b) of aggregates grown in either a conventional dish (a1, b1), an O-shaped dish (a2, b2) or an O-shaped bag (a3, b3).

Figure 3: Cross-sections of aggregates in various vessels. Cross-sections were 12 µm thick and were prepared from frozen samples. Sections were stained with hematoxylin and eosin to visualize the cells. Cell aggregates grown in conventional suspension culture showed necrotic cores (a). Cell aggregates grown in either an O-shaped dish (b) or an O-shaped bag (c) showed very little necrosis in their cores.

Figure 4: Cell viability and growth. Cell viability (a) and cell density (b) of HEK293 cells in various culture vessels after two days of culture (day 2) and five days of culture (day 5). Values shown represent the mean of the results from three independent experiments. Values for each experiment were calculated from trypan blue-stained cells counted with an automatic cell counter. Error bars indicated standard deviation.

DISCUSSION:

In this study, we developed O-shaped vessels and performed HEK293 suspension culture in them for uniform aggregates formation and expansion. In the conventional culture dish, orbital shaking culture produced two different diameters of aggregates, whereas we observed uniform aggregates in the O-shaped vessels (**Figure 1**). According to the observation of the distribution of stained beads in orbital shaking conditions, beads gathered in the center-bottom of the conventional culture dish. That gathering presumably caused the difference in cell density

leading to forming various sizes of aggregates. However, O-shaped vessels did not have the center-bottom region, and, as expected, we observed less variation in cell density (data not shown). Therefore, cells could form uniformly-sized aggregates in O-shaped vessels. To produce uniform aggregates, culturing in microwells is widely used, but this approach also has some problems, such as in supplying culture medium without aggregates dropping out from the microwells[13]. In the case of O-shaped vessels, uniform aggregates can be produced in a simple suspension culture.

Commented [VB5]: We cannot have data not shown. This sentence is important, and it will be good if data is shown here.

Orbital shaking culture is the popular culture system utilized for mammalian cells. There are various culture methods for mass production; stirred tank bioreactor[14], wave motioned bags[15] and rotating bottles. Orbital shaking culture does not include an inner impeller for stirring medium unlike stirred tank bioreactor. This feature is similar to wave motioned bags and rotating bottles. These impeller-free culture systems can avoid cellular damage from shear force surrounding impellers and realize low shear stress in suspension culture. Especially, orbital shaking culture systems are effective for mass production of sensitive cells such as mammalian cells because of their high scalability and low shear stress.

O-shaped vessels can improve remaining problem of orbital shaking culture in aggregate formation. In orbital shaking culture systems, floating cells migrate into a center and bottom of the vessel, which is known as “Einstein’s tea leaves” problem[16]. This migration caused inhomogeneous aggregation and non-uniform aggregate production in conventional orbital shaking vessels. In this study, O-shaped vessels could prevent concentration of cells into the center-bottom of the vessels, which is speculated as a reason of uniform aggregation in orbital shaking O-shaped vessels.

Histological analyses showed that aggregates in the conventional dish contained denucleated cells (**Figure 2a**). In contrast, these denucleated cells did not appear in the aggregates from O-shape vessels (**Figure 2b and 2c**). It is possible that these denucleated cells were caused by the shortage of substrates such as glucose, glutamine and oxygen[9]. According to size measurement, aggregates in the O-shaped vessels had homogeneous diameters lower than 400 µm. In contrast, in conventional dish, some aggregates had the diameter larger than 400 µm and these aggregates include denucleated cells. This result suggests that creating homogeneous-sized aggregates in O-shaped vessels is effective to control the quality of aggregates. In addition, it is also speculated that oxygenation through the gas permeable polyethylene film prevent the appearance of denucleated cells in O-shaped bag.

These experiments showed the possibility of these O-shaped vessels as a simple system for producing uniform aggregates. Although other culture bags for suspension culture have been developed[15], those culture bags are square-shaped, which prevents effective mixture in orbital shaking. The bag in this study has a novel round shape suitable for orbital shaking to produce aggregates with homogeneous size. This character of vessels is important for controlling the conditions of cells in mass production and the high reproducibility. The possible application of the O-shaped vessels is widespread; producing recombinant proteins from cells and regenerative medicine by using stem cells.

In conclusion, we developed a novel O-shaped bag suitable for producing uniform cell aggregates with orbital shaking culture. The bag showed possibilities for various biomedical applications such as in regenerative medicine.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Supplemental Figure 1

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