**TITLE:**

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

**AUTHORS & AFFILIATIONS:**

Ikki Horiguchi1,2, Ikumi Suzuki3, Takashi Morimura3, Yasuyuki Sakai1,4

1 Department of Chemical System Engineering, The University of Tokyo, Tokyo, Japan

2Department 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.

**INTRODUCTION:**

Suspension cell culture plays an important role in cell production for regenerative medicine1 and recombinant protein production2 because it is easy to scale up and achieve high cell densities, sometimes up to more than 107 cells/mL5. Chinese hamster ovary (CHO) cells3, 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 therapies1, 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 culture8. 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 aggregates9. 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 systems2,10.

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.

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/cm2

**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 × 105 cells/mL).

2.4. Connect the inlet of the O-shaped bag (**Figure 1a**) to a clamped 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 on the tube.

2.8. Incubate cells in the O-shaped bag by shaking at 45 rpm in conditions of 37 oC and 5% CO2.

**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 ×*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 oC and 5% CO2.

**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.

[**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 × 106 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 microwells13. In the case of O-shaped vessels, uniform aggregates can be produced in a simple suspension culture.

Orbital shaking culture is the popular culture system utilized for mammalian cells. There are various culture methods for mass production; stirred tank bioreactor14, wave motioned bags15 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” problem16. 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 oxygen9. 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 developed15, 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.

**ACKNOWLEDGMENTS:**

This research is supported by a collaboration with FUKOKU, CO., Ltd. Takao Yoshida from FUKOKU, CO., Ltd., provided the idea of the O-shaped culture bag. Takamasa Sato from FUKOKU, CO., Ltd., supported this research in terms of computational simulation for developing O-shaped culture bag. We would like to appreciate the corresponding author’s current affiliation, Osaka University, for allowing us to work for the publication.

**DISCLOSURES:**

The authors declare that they have no competing financial interests.

**REFERENCES:**

1. Mihara, Y., Matsuura, K., Sakamoto, Y., Okano, T., Kokudo, N., Shimizu, T. Production of pancreatic progenitor cells from human induced pluripotent stem cells using a three-dimensional suspension bioreactor system. *J. Tissue Eng. Regen. Med.,* *In press,* DOI:10.1002/term.2228
2. Raven, N., Rasche, S., Kuehn, C., Anderlei, T., Klöckner, W., Schuster, F., et al. Scaled-up manufacturing of recombinant antibodies produced by plant cells in a 200-L orbitally-shaken disposable bioreactor. *Biotechnol. Bioeng.* **112** (2), 308-321 (2015)
3. Han, Y., Liu, X., Liu, H., Li, S., Wu, B., Ye, L., et al. Cultivation of recombinant chinese hamster ovary cells grown as suspended aggregates in stirred vessels. *J. Biosci. Bioeng.* **102** (5), 430-435 (2006)
4. Portolano, N., Watson, P.J., Fairall, L., Millard, C.J., Milano, C.P., Song, Y. et al. Recombinant Protein Expression for Structural Biology in HEK 293F Suspension Cells: A Novel and Accessible Approach. *J. Vis. Exp.* (92), e51897, doi:10.3791/51897 (2014).
5. Himmelfarb, P., Thayer, P.S., Martin, H.E. Spin filter culture: the propagation of mammalian cells in suspension. *Science* **164** (3879), 555-557 (1969)
6. Kropp, C., Massai, D., Zweigerdt, R. Progress and challenges in large-scale expansion of human pluripotent stem cells. *Process Biochem.* **59**, 244-254 (2017)
7. Singh, H., Mok, P., Balakrishnan, T., Rahmat, S. N. B., Zweigerdt, R. Up-scaling single cell-inoculated suspension culture of human embryonic stem cells. *Stem. Cell. Res*. **4** (3), 165-179 (2010)
8. Horiguchi, I. and Sakai, Y. Serum replacement with albumin-associated lipids prevents excess aggregation and enhances growth of induced pluripotent stem cells in suspension culture. *Biotechnol. Prog.* **32** (4), 1009-1016 (2016)
9. Sutherland, R.M., Sordat, B., Bamat, J., Gabbert, H., Bourrat, B., Mueller-Klieser, W. Oxygenation and differentiation in multicellular spheroids of human colon carcinoma. *Cancer Res*. **46** (10), 5320-5329 (1986)
10. Hang, H., Guo, Y., Liu, J., Bai, L., Xia, J., Guo, M. et al. Computational fluid dynamics modeling of an inverted frustoconical shaking bioreactor for mammalian cell suspension culture. *Biotechnol. Bioprocess Eng.* **16** (3), 567-575 (2011)
11. Einstein, A., The cause of the formation of meanders in the courses of rivers annd of the so-called bear’s law. *Die Naturwissenschaften.* **14** (1926)
12. Tandon, A., Dartmouth, U., Marshall, J. Einstein’s tea leaves and pressure systems in the atmosphere. *Phys. Teach.* **48**, 292-295 (2010)
13. Miyamoto, D., Nakazawa, K. Differentiation of mouse iPS cells is dependent on embryoid body size in microwell chip culture. *J. Biosci. Bioeng.* **122** (4), 507-512 (2016)
14. Olmer, R., Lange, A., Seizer, S., Kasper, C., Haverich, A., Martin, U. et al. Suspension culture of human pluripotent stem cells in cotrolled, stirred bioreactors. *Tissue Eng Part C*. **18** (10), 772-784 (2012)
15. Singh, V. Disposable bioreactor for cell culture using wave-induced agitation. *Cytotechnology*. **30**, 149-158 (1999)
16. Einstein, A. The cause of the formation of meanders in the courses of rivers and of the so-called bear’s law. *Die Naturwissenschaften*. **14**, (1926)