Particle-tracking-based estimation of shear stress on cell aggregates in shaking vessel with different shaking methods/speeds

Ikki Horiguchi (horiguchi.ikki.es@osaka-u.ac.jp)
Osaka University
Hotaka Nagate
The University of Tokyo
Yasuyuki Sakai
The University of Tokyo

Article

Keywords: pluripotent stem cells, suspension culture, shear stress, particle tracking

Posted Date: July 12th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1835006/v1

Abstract

Suspension cultures are widely used for cell expansion in regenerative medicine and production. Shear stress caused by the flow of medium in cultures causes cell death and delayed growth; thus, the shear stress on the cell surface must be minimized for successful suspension culture. We established a particle tracking system for estimating the shear stress on induced pluripotent stem cell aggregates during shaking culture and utilized it to optimize shaking conditions (orbital and reciprocating shaking). When the average accelerations of aggregates were calculated, high acceleration occurred periodically, and acceleration on the aggregates in orbital shaking was stable. Furthermore, the number of dead cells correlated with the time average of acceleration. In the case of growth, there was optimal acceleration due to various events, such as aggregate formation and cell death. These results indicated that image-based analyses of acceleration are helpful for estimating the shear stress on the aggregates in suspension culture to optimize agitation.

Introduction

Suspension culture systems are used for mass production of cells because of their scalability compared to that of adherent cultures. Suspension culture is widely used for various purposes, such as the production of antibiotics, foods, and cells. Recently, various attempts have been made to mass-produce pluripotent stem cells as cell sources for regenerative medicine and drug screening[1,2]. Generally, a suspension culture system includes a dynamic system to agitate the medium for supplying oxygen, realizing uniform conditions in the medium, keeping cells floating, etc.[1]. There are various types of agitation systems, including impeller shearing, shaking bags[3,4], and rolling bottles.

However, mammalian cells—especially pluripotent stem cells—are sensitive to shear stress due to the medium flow of the suspension culture, which limits the scalability of their suspension culture[5]. Therefore, scaling-up of culture vessels for mammalian cells requires gentler agitation than that for other cells such as bacteria and plants. In previous studies, researchers attempted to reduce the shear stress using various approaches[6]. One method is to improve the impeller for effective mixing with lower shear stress. Another representative approach is to improve the viscosity of the medium to reduce the flow dynamics and shear stress on floating cells. Some researchers reported that high medium viscosity achieved by adding polymers such as polysaccharides and poloxamer can overcome cell damage from shear stress[7,8]. Some reports showed that not only increasing viscosity but also elevating yield stress (changing the medium to Bingham plastic) can effectively prevent cell damage due to shear stress[7]. These attempts successfully avoided cell death due to shear stress from the culture medium or impeller; however, to apply these technologies to the actual process, the trade-off between the effect of the mixture and the protection from shear stress must be considered.

For designing a scalable mammalian cell culture system based on suspension culture, the shear stress acting on cells must be estimated while considering the trade-off. Computational flow dynamics (CFD), which is based on the Navier–Stokes equation, is an effective method for estimating the shear stress

from a culture medium. It is an important tool for estimating shear stress without experiments; however, the location of the cells in the vessel during suspension culture must be estimated for this method, which is a challenging task. Indeed, previous research on CFD has shown that the shear stress in a culture vessel is not uniform and that the share stress acting on cells depends on their location. Previous studies have used CFD analysis with particles to calculate the shear stress. A gradient flow rate on the particle is required to calculate the shear stress, which requires a large amount of calculations.

In this study, we developed an image-based methodology to estimate the shear stress acting on cell aggregates in suspension culture using particle-tracking velocimetry (PTV). For the last few decades, PTV, or particle image velocimetry (PIV), has been utilized to visualize fluidic flows such as air and water. In the bioengineering/biomedical field, numerous researchers have applied PTV to visualize blood flow in the body[9,10] and flow dynamics in artificial organs[11], microfluidic devices[12], and bioreactors[13]. PTV is a Lagrangian approach used to measure the velocities and trajectories of particles moving in liquid flow. In this study, we applied the PTV method to a shaking vessel to estimate the shear force acting on the cell aggregates from acceleration based on Newton's equation of motion. In addition, we compared different shaking speeds/methods to determine the optimal shaking conditions and important parameters for optimization. Through the experiment, we suggested the shear force estimation method to optimize culture condition in suspension culture.

Results

Trajectories of induced pluripotent stem cell (iPSC) aggregates in various shaking condition

Particle tracking showed the trajectories of iPSC aggregates in two different types of shaking operations (reciprocating and orbital shaking) at various shaking rates (Fig. 1). In the reciprocating conditions with a lower agitation rate (< 90 rpm), trajectories were regular, and aggregates moved along with the flow of the medium, whereas aggregate movement became intense and irregular with increasing agitation rate (> 90 rpm), which indicated that turbulent flow occurred around the aggregates. In the case of orbital shaking conditions, the aggregates showed spiral trajectories, and the trajectory became intense with a higher agitation rate.

Focusing on the position of aggregates, aggregates tended to be the center of culture vessels under orbital shaking conditions with a low agitation rate (< 90 rpm), and reciprocating shaking gathered aggregates along with movement. These gathering phenomena did not occur, and the aggregates spread at a high agitation rate (120 rpm).

Acceleration of aggregates in various shaking conditions

Acceleration rates of the aggregates were derived from the time profile of the trajectory (Fig. 2). The pattern of the acceleration time profile differed between the reciprocating and orbital shaking conditions. In reciprocating shaking, periodic high acceleration is applied to aggregates, whereas in orbital shaking,

time-stable acceleration is applied to aggregates. The calculation of the time average of acceleration showed that a higher average acceleration was applied at a higher shaking rate. Compared with the time average of acceleration at the same shaking rate in orbital shaking, a lower time-average acceleration was applied in reciprocating shaking.

Cell growth and death during suspension culture

Using microscopic observations, we suggested the optimal shaking rate for the suspension culture (Fig. 3). Cells over aggregated under slow shaking conditions (60 rpm), and the diameter of the formed aggregates decreased as the shaking rate increased. In addition, debris appeared under high shaking conditions (120 rpm).

Evaluation of cell growth and death showed that the measured acceleration reflected the damage to cells due to shear stress (Fig. 4). We evaluated two different shaking methods—orbital and reciprocating shaking—to clarify which parameter of acceleration reflected the shear stress during suspension culture (Fig. 4a and 4b). Figure 4a1 shows that an optimal average acceleration existed between 500 and 700 mm/s^2 in terms of cell growth, regardless of the shaking method. In addition, Fig. 4a2 shows that an almost linear relationship existed between the average acceleration and number of dead cells based on the leaked lactate dehydrogenase (LDH). In contrast, when we rearranged the data by maximum acceleration, the data points were separated into two groups with different shaking conditions in both fold increase and number of dead cells.

Discussion

In this study, we estimated cell damage caused by shear stress using image capturing and tracking. Shear stress causes cell damage and delays or limits cell growth in suspension cultures[14]. Thus, the shear stress must be estimated for developing a bioreactor system. However, the experimental measurement of shear stress acting on cells in suspension culture is still challenging because measuring the gradient of the shear rate on the surface is difficult. Generally, while designing bioreactors, CFD is used to estimate the medium flow and shear stress in a bioreactor; however, this estimation of shear stress does not consider the cellular location. Recently, the Lagrangian approach was applied to estimate the location of cells and the shear stress acting on them; however, the problem of accuracy and validation from experimental data still exists. Therefore, we estimated the shear stress acting on cells by tracking the trajectory of the cell aggregates.

When shaking operations were compared, cells showed higher acceleration in the reciprocating condition than in the orbital condition. In orbital and reciprocating shaking with a moderate shaking rate (< 90 rpm), the trajectories of the aggregates were regular, which indicated that laminar flow was dominant in the vessel. In contrast, in reciprocating shaking with a high shaking rate (120 rpm), the trajectory was irregular, indicating turbulent flow in the vessel. The trajectory suggested that reciprocating was more likely to generate turbulent flow around cell aggregates in the suspension culture. In terms of acceleration,

orbital shaking led to a stable and low acceleration of cell aggregates, whereas reciprocating shaking led to periodic fluctuations in acceleration. This is reasonable because cell aggregates turned back in reciprocating vessels and the cell aggregates were subjected to a large acceleration. Generally, turbulent flow promotes mass transfer, and there are many suspension culture systems with reciprocating medium flow, known as WAVE bioreactors. These analyses showed that the reciprocating culture system not only effectively promoted mass transfer, such as that of oxygen, but also applied high and periodic shear stress to the cells.

According to the scattering plot of cell growth/death and average/maximum acceleration, cell death/growth was correlated with time-averaged acceleration. The growth plot exhibited a trade-off, and there was an optimum average acceleration of 500–700 mm/s^2 regardless of the method of shaking (Fig. 4a1). Interestingly, the average acceleration and total number of dead cells had a linear correlation with the maximum acceleration and number of dead cells (Fig. 4a2 and 4b2). Various types of shear stress act on cells: vascular flow in a body[15], flow in the nozzle of a bio three-dimensional printer, and medium flow in bioreactors or bioprinters[16,17]. Shear stress has long been studied as a physical factor that causes cell damage and delayed growth[17]. In addition, shear stress has been reported to increase plasma membrane fluidity[18,19] and induce mechanotransduction[20,21].

Our results showed that the average acceleration from the captured video indicate shear stress in the suspension culture. The relationship between acceleration and force is known to be linear according to Newton's equation of motion; thus, the force acting on the particle can be estimated. In the shaking vessel, the particles were subjected to centrifugal force and the force from the fluid; however, in this case, the force from the liquid was dominant compared to the centrifugal force on the particles. This is because the particles should move to the wall of the vessel owing to centrifugal force if the centrifugal force is dominant.

Although particle-tracking-based acceleration estimation has a high potential to optimization of culture condition, it requires further improvement to use the measured acceleration as a parameter to design bioreactors. For example, one limitation is that current method is ignoring z-axis acceleration. In this study, vessel diameter (34 mm) was large comparing with medium height (approx. 4 mm), thus we considered that movement on x-y dimension was dominant. To apply the estimation method to larger scale culture, considering on z-axis is necessary and adding a camera to track the trajectory of z-axis is required.

In this study, we proposed a methodology for determining the optimal conditions for shaking vessel culture using particle tracking. Particle tracking showed that the shear stress in orbital shaking was lower than that in reciprocating shaking at the same shaking rate, and there was an optimal average acceleration in terms of cell growth. Although further improvements are still required, this study demonstrated the feasibility of particle-tracking-based estimation of shear stress in suspension cultures.

Material And Methods

Maintenance of human iPSCs (hiPSCs)

The HiPSC line, TkDN4-M, was provided by the Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, University of Tokyo, Tokyo, Japan. They were harvested on a tissue-culture-treated 60 mm dish (Iwaki, Shizuoka, Japan) coated with vitronectin fragments and maintained in Essential 8 medium (Thermo Fisher Scientific, Waltham, MA) containing antibiotic-antimycotic (Thermo Fisher Scientific). The culture medium was replaced every 24 h. Calcium- and magnesium-free phosphate-buffered saline (PBS, FUJIFILM Wako Pure Chemical, Osaka, Japan) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA, Dojindo, Kumamoto, Japan) was used for passage of TkDN-4M cells. The number of passages was lower than 50 for experimental use.

Capturing movement of iPSC aggregates in various types of shaking culture

iPSC aggregates were prepared by suspension culture for four days under orbital shaking at 90 rpm. The aggregates were stained with Coomassie Brilliant Blue (Quick-CBB Plus, Fujifilm Wako Pure Chemical). Stained aggregates were suspended in 4 mL of phenol-red-free Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical). The culture vessel was loaded onto the capture system for aggregates under various shaking conditions.

Figure 5 shows an assembly of the capturing system for aggregates in shaking culture. A shaker (CS-LR, Titec, Saitama, Japan) that can switch the shaking operation between orbital and reciprocating was utilized to apply various shaking operations to the culture vessels during the capturing system. A small high-speed camera (Q1m, NAC image technology, Tokyo, Japan) was used to capture the movement of iPSC aggregates in the shaking vessels. As a light source, an LED plate light (TMD100x120-22WD-4, Aitec system, Yokohama, Japan) was placed on the shaker. The aggregates were captured every 1 ms (1000 fps) for 2 s.

Captured aggregate motions were analyzed using a tracking software (MOVIAS Neo, Nac Image Technology, Tokyo, Japan). Aggregate trajectories were captured in the software, and the time profile of acceleration was calculated by the moving average of 100 time points.

Suspension culture of hiPSCs

HiPSCs were dissociated into single cells by soaking in EDTA for 5 min and were collected in Essential 8 medium containing 10 μM Y-27632 (FUJIFILM Wako Pure Chemical). Following the trypan blue extrusion test for cell counting, 8×10^5 cells were seeded in 4 mL of StemFit AK02N medium (final inoculum cell density was 2×10^5 cells/mL) with 10 μM Y-27632 in a low-cell-attachment-treated 6 well plate (Thermo Fisher Scientific). The seeded cells were incubated at 37°C and 5% CO_2 and agitated by orbital shaking or reciprocating at various shaking rates (60, 75, 90, 105, and 120 rpm). After 4 days of culture, the aggregate morphologies were observed by phase-contrast microscopy.

The culture medium was replaced with Y-27632-free Essential 8 medium on day 2. After replacement of the culture medium, the medium was renewed on day 3, and the cells were collected for analyses on day 4. Medium samples were collected on days 2, 3, and 4 for LDH measurements.

Measurement of cell growth and death

After four days of suspension culture, the cells were collected and dissociated into single cells by incubation in TrypLE Express (Thermo Fisher Scientific) for 5 min. Following pipetting for dissociation, the viable cell number was counted using the trypan blue extrusion test with an eosinophil counter.

The number of dead cells was estimated by measuring the amount of LDH released from the cells in the collected medium samples. Medium samples were collected at every medium change on days 2 and 3, and after harvest on day 4. LDH concentrations of the samples were measured using the LDH-Cytotoxic Test Wako (FUJIFILM Wako Pure Chemical) and a fluorescence microplate reader (Arvo-SX, PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. A standard curve was obtained by measuring the amount of LDH from various densities of iPSC suspension (1×10^3, 1×10^4, 1×10^5, 2×10^5, and 3×10^5 cells/mL) treated with phosphate buffered saline containing 0.2% Tween 20.

Declarations

Acknowledgements

This work was financially supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Early-Career Scientists, Grant Number JP18033899. The capture of the motion of aggregates in this study was also technically supported by Susumu Someya and Kensuke Kobori, the technical staff of Nac Image Technology Inc., Tokyo, Japan. Editatge provided by Cactus communications, Tokyo, Japan, proof-readed and grammatical checked the manuscript.

Author contributions

IH contributed to the conception and design of the study, interpretation of data, and preparation of the manuscript and all figures. HN contributed to the design of the study and the acquisition, analysis, and interpretation of the data. YS contributed to the conception and design of the work and interpretation of the data. All authors have reviewed the manuscript.

Data availability statement

The datasets generated during and/or analyzed during current study are available in this article or from the corresponding author on request.

Additional information

Competing interests statement

The authors declare no competing interests.

References

1. Kropp, C., Massai, D. & Zweigerdt, R. Progress and challenges in large-scale expansion of human pluripotent stem cells. Process Biochemistry **59**, 244–254 (2017).
2. Torizal, F. G., Horiguchi, I. & Sakai, Y. Physiological Microenvironmental Conditions in Different Scalable Culture Systems for Pluripotent Stem Cell Expansion and Differentiation. The Open Biomedical Engineering Journal **13**, 41–54 (2019).
3. Torizal, F. G. *et al.* Production of homogenous size-controlled human induced pluripotent stem cell aggregates using ring-shaped culture vessel. Journal of Tissue Engineering and Regenerative Medicine **16**, 254–266 (2022).
4. Horiguchi, I., Suzuki, I., Morimura, T. & Sakai, Y. An orbital shaking culture of mammalian cells in O-shaped vessels to produce uniform aggregates. *Journal of Visualized Experiments* 2019, (2019).
5. Zhan, C. *et al.* Low Shear Stress Increases Recombinant Protein Production and High Shear Stress Increases Apoptosis in Human Cells. iScience **23**, 101653 (2020).
6. Horiguchi, I. & Kino-oka, M. Current Developments in the Stable Production of Human Induced Pluripotent Stem Cells. Engineering **7**, 144–152 (2021).
7. Horiguchi, I. *et al.* Protection of human induced pluripotent stem cells against shear stress in suspension culture by Bingham plastic fluid. Biotechnology Progress **37**, 1–8 (2021).
8. Otsuji, T. G. *et al.* A 3D sphere culture system containing functional polymers for large-scale human pluripotent stem cell production. Stem Cell Reports **2**, 734–745 (2014).
9. Hong, G. R. *et al.* Characterization and Quantification of Vortex Flow in the Human Left Ventricle by Contrast Echocardiography Using Vector Particle Image Velocimetry. JACC: Cardiovascular Imaging **1**, 705–717 (2008).
10. Sengupta, P. P., Pedrizetti, G. & Narula, J. Multiplanar visualization of blood flow using echocardiographic particle imaging velocimetry. JACC: Cardiovascular Imaging **5**, 566–569 (2012).
11. Bonalumi, F. *et al.* Bioengineering a cryogel-derived bioartificial liver using particle image velocimetry defined fluid dynamics. Materials Science and Engineering C **123**, 111983 (2021).
12. Ergin, F. G., Watz, B. B. & Gade-Nielsen, N. F. A review of planar PIV systems and image processing tools for lab-on-chip microfluidics. Sensors (Switzerland) **18**, 1–23 (2018).
13. Odeleye, A. O. O., Marsh, D. T. J., Osborne, M. D., Lye, G. J. & Micheletti, M. On the fluid dynamics of a laboratory scale single-use stirred bioreactor. Chemical Engineering Science **111**, 299–312 (2014).
14. Schroeder, M. *et al.* Differentiation and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. Biotechnology and Bioengineering **92**, 920–933 (2005).
15. Hospital, H. G. & Medical, A. Vascular Wall Shear Stress: Basic Principles and Methods THEODOROS G. PAPAIOANNOU. Hellenic J Cardiol **46**, 9–15 (2005).

16. Ning, L., Betancourt, N., Schreyer, D. J. & Chen, X. Characterization of Cell Damage and Proliferative Ability during and after Bioprinting. ACS Biomaterials Science and Engineering **4**, 3906–3918 (2018).
17. Shi, J. *et al.* Shear stress analysis and its effects on cell viability and cell proliferation in drop-on-demand bioprinting. Biomedical Physics and Engineering Express **4**, (2018).
18. Butler, P. J., Norwich, G., Weinbaum, S. & Chien, S. Shear stress induces a time- and position-dependent increase in endothelial cell membrane fluidity. American Journal of Physiology - Cell Physiology **280**, 962–969 (2001).
19. Butler, P. J., Norwich, G., Weinbaum, S. & Chien, S. Shear stress increases endothelial cell-membrane fluidity. *Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings* **1**, 6 (1999).
20. White, C. R. & Frangos, J. A. The shear stress of it all: The cell membrane and mechanochemical transduction. Philosophical Transactions of the Royal Society B: Biological Sciences **362**, 1459–1467 (2007).
21. Aron, B., Chien, S., Barakat, A. I. & Robert, M. EB2001 symposium report Endothelial cellular response to altered shear stress. 529–533 (2001).

Figures

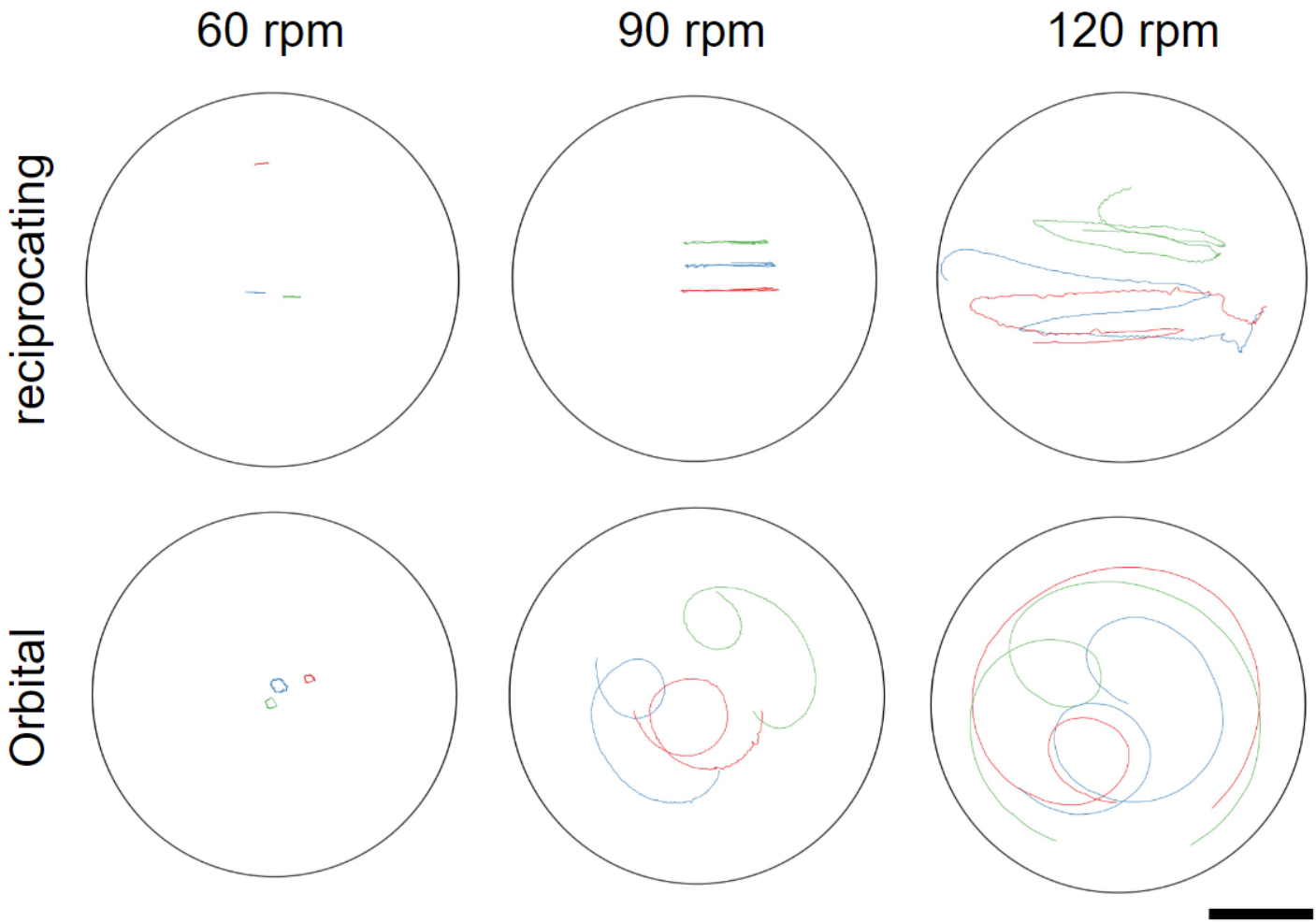

Figure 1

Trajectory of three representative cell aggregates in suspension culture with various shaking conditions for 1 s. Scale bar indicates 10 mm.

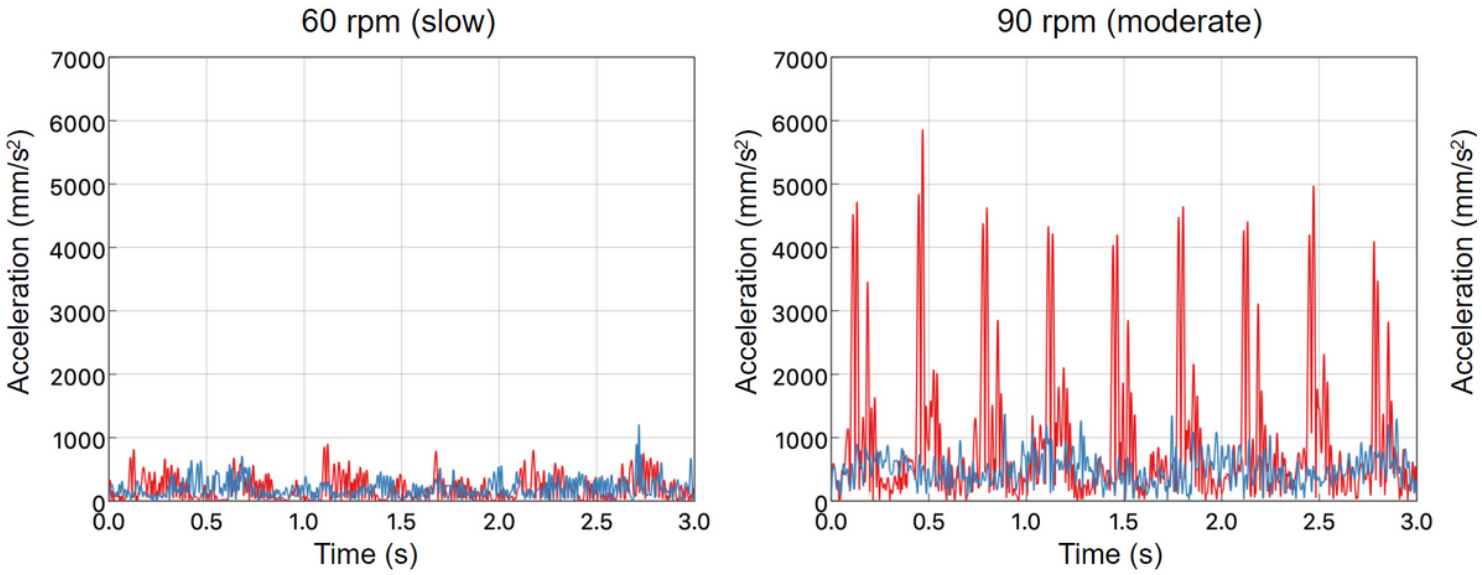

Figure 2

Representative time profiles of the acceleration of the tracked aggregates in shaking vessels with various shaking conditions. Red line: acceleration in reciprocating vessel; blue line: acceleration in orbital shaking.

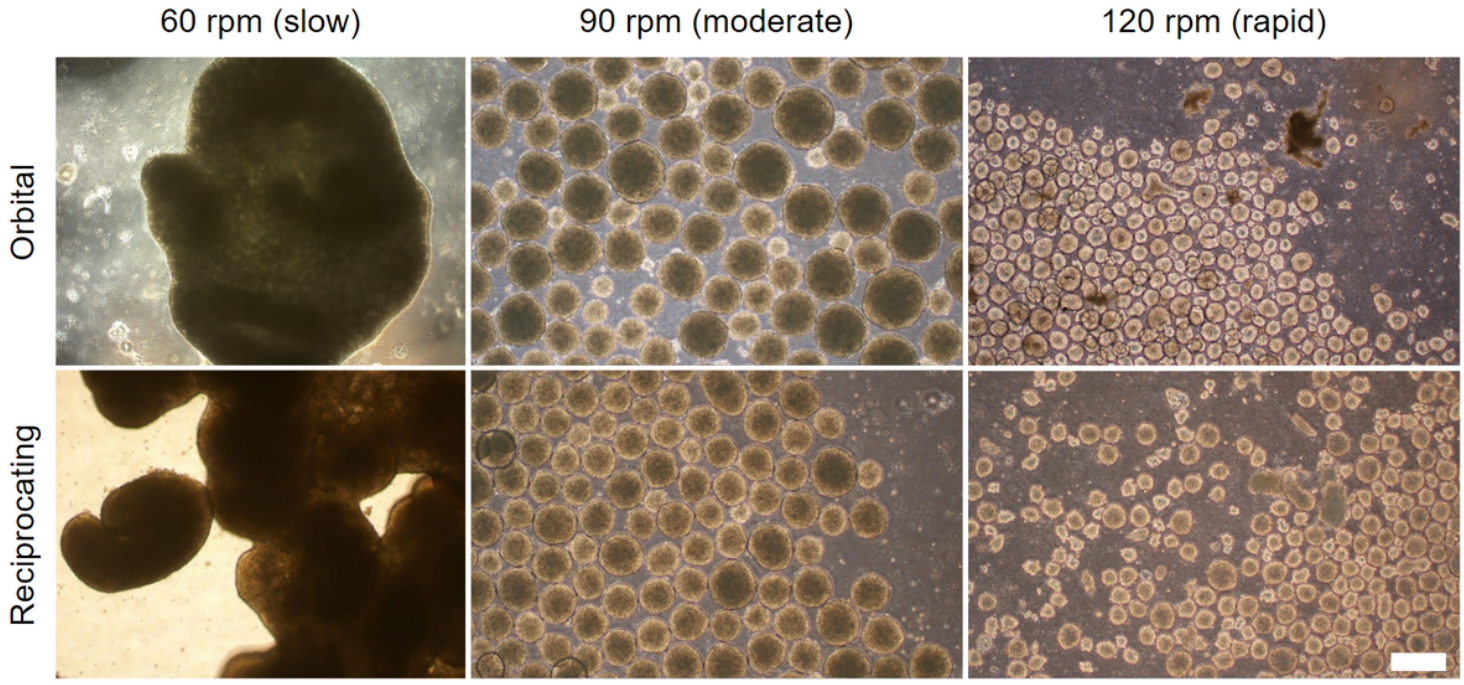

Figure 3

Morphologies of aggregates after suspension culture with various shaking methods and rates for 4 days. Scale bar represents 400 μm.

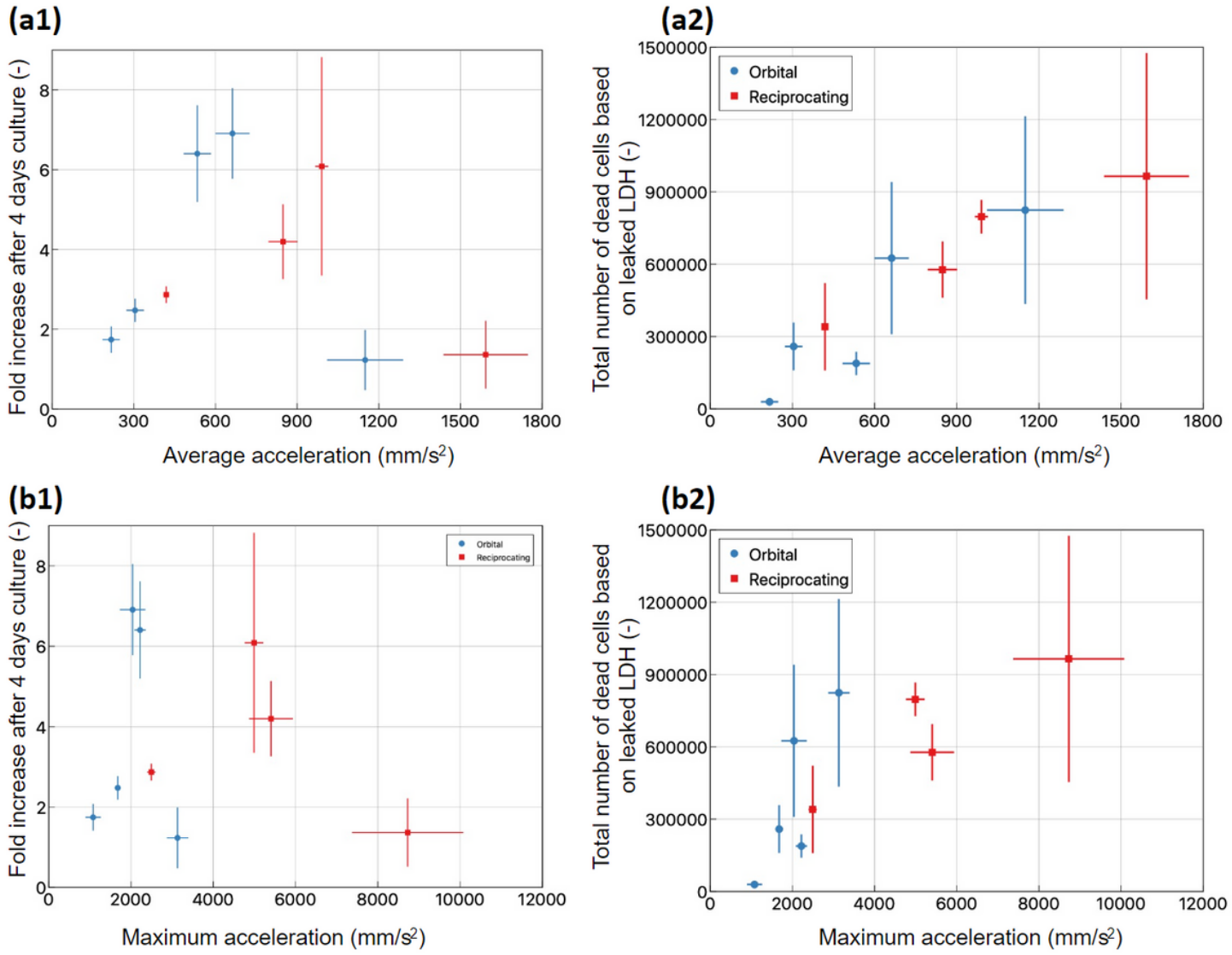

Figure 4

Correlation between the cell growth/death and average/maximum acceleration. (1) Fold increase after 4 days suspension culture as an indicator of growth, (2) total number of dead cells during 4 days suspension culture estimated by the amount of leaked LDH. These data were arranged by (**a**) average acceleration per second and (**b**) maximum acceleration during measurement. Error bars indicate S.D. n = 6.

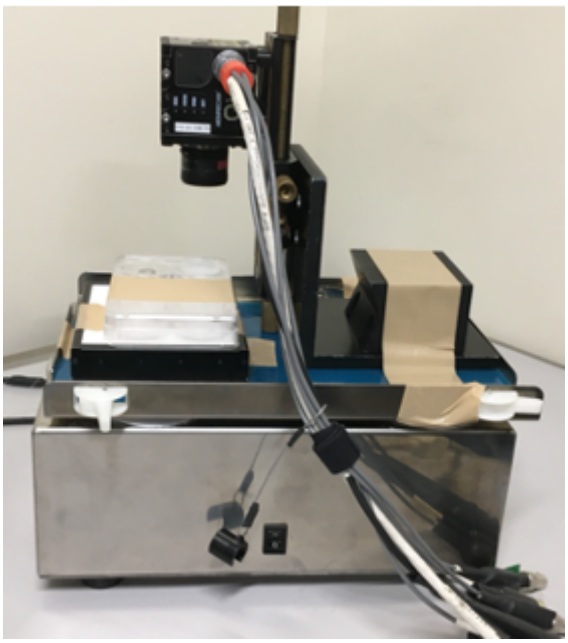

Figure 5

Experimental setup for capturing the trajectory of aggregates in a shaking vessel.