

Stem Cell Research

Considerations for efficient scale-up of PSCs in suspension culture

Keywords

Cell therapy, downstream workflows, large-scale expansion, scale-up, small-scale expansion, spheroids, suspension culture

Summary

Here we highlight some key considerations for pluripotent stem cell (PSC) scale-up in suspension culture systems. Since suspension culture systems require PSCs to float in a three-dimensional (3D) environment, these culture systems behave differently than adherent culture systems. Consequently, the transition from adherent to suspension culture can be a challenge for some researchers. In order to ease this transition, we will discuss some useful strategies to employ when starting a new PSC line in a suspension culture. We start by covering the most common suspension culture formats used to nucleate and grow 3D PSC spheroids. Next, we examine experimental variables that can be controlled to advantageously manipulate spheroid growth and size. These include parameters like culture vessel volume, cell seeding density, and shake/agitation speed. In addition, efficient cell passaging strategies for PSC spheroid dissociation will also be discussed.

Introduction and benefits

Over the years, the use of PSCs in therapeutic and screening applications has become an important consideration within the stem cell field [1]. Accordingly, large numbers of high-quality PSCs must be generated for these downstream applications. For example, to yield sufficient quantities of beta islets [2] or cardiomyocytes [3], an individual patient will require billions of differentiated stem cells. Many researchers attempt to achieve these cell numbers by leveraging traditional two-dimensional (2D) adherent cultures due to the familiarity and ease of use of these cultures. Importantly, although scaling up through the use of adherent PSC cultures can be accomplished, there are several drawbacks to this approach.

The main limitation is the 2D surface area required for PSC expansion. As PSCs continue to expand in adherent cultures, they require increasingly large surface areas to support their growth. While methods exist to mitigate this large surface area requirement, such as the Thermo Scientific™ Nunc™ Cell Factory™ Systems, researchers are subsequently limited by the number of vessels that appropriately fit into a single incubator [4]. Consequently, scaling up in adherent cultures requires a significantly large footprint if cell numbers in the billions are to be achieved.

Therefore, in order to avoid the restrictions resulting from the large surface area and footprints faced in adherent workflows, culturing PSCs in suspension becomes a preferable method for cell number expansion and scale-up [5]. Growing PSCs in 3D suspension culture vessels overcomes surface area limitations by utilizing the full volume of the culture vessel. This enables suspension cultures to be seeded at higher densities than adherent cultures, allowing for greater numbers of cells to be generated in a shorter amount of time. Furthermore, compared to adherent cultures, this significantly reduces the plastic consumables required to achieve a density of billions of cells in suspension cultures (Figure 1).

In addition to reducing plastic consumables, suspension cultures also demonstrate greater efficiency with regard to the number of cells generated per milliliter of the medium. For example, assuming daily medium exchange with Gibco™ StemScale™ PSC Suspension Medium, a 3 L bioreactor requires up to 10 L of culture medium to grow approximately 4.5 billion cells (Figure 1). Accordingly, this results in 450,000 cells generated per milliliter of StemScale medium in a suspension culture system. In contrast, adherent culture systems that utilize multiple 10-stack Nunc Cell Factory System require up to 18 L of Gibco™ StemFlex™ Medium

to grow the same cell yield (Figure 1). Accordingly, this results in 250,000 cells generated per milliliter of StemFlex medium in an adherent culture system, indicating suspension culture systems can support greater cell densities when scaling up to large cell yields. Overall, due to consumption of less media and plastics, suspension culture protocols can be developed to reduce cost over time [6].

Suspension culture systems offer additional PSC expansion benefits that, compared to adherent culture workflows, are much easier to employ. For instance, adherent cultures produced through Nunc Cell Factory Systems are labor intensive and can introduce the potential for contamination when harvesting cells. However, large-scale stirred bioreactors utilized in suspension culture maintain a closed environment and can enable users to take advantage of automated cell processing techniques to reduce manual labor. Additionally, stirred bioreactors enable daily sampling to monitor spheroid growth and culture health. Nunc Cell Factory Systems, in contrast, render only the bottom layer visible under a microscope, thus limiting the ability to assess cell growth throughout subsequent Nunc Cell Factory Systems layers.

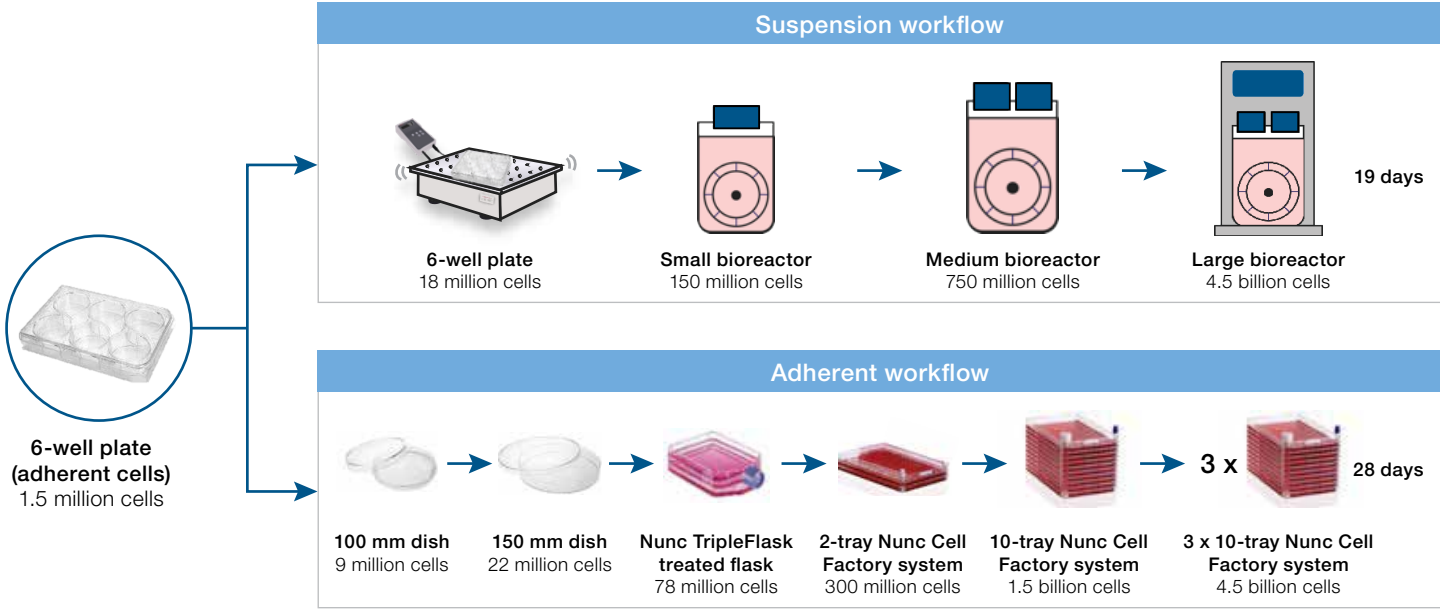


Figure 1. Estimation of the time required to generate 4.5 billion cells in adherent versus suspension culture systems.

Notably, suspension cultures are easily scalable across multiple types of vessel formats (Figure 2A). Small-scale suspension cultures can be grown in 6-well plates or shake flasks on an orbital shaker platform, while large-scale suspension cultures can be grown in spinner flasks or stirred bioreactors. Additionally, while overall performance in suspension cultures is cell line–dependent, cell growth rates are consistent between passages for established suspension cultures grown in StemScale medium (Figure 2B).

In the following sections, we present technical considerations for the scale-up of suspension cultures. In particular, we focus on utilizing spheroid cultures grown in StemScale medium for rapid scale-up. Spheroid culture workflows are relatively easy to grasp, allowing even inexperienced researchers to learn the basics of suspension culture quickly. Since PSC spheroids are formed from the spontaneous nucleation of single cells into larger aggregates, no additional components (such as microcarriers) are required to initiate spheroid suspension cultures. Furthermore, passaging of spheroids is limited only by their overall size, meaning a flexible passaging schedule is easy to maintain.

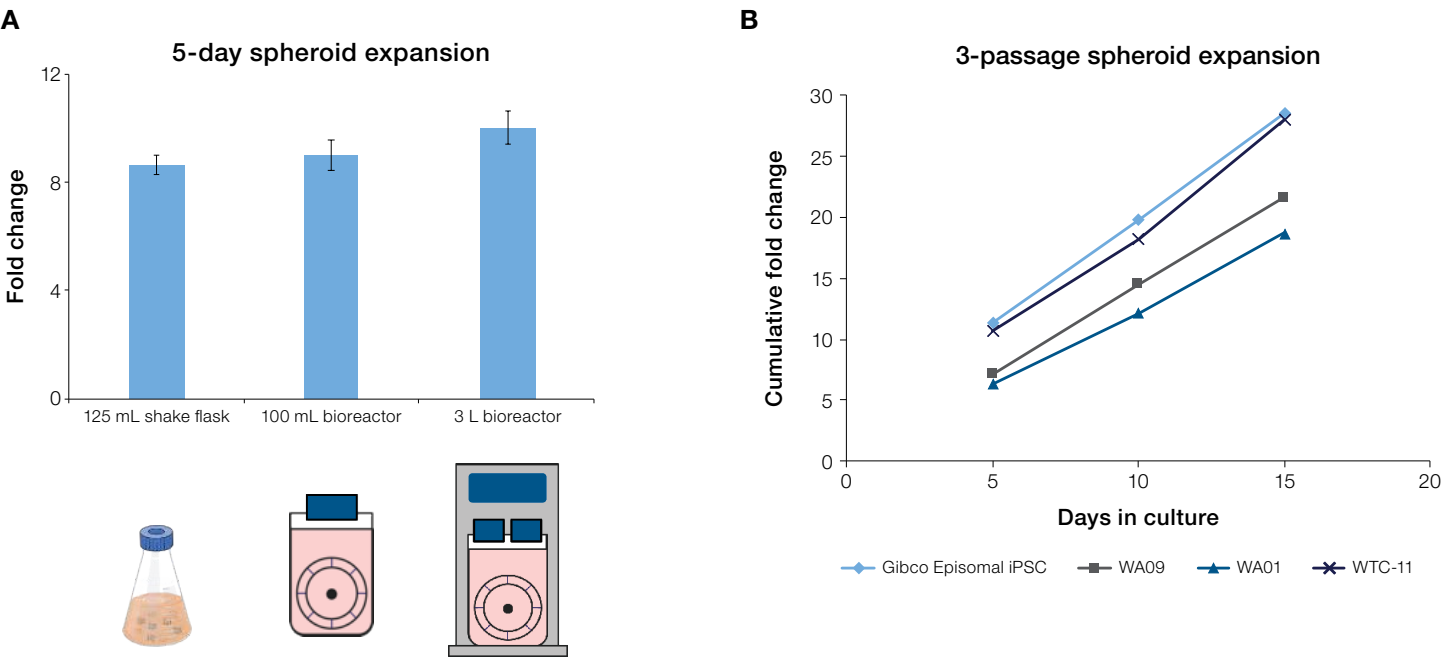


Figure 2. Suspension culture systems enable consistent spheroid growth across multiple vessel formats and different cell lines, from induced pluripotent stem cells (iPSCs) to embryonic stem cells (ESCs).

Suspension culture vessels and shear force

One of the more significant factors affecting growth of PSC suspension cultures is the shear force typically caused by the fluid dynamics within a suspension culture vessel [7]. Due to the need for constant agitation, the fluid inside a suspension culture vessel is perpetually in motion. The relative movement of these fluid particles induces shear forces, which in turn act upon spheroids at notably different vectors. Importantly, the dynamics of shear force vary depending on the type of suspension culture vessel utilized. Orbital shaker platform cultures, for example, maintain a significantly different shear stress environment than stirred-tank bioreactors.

Since shear forces are caused by the fluid dynamics within a vessel, the volume of fluid becomes an important governing factor of shear forces. In particular, suspension culture vessels placed on orbital shaker platforms must have a precise volume to support a consistent suspension culture environment. Due to movement of the orbital shaker platform, these vessels experience a swirling motion that moves fluid around the vessel walls. The range of motion of these fluids varies greatly depending on the volume inside the vessel. To ensure consistent spheroid agitation, the fluid fill within the orbital shaker platform cultures must be optimal to ensure that spheroids experience a full range of motion around the vessel (Figure 3A). If the volume is too high, fluid at the vessel's center will experience little-to-no movement, therefore preventing spheroids from being fully maintained in suspension (Figure 3B).

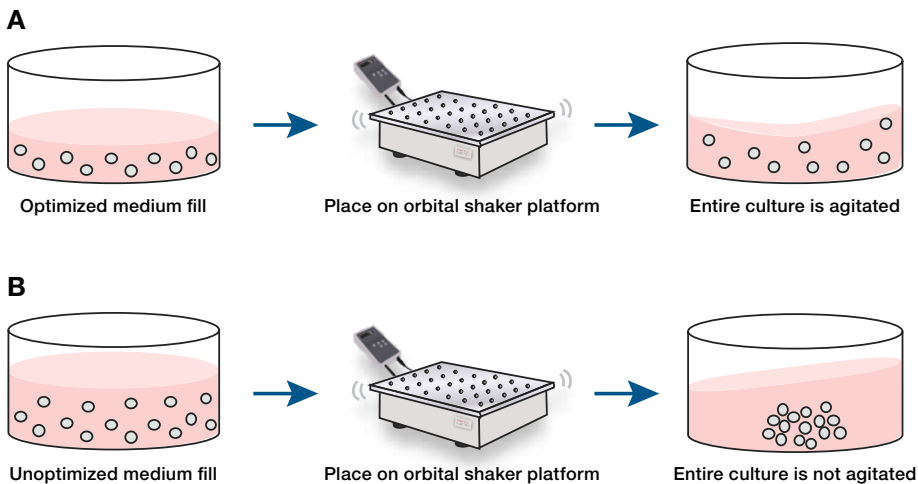


Figure 3. Medium volume and agitation speed play a significant role in orbital shaker platform suspension cultures.

Stirred-tank bioreactors are commonly utilized at the liter-scale due to their many advantages. However, there are some potential drawbacks to the horizontal-blade impeller design that these particular bioreactors employ. As the size of the stirred-tank bioreactor increases, shear stress applied to spheroids by the horizontal-blade impeller will also increase [8], particularly at the impeller tip (Figure 4A). This makes it difficult to ensure a consistent suspension culture environment for spheroids while scaling up, potentially causing issues for culture expansion within the largest bioreactors. Unfortunately, this elevated shear stress environment may even prevent sensitive PSC lines from nucleating into spheroids.

Alternatively, one might consider utilizing a different impeller design during expansion to mitigate the high shear stress encountered in a stirred-tank bioreactor. Unlike the horizontal-blade design, a vertical-wheel impeller design minimizes the shear stress that spheroids will be exposed to in suspension [9]. As such, due to the nature of the vertical-wheel design, hydrodynamic forces in the vessel are more evenly distributed and lead to an overall lower shear stress environment (Figure 4B). Minimization of shear stress ensures that spheroids expand as viable aggregates.

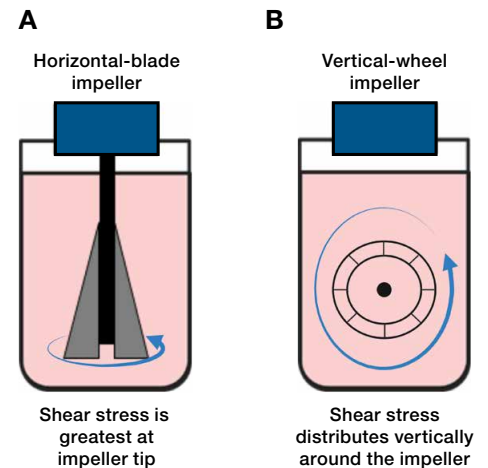


Figure 4. Horizontal-blade impeller versus vertical-wheel impeller.

Suspension culture seeding density

An experimental variable that is easy to manipulate and that readily improves suspension culture yields is the initial seeding density. For spheroids grown in StemScale medium, an initial seeding density of 150,000 cells/mL when initiating suspension cultures in the presence of a ROCK inhibitor is recommended [10]. Due to the number of nucleated spheroids present in the culture, this seeding density enables high cell yields while allowing medium exchanges to be performed at every-other-day intervals. However, some sensitive PSC lines may have difficulty nucleating into spheroids. For these sensitive lines, the initial seeding density can be increased above 150,000 cells/mL to account for potential challenges encountered when nucleating spheroids. By increasing the seeding density, a greater number of spheroids are likely to nucleate due to the larger number of cells present, making it easier for sensitive PSC lines to expand in suspension culture.

Although increasing seeding density is a useful tool for improving cell yields, seeding density must not be increased too drastically. The increased number of nucleated spheroids may increase the incidence of random spheroid fusion, subsequently resulting in a wider range of spheroid sizes within the suspension culture [11]. Furthermore, a user may encounter difficulty maintaining both the pluripotency and survival of PSC spheroids if too many are present in a suspension culture [12]. Increasing the suspension culture seeding density elevates the number of nucleating spheroids, meaning a greater number of cells are expanding in the culture. Due to this greater increase in overall cell number, the medium is consumed at an accelerated rate, in turn requiring more frequent medium exchanges to

compensate. Therefore, while flexibility in feeding schedule exists for spheroids grown in StemScale medium at densities below 150,000 cells/mL, daily medium exchanges are necessary when seeding spheroids at densities between 150,000 cells/mL and 300,000 cells/mL. It is not recommended to seed suspension cultures at densities higher than 300,000 cells/mL, as the culture medium will be consumed too rapidly to ensure cell survival and the maintenance of pluripotency.

Notably, seeding density can also be increased (within suggested guidelines) if suspension cultures are initiated from cells removed from cryopreservation. When using StemScale medium, spheroids can be nucleated from thawed PSCs seeded directly into suspension culture vessels. It is important to note that these thawed cells typically experience some difficulty when first nucleating into spheroids [13]—oftentimes resulting in the formation of significantly fewer spheroids during the first passage post-cryopreservation. Beyond this initial passage, cells recover and are readily capable of spheroid nucleation for subsequent passages. Importantly, increasing seeding density during the initial recovery passage enables a greater number of spheroids to nucleate, despite the difficulties encountered immediately following cryopreservation. As a result, this ensures that sufficient quantities of spheroids are present to continue suspension culture through subsequent passages. Additionally, pairing this strategy with other methods, such as increasing the ROCK inhibitor concentration during the spheroid nucleation phase [10], further ensures that thawed PSCs easily form spheroids following cryopreservation.

Medium exchange strategies

Performing medium exchanges on suspension cultures may seem problematic at first due to the presence of suspended spheroids. However, by removing the vessel from agitation, spheroids will no longer remain in suspension, and instead begin to settle via gravity sedimentation within 5 minutes of the vessel being removed from the source of agitation (Figure 5). This causes spheroids to collect at the bottom of the suspension culture vessel. At this point, spent medium in the suspension culture vessel can be carefully aspirated from the top of the vessel. When growing spheroids in StemScale medium, removing at least 50% of the spent medium every other day is recommended to ensure that suspension cultures remain highly viable. Optionally, medium replacement can occur more frequently (i.e., daily), or with a replacement volume greater than 50%. Once spent medium has been removed, it should be replaced with an equal volume of fresh medium. It is important to ensure that equal amounts of medium volumes are utilized for removal and addition in order to maintain a constant volume within the culture vessel. Provided the volume is constant, shear

forces will also remain consistent within the suspension culture vessel, ensuring PSC spheroids remain viable. Once the medium exchange is complete, the suspension culture vessel should be placed back onto the source of agitation to ensure that spheroids are quickly resuspended.

Medium exchanges should be performed rapidly to prevent spheroids from fusing while settled at the bottom of the suspension culture vessel [7]. To prevent undesirable spheroid aggregation, it is recommended that spheroids do not remain sedimented for more than 15 minutes (Figure 6). Beyond the 15-minute mark, smaller spheroids will adhere to one another, even when reintroduced to agitation. Upwards of 30 minutes, large spheroids will also remain in a state of unwanted adherence. Both orbital shaker platform and small spinner flask cultures can have medium exchange performed within this 15-minute window without any negative consequences. For large bioreactor systems, however, preventing unwanted aggregation can be a considerable challenge.

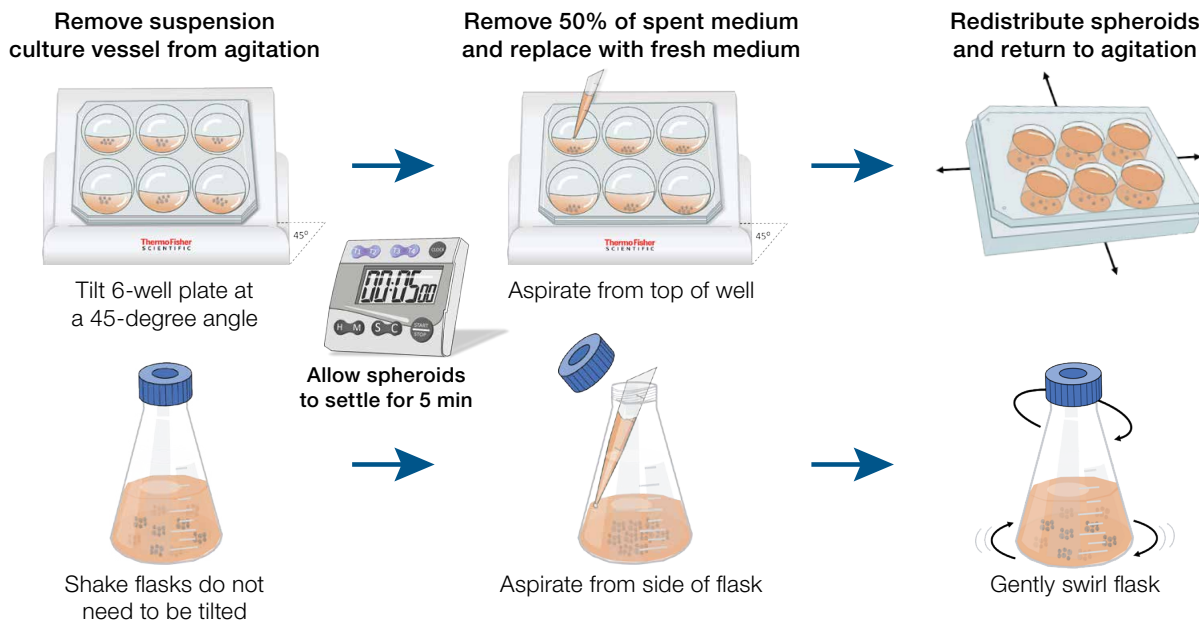


Figure 5. Suspension culture feeding protocol via gravity sedimentation of spheroids for 5 min.

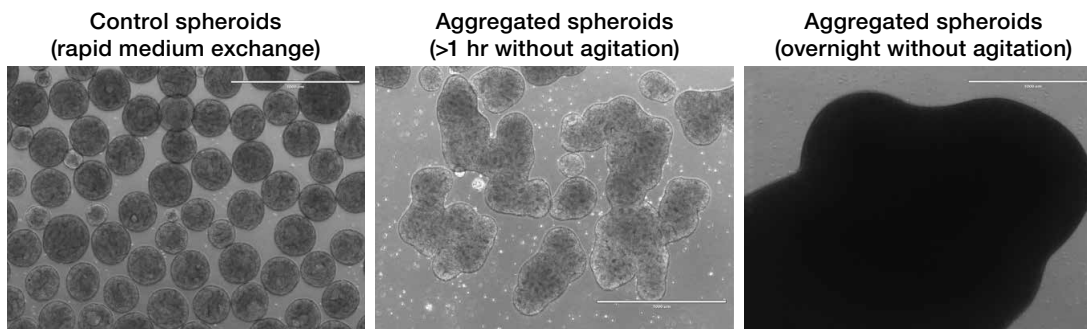


Figure 6. Spheroids removed from agitation for extended periods of time may aggregate into large clumps.

Researchers utilizing large bioreactor systems (i.e., >3 L volumes) often have difficulty performing a 50% medium replacement in a brief period of time due to the medium exchange volumes required at these scales. In these large bioreactor systems, several options may be considered to ensure that spheroids do not fuse during medium changes. The first is to perform the medium exchange in a stepwise manner. This can be accomplished by temporarily returning cultures to the source of agitation halfway through the 50% medium exchange to briefly resuspend spheroids (Figure 7). By initially removing 25% of the spent medium and resuspending spheroids, it is possible to minimize the adhesion of newly fused PSC spheroids. Spheroid resuspension will occur within 5–10 minutes of

reinitiating agitation. These resuspended spheroids can then be settled via 5-minute gravity sedimentation again, at which point the remaining 25% of the spent medium can be removed. Another method to prevent spheroid fusion is to use an external peristaltic pump capable of rapidly aspirating the spent medium. Oftentimes, built-in peristaltic pumps on bioreactor units may not be capable of achieving the pump speeds required for rapid medium aspiration. To work around this issue, external peristaltic pump sources can be utilized to achieve the necessary aspiration speed. Lastly, researchers may choose to utilize a constant perfusion system [14]. In this modality, there is no need to settle spheroids for removing medium, thus eliminating concerns surrounding undesirable spheroid aggregation.

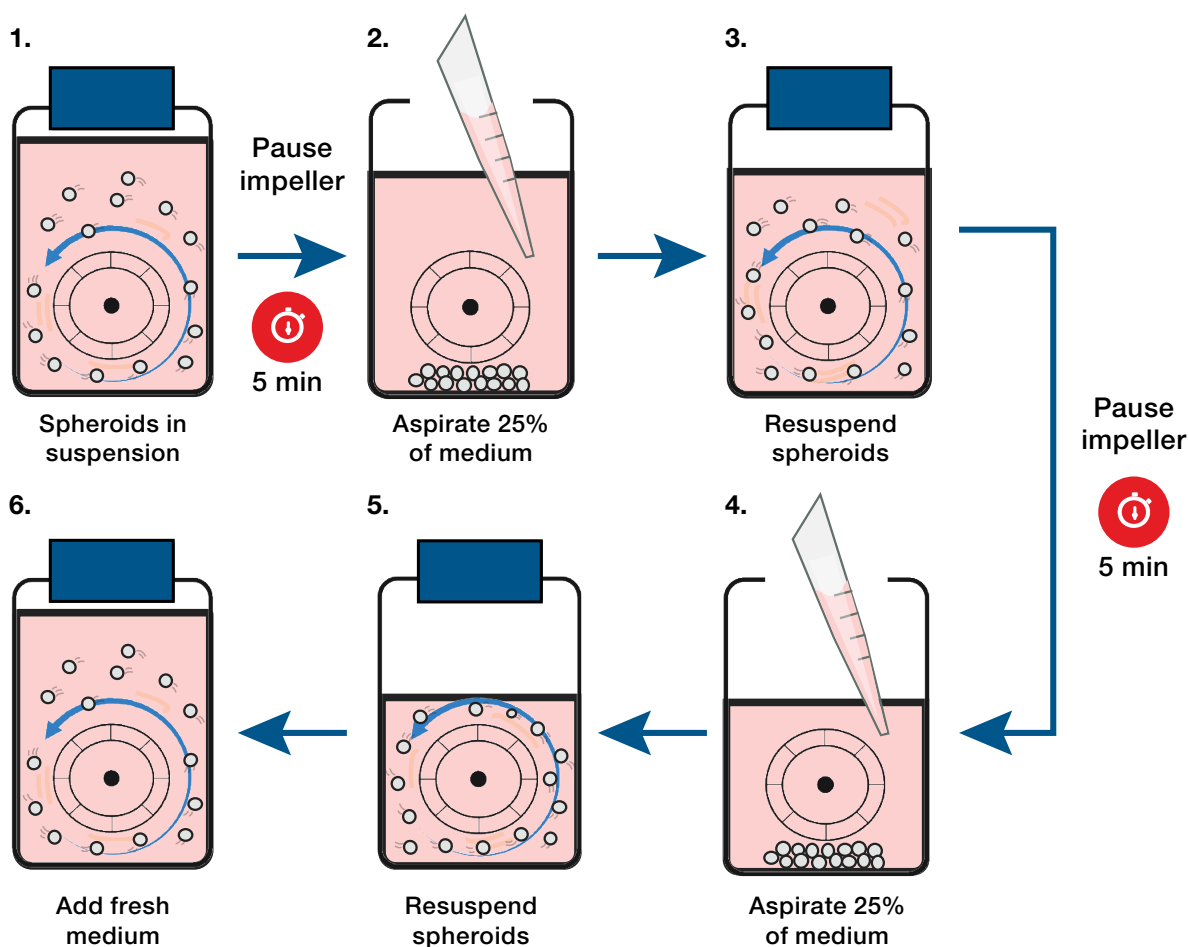


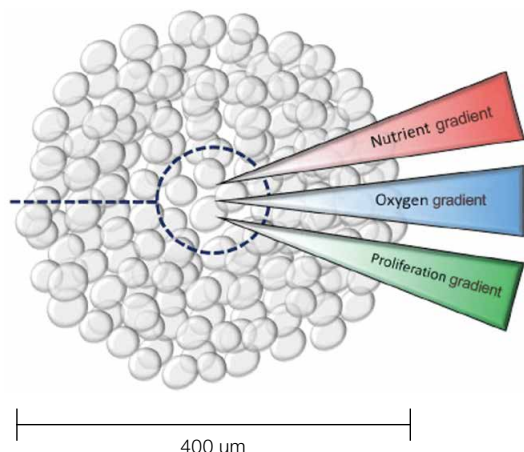
Figure 7. Temporary resuspension of spheroids while feeding liter-scale bioreactors can minimize potential spheroid aggregation issues.

Suspension culture passaging strategies

Once spheroids reach a certain size, they are ready to be passaged. Importantly, spheroids should be passaged before the formation of a necrotic core, as this will negatively impact overall cell viability following spheroid dissociation [15]. Passaging should occur once spheroids reach 300–400 μm in diameter (Figure 8). This optimal size is typically achieved within 4–5 days of suspension culture. Passaging spheroids at sizes less than 400 μm in diameter will consistently minimize the formation of a necrotic core.

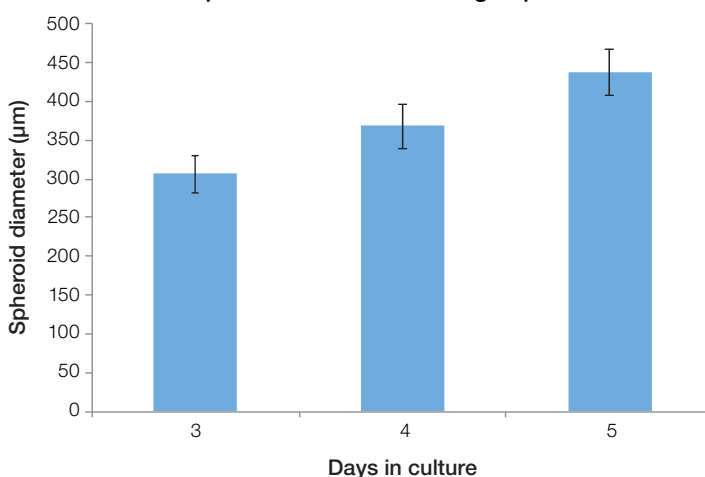
Spheroids are easily passaged into single cells by using the Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Figure 9). Once spheroids are suspended in StemPro Accutase reagent, they are subsequently placed in a 37°C water bath to promote dissociation. Of note, spheroids should be gently agitated every 2–3 minutes to assess the level of dissociation. As more spheroids become dissociated over time, the StemPro Accutase reagent will become cloudier. Gentle agitation of the tube will resuspend and further promote dissociation of the spheroids.

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Spheroid diameter during expansion



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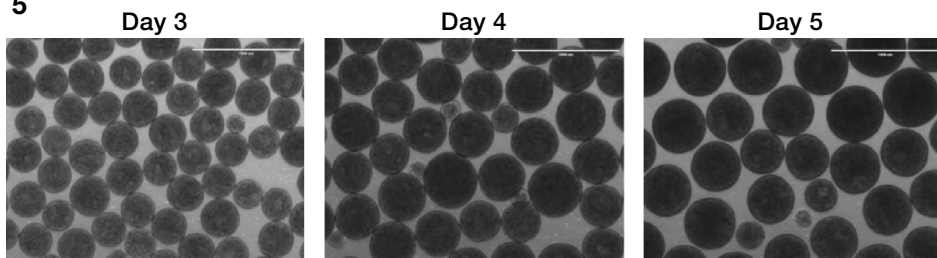


Figure 8. Spheroids are ready to be passaged after 4–5 days of growth, when the average spheroid diameter reaches 300–400 μm in size.

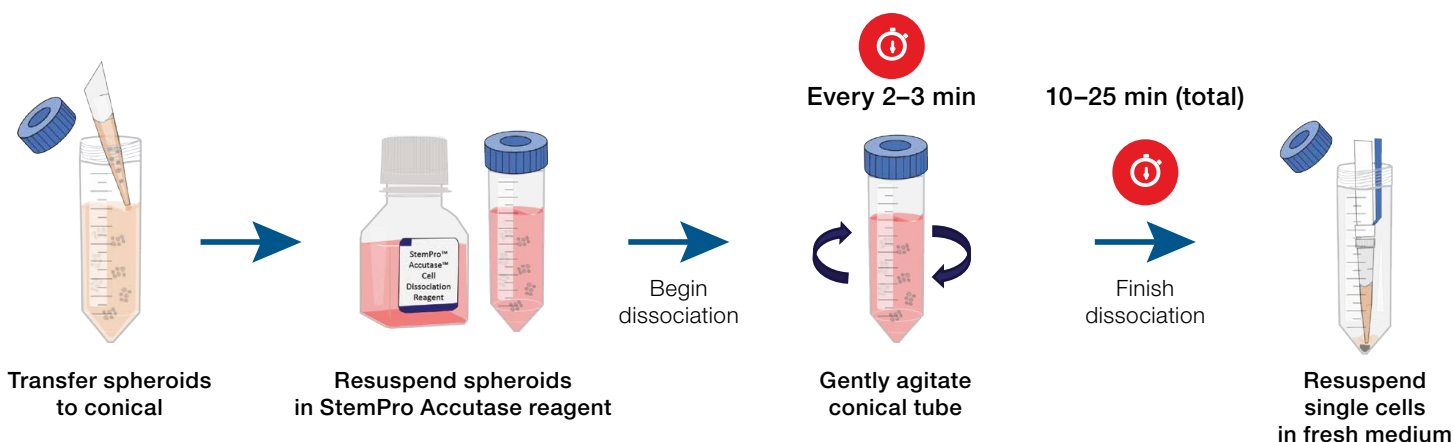


Figure 9. Dissociation of spheroids into single cells by use of StemPro Accutase reagent.

The amount of time that spheroids require for dissociation depends upon the total number of spheroids present. Greater numbers of spheroids require extended exposure to StemPro Accutase reagent to ensure complete dissociation. In orbital shaker platform cultures, spheroids can be dissociated within 10–15 minutes. Spinner flask cultures may require 15–20 minutes for dissociation. At large-scale bioreactor cultures (i.e., >3 L volumes), however, up to 25 minutes may be required for adequate spheroid dissociation. Accordingly, the volume of StemPro Accutase reagent should be increased to ensure that the entire spheroid pellet remains fully immersed in dissociation reagent (Table 1).

Table 1. Dissociation using StemPro Accutase reagent in multiple vessel formats.

Vessel format	StemPro Accutase reagent volume	Dissociation time
6-well plate	1 mL per well	10 min
125 mL shake flask	5 mL per flask	10–15 min
250 mL shake flask	10 mL per flask	15 min
500 mL shake flask	20 mL per flask	15–20 min
100 mL bioreactor	15 mL per vessel	15–20 min
500 mL bioreactor	30 mL per vessel	20 min
3 L bioreactor	80 mL per vessel	25–30 min

Cell therapy workflow considerations for suspension culture

There are several important considerations to make when planning for the transition of suspension cultures from a research use only (RUO) workflow to cell therapy use. For example, PSC spheroids cultured in a cell therapy-compatible medium may experience a reduction in growth rates and/or spheroid nucleation, due to formulation changes intended to reduce risk and minimize the use of animal products. If reduced growth is apparent, these difficulties can be suitably mitigated by simple protocol modifications. For example, increasing the seeding density from 150,000 cells/mL to 200,000 cells/mL ensures that a greater number of spheroids nucleate in a cell therapy-compatible medium. Furthermore, extending culture duration from 4–5 days to 5–6 days offers spheroids an additional day to expand. These modifications ensure that cell therapy-compatible cultures maximize cell yields while minimizing protocol modification efforts.

Another important consideration associated with cell therapy is the dissociation reagent utilized when passaging spheroids. StemPro Accutase reagent is an RUO-compatible dissociation reagent, and therefore cannot be utilized in cell therapy workflows. Instead, Gibco™ Cell Therapy Systems™ (CTS™) TrypLE™ Select Enzyme is recommended, which is intended to support transition from the research bench to the clinic. For this reason, CTS TrypLE Select Enzyme can be utilized for dissociating spheroids into single cells.

CTS TrypLE Select Enzyme dissociates spheroids into single cells in a similar amount of time as the StemPro Accutase reagent. However, the CTS TrypLE Select Enzyme is far more potent than StemPro Accutase reagent during the spheroid dissociation step, often resulting in cellular lysis. Consequently, difficulty may be encountered when nucleating spheroids digested in CTS TrypLE Select Enzyme due to the presence of genomic DNA (gDNA) released from these lysed cells (Figure 10). To avoid this issue, one should supplement suspension cultures with small quantities of DNase I. The presence of DNase prevents gDNA from interfering with the spheroid nucleation process.

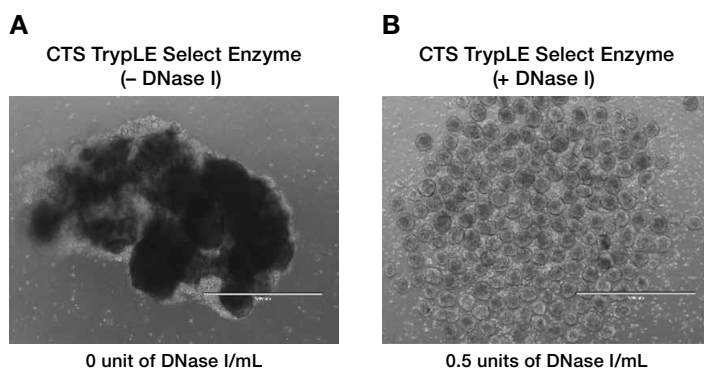


Figure 10. gDNA from cells lysed using CTS TrypLE Select Enzyme can interfere with spheroid nucleation.

Lastly, many cell therapy-compatible suspension cultures necessitate the use of closed-system environments. Typically, large-scale bioreactors employ entirely closed systems, thus requiring a method to dissociate spheroids from within these closed-system environments. While spheroid dissociation in a water bath is simple to perform, it increases the risk of contamination of cells and is therefore not ideal for maintaining closed systems. Instead, automated systems can be utilized to maintain and process spheroids in a closed environment and minimize contamination risk. Along these lines, automated spheroid dissociation is possible through the use of the Thermo Scientific™ Rotea™ Counterflow Centrifugation system. The Rotea system maintains spheroids in a closed system while promoting their rapid dissociation. Currently, the Rotea system can process the cell yields grown in a wide range of vessel sizes, from 100 mL spinner flasks up to 3 L bioreactors.

Due to the nature of counterflow centrifugation, spheroids dissociate more rapidly in the Rotea system as compared to the water bath method (Table 2). Importantly, DNase I is still recommended during Rotea system-associated dissociation with CTS TrypLE Select Enzyme, in order to prevent undesired aggregation of spheroids from gDNA during the nucleation phase of downstream suspension cultures.

Table 2. The Rotea Counterflow Centrifugation System promotes rapid dissociation of spheroids.

500 mL bioreactor dissociation format	Dissociation time	StemPro Accutase reagent volume	Estimated total cell yield
Rotea Counterflow Centrifugation System	6 min	20 mL	750 million cells
37°C water bath	20 min	30 mL	750 million cells

Closing thoughts

Overall, suspension culture systems are a useful method for researchers aiming to scale up to large quantities of PSCs in a short amount of time. The spheroids expanded in suspension culture can then be utilized for a variety of downstream applications, such as PSC differentiation. In fact, 3D spheroid differentiation often enables researchers to save additional time during their downstream research workflows. Instead of dissociating spheroids into single cells, replating them into well plates, and then initiating differentiation in adherent culture, 3D spheroids can be directly differentiated as aggregates. While some protocols for suspension culture differentiation require optimization, current protocols for 3D neural and cardiomyocyte differentiations can be found in application notes at thermofisher.com/stemscale.

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