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Cell therapy development

Rapid dissociation of PSC spheroids in a closed environment using the CTS Rotea system

Introduction

When scaling up to large quantities of pluripotent stem cells (PSCs), it is important to consider how these cells will be processed and utilized in downstream studies. For clinical applications, this often means strict protocols that differ from standard methods to minimize errors during PSC handling. One such example involves the use of a water bath to dissociate PSC aggregates grown in suspension culture. These aggregates include spheroids or embryoid bodies ranging from 300 to 400 µm in diameter. Although the water bath dissociation method is simple to utilize, the risk of contamination is a significant disadvantage. A preferable dissociation method would eliminate the use of a water bath, allowing PSC spheroid dissociation to be maintained in an entirely closed-system environment. Therefore, most clinical applications would benefit from a method to efficiently dissociate cell cultures without placing them inside a water bath. Here we demonstrate how the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System can be utilized for the rapid dissociation of PSC spheroids while maintaining them within a closed-system environment.

Methods

Spheroids utilized in this study were grown in Gibco™ StemScale™ PSC Suspension Medium. Refer to thermofisher.com/stemscale for details on how to establish suspension cultures in StemScale medium. Once the average spheroid diameter of a suspension culture was between 300 and 400 µm in size, the spheroids were collected and transferred into a 600 mL transfer bag. Additional 600 mL transfer bags were filled with the appropriate volumes indicated in Figure 1. All transfer bags were then connected to the CTS Rotea system. Using the spheroid dissociation protocol described in Table 1, the spheroids were dissociated into single cells and harvested for cell counting. The resulting single cells were then reseeded into new suspension cultures.

The spheroid dissociation protocol described in Table 1 can be summarized as follows:

1. Initiate priming steps.
2. Load spheroids to create fluidized cell bed.
3. Wash spheroids with PBS.
4. Add Gibco™ StemPro™ Accutase Cell Dissociation Reagent to spheroids.
5. Dissociate spheroids during the pause loop on the CTS Rotea system.
6. Wash single cells with PBS.
7. Add fresh medium to single cells.
8. Harvest single cells.

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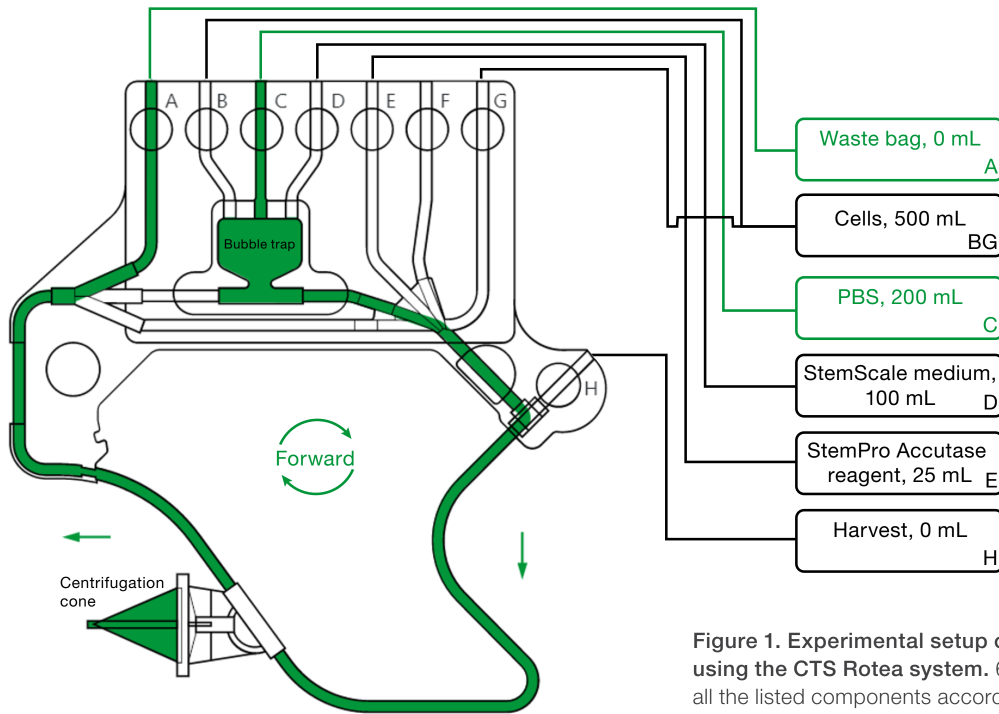

Figure 1. Experimental setup of the spheroid dissociation protocol using the CTS Rotea system. 600 mL transfer bags are used to store all the listed components according to the volumes in this image.

Table 1. Spheroid dissociation protocol using the CTS Rotea system.

Step*	Description	Flow path	Centrifugation force (x *g*)	Pump (mL/min)	Step type	Trigger(s)
Priming sequence						
1	Pre-prime	C to A	0	100	Normal	Bubble sensor input start of flow (dry to wet)
2	Lubricate rotary coupling	C to A	0	100	Normal	Volume (30 mL)
3	Prime chamber and line A	C to A	10	100	Normal	Volume (30 mL)
4	Prime bubble trap and line B	A to B	10	100	Normal	Volume (15 mL)
5	Prime line D	A to D	10	100	Normal	Volume (5 mL)
6	Prime line E	A to E	10	100	Normal	Volume (5 mL)
7	Pressure prime	A to F	10	0	Pressure prime	—
8	Prime pause	J to K	10	40	Pause	Timer (5 sec)
9	Ramp speed to initiate bed	J to K	75	80	Pause	Timer (10 sec)
Loading, washing, and dissociating the spheroids						
10	Initiate bed	B to G	75	80	Normal	Timer (60 sec)
11	Load input material	B to A	75	80	Normal	Volume or timer**
12	Wash	C to A	75	40	Normal	Volume (30 mL)†
13	Add StemPro Accutase reagent	E to A	75	40	Normal	Volume (20 mL)†
14	Dissociate spheroids	J to K	75	40	Pause	Timer**
15	Concentrate single cells	J to K	650	15	Pause	Timer (10 sec)
16	Wash	C to A	650	15	Normal	Volume (30 mL)†
17	Add fresh medium	D to A	650	15	Normal	Volume (10 mL)†
18	Concentrate bed for harvest	J to K	800	15	Pause	Timer (20 sec)
19	Harvest	D to H	600	15	Harvest	Volume**

* The steps listed in this protocol assume a suspension culture vessel size of 500 mL and an average spheroid diameter between 300 and 400 µm. Modifications to flow rate and centrifugation speed may be necessary when using suspension culture vessels or spheroids of different sizes. Furthermore, the dissociation time for StemPro Accutase reagent will be dependent on both the specific cell line and the number of spheroids present inside the centrifugation cone of the CTS Rotea system. For initial evaluations, it is useful to start with dissociation times between 5 and 10 min.

** Trigger defined according to the requirements of the user.

† The volumes listed here will work for larger vessels, but they can still be increased as necessary.

Results

Figure 2A shows the single-cell yields obtained from dissociating spheroids grown in StemScale medium and harvested from a 100 mL suspension culture vessel. These spheroids were dissociated in both the CTS Rotea system and a water bath once the average spheroid size was approximately 300 to 400 µm in diameter. The single-cell output was found to be comparable between the CTS Rotea system (9-fold expansion) and the water bath (10.5-fold expansion). Notably, the time to dissociate the spheroids was significantly faster in the CTS Rotea system (5 min) compared to the water bath (15 min). Cell viability was also comparable between the CTS Rotea system (98% viable) and the water bath (98.5% viable), as shown in Figure 2B.

The single cells obtained using the CTS Rotea system and water bath were reseeded into new suspension culture vessels. These cells were able to nucleate into spheroids and successfully expand over 5 days of growth, indicating the CTS Rotea system was able to dissociate spheroids with no impact on downstream suspension culture performance (Figures 2C and 2D). Spheroid morphology was also comparable between the spheroids dissociated using the CTS Rotea system and water bath method.

Spheroid dissociation within the CTS Rotea system is also scalable to larger suspension culture vessels. Figure 3 shows the results obtained by dissociating spheroids grown in StemScale medium and harvested from a 3 L bioreactor. The 3 L bioreactor harvest line was able to be connected to the CTS Rotea system, enabling spheroids to be harvested directly into the centrifugation cone of the CTS Rotea system. Similar to the 100 mL suspension culture vessel, the single-cell yields and viabilities obtained from dissociation using the CTS Rotea system (8.5-fold expansion; 93% viable) are comparable to dissociation using the water bath method (9.0-fold expansion; 96% viable) (Figures 3A and 3B). Notably, the time to dissociate the spheroids from the 3 L bioreactor was significantly faster in the CTS Rotea system (10 min) compared to the water bath (30 min). After the single cells were reseeded, the resulting spheroids were able to nucleate and expand over 5 days of growth with no impact on downstream suspension culture performance (Figures 3C and 3D). Due to the centrifugation cone of the CTS Rotea system almost being filled to capacity during dissociation, the output from a 3 L bioreactor system is the approximate upper limit for loading spheroids into the CTS Rotea system.

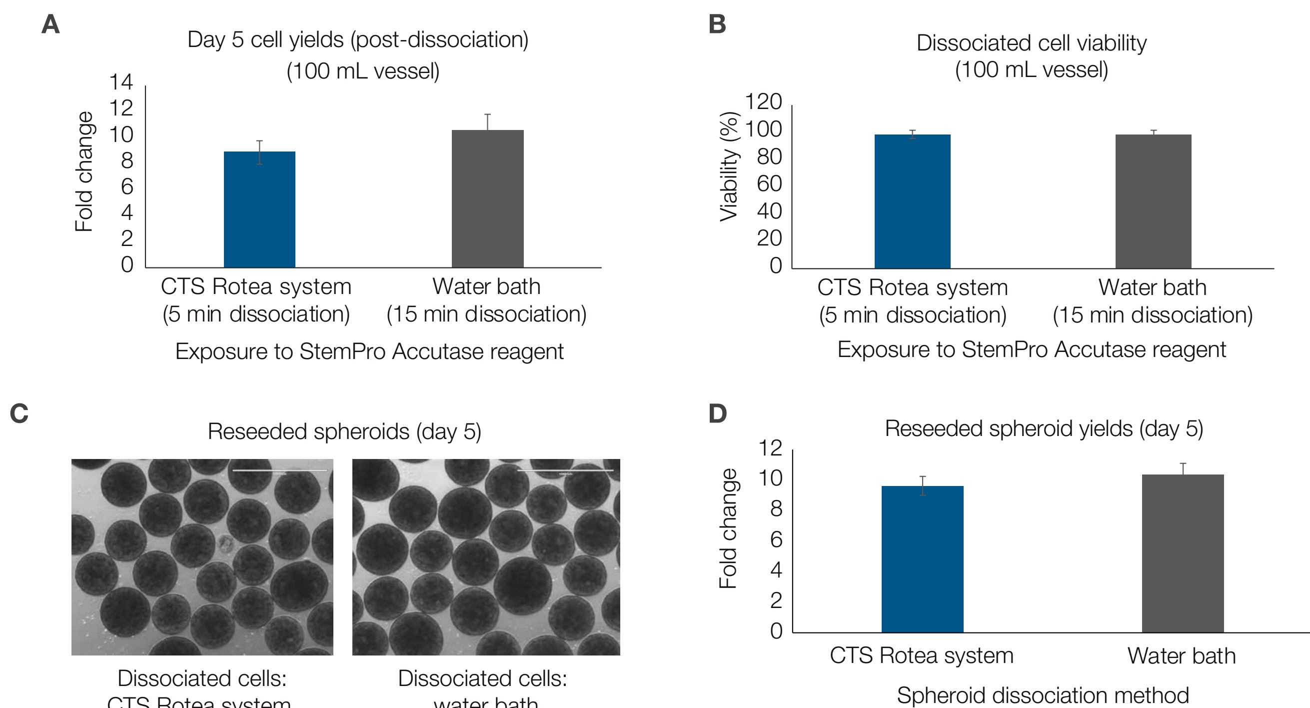

Figure 2. A comparison of spheroid dissociation from a 100 mL suspension culture vessel using the CTS Rotea system and a water bath. Spheroids grown for 5 days and dissociated using both the CTS Rotea system and a water bath method show similar performance in terms of **(A)** total cell yield and **(B)** cell viability. The final cell yields and cell viabilities were obtained from cell counts performed immediately after harvesting the single-cell output from the dissociated spheroids. Notably, the CTS Rotea system can dissociate spheroids significantly faster than the water bath method. **(C)** All single cells harvested from the CTS Rotea system were able to re-nucleate into spheroids when reseeded into new suspension culture vessels. **(D)** After 5 days of growth, the final cell yields of these reseeded spheroids were also shown to be similar, indicating the CTS Rotea system can gently dissociate spheroids into single cells capable of being utilized for downstream applications.

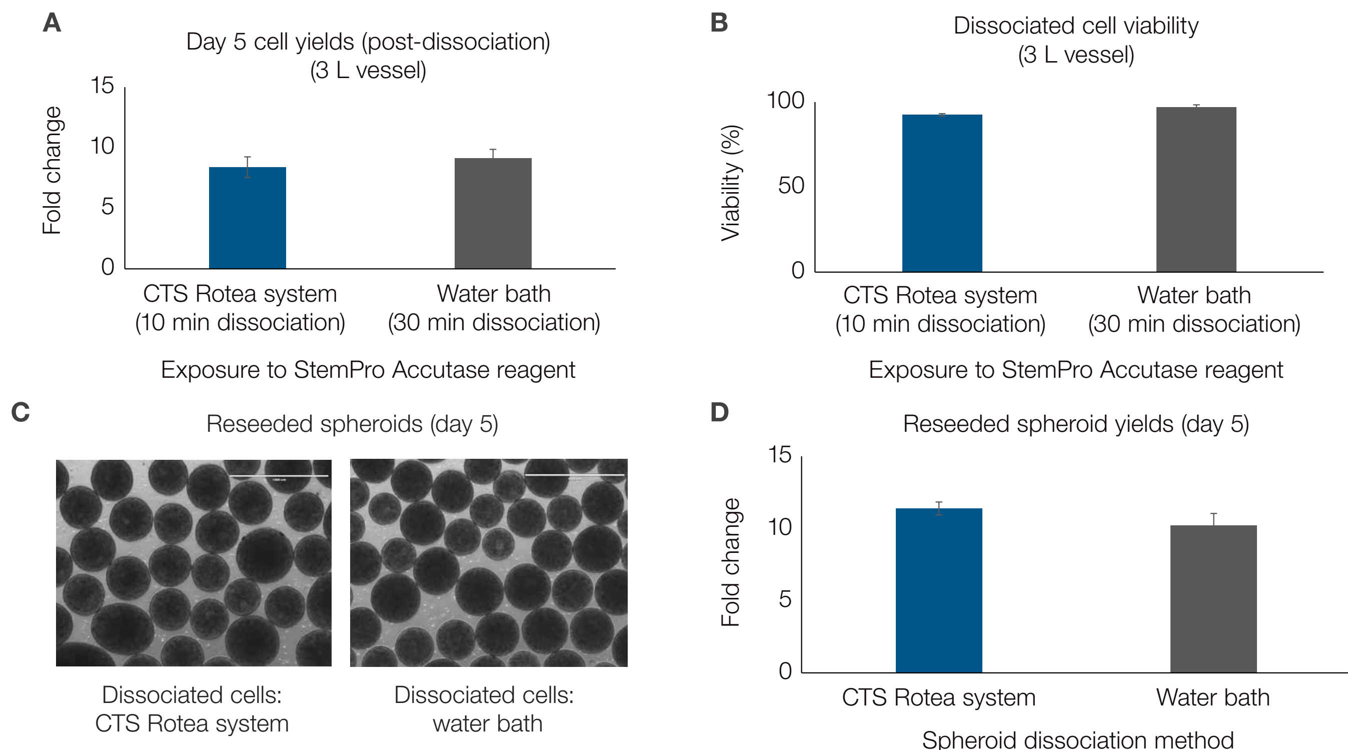

Figure 3. A comparison of spheroid dissociation from a 3 L bioreactor using the CTS Rotea system and a water bath. Spheroids grown for 5 days and dissociated using both the CTS Rotea system and a water bath method show similar performance in terms of **(A)** total cell yield and **(B)** cell viability. The final cell yields and cell viabilities were obtained from cell counts performed immediately after harvesting the single-cell output from the dissociated spheroids. Notably, the CTS Rotea system can dissociate spheroids significantly faster than the water bath method. **(C)** All single cells harvested from the CTS Rotea system were able to re-nucleate into spheroids when reseeded into new suspension culture vessels. **(D)** After 5 days of growth, the final cell yields of these reseeded spheroids were also shown to be similar, indicating the CTS Rotea system can gently dissociate spheroids into single cells capable of being utilized for downstream applications.

Discussion

Overall, the CTS Rotea system can easily be utilized for the rapid dissociation of 3D spheroids in a closed-system environment. We have observed that cells dissociated within the CTS Rotea system behave similarly to those dissociated in a water bath. This indicates that the two key advantages offered by the CTS Rotea system over a water bath method are (1) faster dissociation times and (2) dissociation within a closed system.

While the settings described in Table 1 are useful when performing the first dissociation protocol for large (i.e., 300–400 µm diameter) spheroids, there are many additional factors affecting spheroid dissociation that should be considered. Failure to properly account for these factors could lead to improper spheroid dissociation and reduced cell viability. In this section, we describe the most important factors affecting spheroid dissociation in the CTS Rotea system.

Effect of centrifugation speed on spheroid viability

The correlation between centrifugation speed and flow rate plays an important role when processing PSCs inside the CTS Rotea system. For single cells ranging from 10 to 20 µm in diameter, this balance determines whether PSCs will remain inside or be eluted from the centrifugation cone. Because the CTS Rotea system can gently process single-cell suspensions, the exact values themselves do not significantly affect PSC viability. However, for 3D spheroids ranging from 300 to 400 µm in diameter, more precise consideration of the centrifugation speed and flow rate is necessary to maintain a high spheroid viability. Without properly balancing both centrifugation speed and flow rate, spheroids that flow through the CTS Rotea system could become significantly damaged.

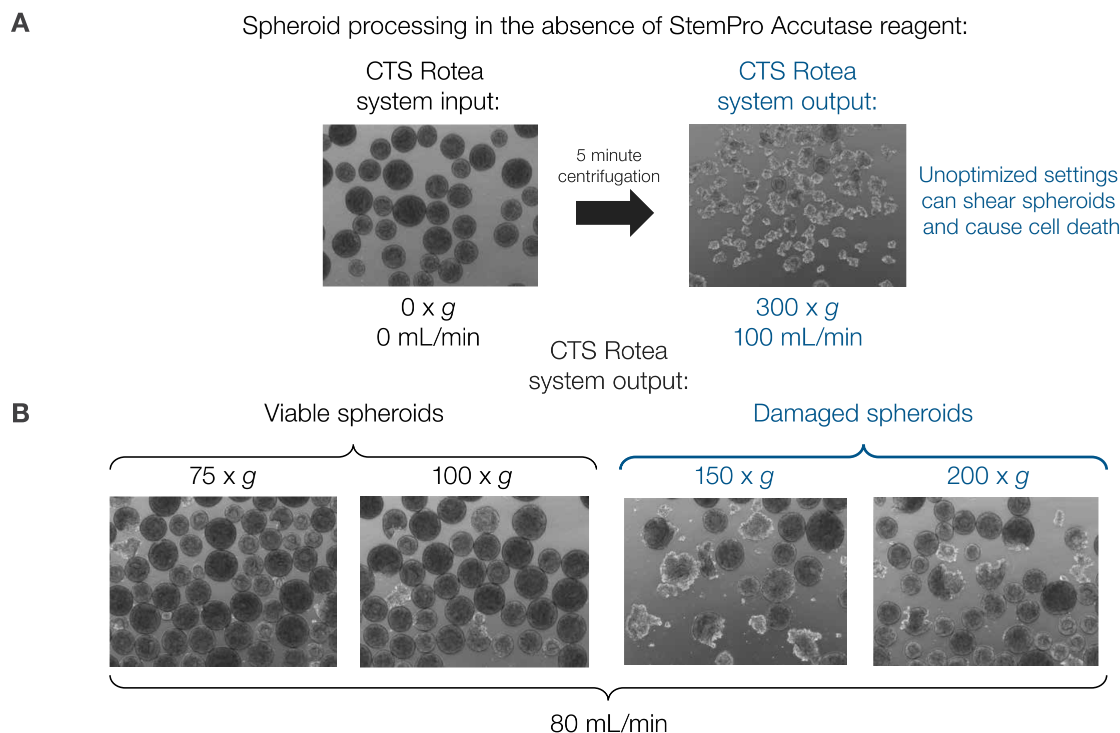

Figure 4. Centrifugation speed has a significant effect on spheroid viability. The spheroids imaged here were input into the CTS Rotea system, centrifuged for 5 min, and harvested for imaging. They were not dissociated into single cells, meaning their output morphology should appear similar to their input morphology. When spheroids are being processed inside the CTS Rotea system, it is important to minimize the centrifugation speed as much as possible. **(A)** Unoptimized settings result in the shearing and death of whole spheroids. **(B)** By minimizing centrifugation speed, the spheroids will remain healthy and viable. Notably, 75 x *g* is the minimum centrifugation speed possible, as spheroids begin to elute into the waste bag below this speed.

Figure 4 demonstrates the effects of centrifugation speed and flow rate on spheroid morphology. The spheroids shown in this figure were loaded into the centrifugation cone of the CTS Rotea system and centrifuged for 5 min before being harvested. Because the spheroids were not exposed to StemPro Accutase reagent, no spheroid dissociation occurred, meaning the most optimal instrument settings are when the input spheroids appear similar to the output spheroids.

In Figure 4A, the effects of unoptimized centrifugation speed and flow rate can be seen. At a high flow rate (100 mL/min) and a centrifugation speed of 300 x *g*, spheroids loaded into the CTS Rotea system become sheared and damaged after 5 min of centrifugation. The overall culture viability is significantly reduced, preventing the spheroids from recovering and expanding in subsequent passages.

To minimize this kind of damage, Figure 4B demonstrates how the settings of the CTS Rotea system can be balanced to gently process 3D spheroids. The spheroids were introduced into the centrifugation cone at a constant flow rate of 80 mL/min and centrifuged at various speeds for 5 min. At centrifugation speeds of 75 and 100 x *g*, the harvested spheroids experienced little to no damage, ensuring PSC viability remained high. However, at centrifugation speeds of 150 and 200 x *g*, the harvested spheroids exhibited significant signs of damage, causing a reduction in PSC viability.

Overall, these results indicate the importance of balancing the centrifugation speed with the flow rate while processing large spheroids inside the CTS Rotea system. Centrifugation speeds of 75–100 x *g* are preferable for spheroid sizes of 300–400 µm to minimize any potential damage that may occur to spheroids flowing through the CTS Rotea system. Notably, spheroids of this size begin to elute into the waste bag at centrifugation speeds below 75 x *g*, preventing the centrifugation speed from being reduced further. By starting with a lower centrifugation speed, the flow rate can then be optimized to ensure spheroid viability remains high. This concept can easily be applied to spheroids of varying sizes in order to obtain viable cells after processing using the CTS Rotea system.

Effect of flow rate on load time

As discussed in the previous section, 3D spheroids are very sensitive to the input settings of the CTS Rotea system and must be gently processed when flowing through the system. Utilizing lower centrifugation speeds and lower flow rates are the simplest methods to minimize any potential spheroid damage. However, this is not always practical when working in large-scale vessels. Table 2 shows the approximate time to complete each step in the spheroid dissociation protocol for a 500 mL vessel. The most significant time investments are required at the spheroid loading step (10 min) and spheroid dissociation step (6 min). Of the two, the spheroid dissociation time does not change much when utilizing different-sized suspension culture vessels. However, the spheroid load time does significantly increase as the suspension culture vessel increases in size.

Table 2. Approximate time to complete each protocol step of the CTS Rotea system for a 500 mL vessel.

Protocol step	Time
Priming	2 min
Load spheroids	10 min
Wash spheroids	3 min
Dissociate spheroids	6 min
Wash cells	3 min
Add fresh medium	3 min
Harvest	3 min

Table 3 shows how flow rate of the CTS Rotea system must be considered when loading spheroids from large-scale suspension culture vessels. Notably, the flow rate is not a significant concern with small-scale suspension culture vessels. The difference between spheroid loading times for a 40 mL/min flow rate and an 80 mL/min flow rate is just 1.25 min for a 100 mL suspension culture vessel. This spheroid loading time difference only increases to 6.25 min for a 500 mL suspension culture vessel. However, a 3 L suspension culture vessel has a load time difference of 37.5 min between a 40 mL/min flow rate and an 80 mL/min flow rate. This is a significant time investment just to load spheroids into the CTS Rotea system, meaning the higher flow rate is preferable to minimize the amount of time spent in the spheroid-loading step.

Table 3. Effect of suspension culture vessel size on load time of the CTS Rotea system.

Vessel size	Flow rate	Load time
100 mL PBS Mini™ Bioreactor	40 mL/min	2.5 min
	80 mL/min	1.25 min
500 mL PBS Mini Bioreactor	40 mL/min	12.5 min
	80 mL/min	6.25 min
3 L PBS 3 Bioreactor*	40 mL/min	75 min
	80 mL/min	37.5 min

* The full cell yield from a 3 L bioreactor is enough to fill the majority of the centrifugation cone of the CTS Rotea system.

Potential undesirable spheroid aggregation within the CTS Rotea system

When single-cell cultures are processed within the CTS Rotea system, it is important for them to form a fluidized bed within the centrifugation cone. The fluidized bed ensures the cells are collected within the cone, while still enabling them to rapidly elute when flow settings are modified. Due to the nature of the fluidized bed, a cell pellet does not form during centrifugation.

Similar to the single-cell cultures, spheroid cultures will also form a fluidized bed within the centrifugation cone. However, this can potentially be problematic for sensitive cell lines, due to the tendency for spheroids to fuse into large aggregates when in contact for long periods of time. Figure 5A shows an example of this spheroid aggregation issue for an orbital shake platform culture. The control spheroids were removed from the orbital shake platform for an extended period. After 1 hour without agitation, the spheroids began to fuse into the large aggregates shown in the image.

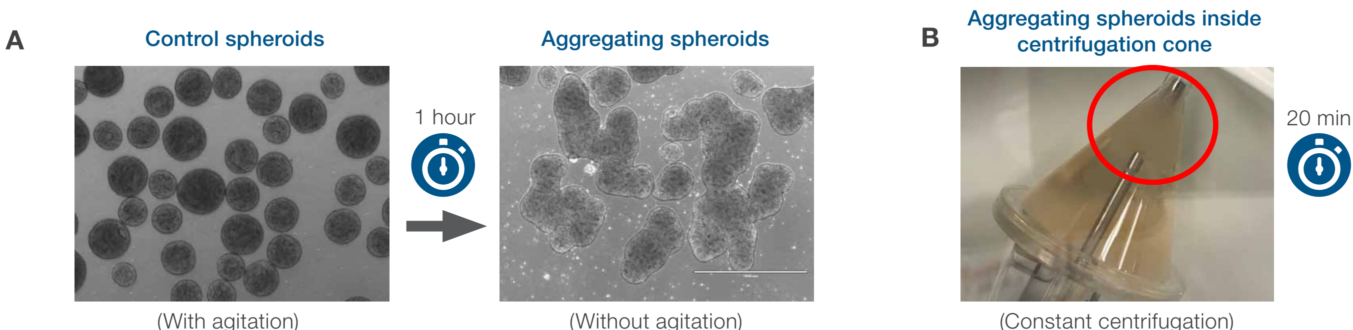

Figure 5. Undesirable spheroid aggregation phenomenon. (A) When spheroids are removed from agitation for extended periods of time, they may attempt to fuse into large aggregates. **(B)** This phenomenon may also occur for sensitive cell lines that remain as a fluidized bed for long durations in the centrifugation cone of the CTS Rotea system.

This same phenomenon can sometimes be observed with the CTS Rotea system as shown in Figure 5B. When spheroids in the centrifugation cone form a fluidized bed, they will also be in close proximity to one another. After constant centrifugation for 20 min, spheroids in the centrifugation cone have sometimes been observed to aggregate together into a large clump. This aggregation impairs spheroid dissociation and ultimately leads to cell death as the large aggregates are forcibly eluted out of the centrifugation cone.

To prevent this undesirable spheroid aggregation, it is possible to insert a temporary spheroid elution loop within the dissociation protocol. Figure 6 illustrates this elution loop in action. The spheroids are partially loaded into the CTS Rotea system for a brief period (2–5 min). After partial loading, the CTS Rotea system enters a pause loop where the centrifugation speed decreases to 0 x *g*. This causes all the spheroids in the centrifugation cone to elute. After a short period of time (15–20 sec), the centrifugation speed increases back to 75 x *g* to reconcentrate spheroids within the centrifugation cone. Once all spheroids have re-entered the cone (15–20 sec), the loop returns to the spheroid-loading step.

The spheroid elution loop enables the rapid loading of large spheroid volumes while still ensuring spheroids do not remain in contact with one another for too long. In doing so, it prevents spheroids from forming undesirable aggregates within the fluidized bed. The loop can also be adapted to work during the spheroid wash and dissociation steps. Once spheroids have dissociated into single cells, the single cells will then be able to form a fluidized bed with no aggregation issues. For an example of the loop steps when loading cells, refer to Table 4.

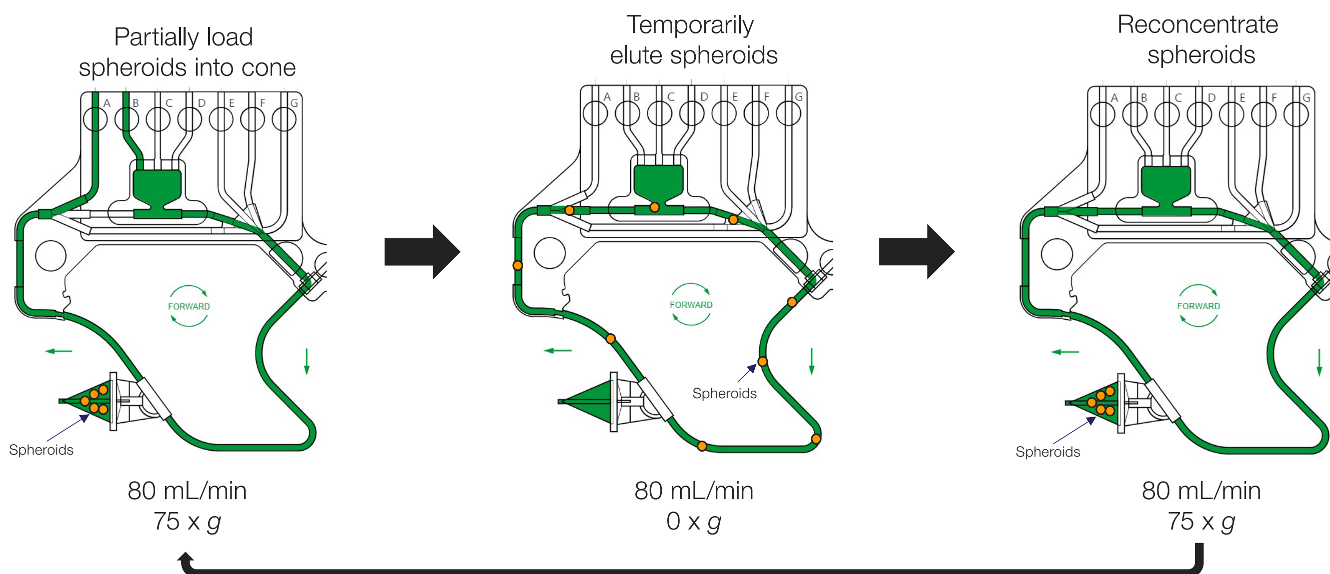

Figure 6. Temporary spheroid elution loop. If spheroid aggregation inside the centrifugation cone of the CTS Rotea system is a problem, this spheroid elution loop can be utilized to prevent spheroids from fusing into a large aggregate. By initiating a pause loop and temporarily stopping centrifugation, the spheroids will be eluted out of the centrifugation cone. After a brief period, centrifugation can be restarted to retain spheroids inside the centrifugation cone. This process can loop as many times as necessary and can be adapted at any point in the dissociation protocol.

Table 4. Example of a spheroid elution loop during initial spheroid loading.

Step*	Description	Flow path	Centrifugation force (x *g*)	Pump (mL/min)	Step type	Trigger(s)
1	Load cells	C to A	75	80	Normal	Timer (2 min)
2	Temporarily elute spheroids	J to K	0	80	Pause	Timer (20 sec)
3	Reconcentrate spheroids	J to K	75	80	Pause	Timer (20 sec)

* Loop steps 1–3 as many times as necessary to load all cells.

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Conclusions

- The CTS Rotea system enables 3D spheroid dissociation within a closed-system environment.
- The centrifugation cone of the CTS Rotea system can process the total cell yields from suspension culture vessels up to 3 L in size.
- Spheroid dissociation inside the CTS Rotea system is significantly faster than inside a water bath.
- Centrifugation speed should be low (75–100 x *g*) to maximize spheroid viability for large (300–400 µm diameter) spheroids.
- Flow rate should be high, but balanced with centrifugation speed, to minimize the amount of time spent loading spheroids into the CTS Rotea system.
- If spheroid aggregation is a problem, spheroids can periodically be eluted from the centrifugation cone to prevent spheroid fusion from occurring.

Ultimately, it is important to consider the effects of centrifugation speed and flow rate on the entire spheroid dissociation protocol. A high flow rate is optimal to minimize spheroid loading time, while a low centrifugation speed is optimal to maximize spheroid viability inside the CTS Rotea system. These two variables must be balanced to ensure a working protocol. If the centrifugation speed is too low compared to the flow rate, then spheroids will be eluted into the waste bag. But if the centrifugation speed is too high compared to the flow rate, then spheroids will have decreased viability due to shearing within the centrifugation cone.

A good starting point for dissociation of 300–400 µm diameter spheroids with the CTS Rotea system is to utilize a centrifugation speed of 75 x *g* and a flow rate of 80 mL/min. This centrifugation speed is low enough to maintain viable spheroids. If spheroids are being eluted into the waste bag due to the 80 mL/min flow rate, then the centrifugation speed can be gradually increased until spheroids are retained inside the centrifugation cone. This method also works for spheroid cultures with smaller spheroid diameters. Alternatively, if the spheroid loading time is not an issue, then the flow rate can instead be decreased to prevent spheroid elution.

More details on how to optimize protocols can be found at thermofisher.com/rotea.

Troubleshooting

Issue	Solution
After dissociating spheroids using the CTS Rotea system, the final cell count is lower than anticipated.	Ensure that spheroids are not settling and becoming trapped inside the bag ports during loading. Gently agitate the bag every few minutes to resuspend any sedimented spheroids.
Spheroids are eluting into the waste bag.	Gradually increase the centrifugation speed until spheroids are retained in the centrifugation cone. Ensure that the centrifugation speed is not damaging the spheroids—smaller spheroids can tolerate higher centrifugation speeds.
Spheroids have formed a solid pellet and have difficulty eluting from the centrifugation cone.	Insert spheroid elution loops into the protocol, or execute the elution loop more frequently.
Damaged or dead cell clumps are present in the cell yield during harvest.	Reduce the centrifugation speed in all steps prior to spheroid dissociation to minimize spheroid damage.
	Insert spheroid elution loops into the protocol to prevent spheroids from fusing into a large clump.
Single cells have difficulty nucleating into spheroids after dissociation inside the CTS Rotea system.	Reduce the centrifugation speed in all steps prior to spheroid dissociation to minimize spheroid damage.
	Reduce the amount of time that spheroids are exposed to the dissociation reagent.

Learn more at **thermofisher.com/rotea**

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