Troubleshooting Guide

Problems	Potential causes	Solutions
Nonuniform cell and spheroid loading on the pillar plate	Precipitation of cells and spheroids in hydrogels during manual cell loading, pillar plate stamping, and cell printing	Single cells and spheroids suspended in hydrogels can precipitate within 1 to 6 minutes, depending on factors such as cell/spheroid size, density, and hydrogel viscosity. Therefore, manual cell loading, pillar plate stamping, 3D bioprinting should be completed while cells/spheroids remain well-suspended in hydrogels. Frequent resuspension of cells and spheroids in hydrogels helps to prevent precipitation.
Cells in hydrogels on the pillar plate detached during culture	Too low concentration of hydrogel used for cell encapsulation	When working with Matrigel-encapsulated cells on the pillar plate, it's essential to consider matrix metalloproteinases (MMPs) that can degrade Matrigel, potentially causing premature cell detachment. To optimize cell encapsulation, use a final concentration of either 70% Matrigel (5.6 - 8.4 mg/mL) or non-diluted Matrigel (8 - 12 mg/mL).
		When working with alginate-encapsulated cells on the pillar plate, it's crucial to consider the impact of excessive phosphate ions in cell growth media, potentially leading to premature cell detachment. To optimize cell encapsulation, use a final concentration of 0.75 - 1.5% (w/v) alginate and cell growth media containing 25 mM $CaCl_2$ for gelation.
	Incomplete gelation of hydrogel	To ensure complete gelation of Matrigel, incubate the mixture of cells and Matrigel at 37°C for 15 - 20 minutes.
		To achieve complete gelation of alginate, incubate the mixture of cells and alginate in cell growth media containing 25 mM $CaCl_2$ for 5 minutes to allow strong ionic crosslinking.
	Multiple freeze-thaw cycles of Matrigel	Prepare 500 µL aliquots of Matrigel and store them in a -20°C freezer. Thaw Matrigel in a refrigerator 1 day before the experiment and discard any remaining Matrigel after use.
Small bubbles entrapped on the pillars	Dry pillar surface	When the surface of the pillar plate is dry, small bubbles can form. However, these bubbles typically disappear after 1 - 2 days of culture. To prevent bubble formation, hydrate the surface of the pillar plate by incubating it in a petri dish with 500 µL distilled water at 37°C for 30 minutes, followed by chilling at 4°C for 5 minutes. This process creates water condensation on the surface of the pillars. Use the pillar plate while it's still hydrated to avoid drying.
	Air bubbles introduced while mixing hydrogels with cell growth media	Gently mix hydrogels with cell growth media to avoid bubble formation. Centrifuge the diluted hydrogel for 1 minute at 3,000 rpm to remove big air bubbles.
	Air bubbles introduced while mixing cells with hydrogels	Centrifuge the hydrogel for 1 minute at 3,000 rpm before mixing with cells. Gently mix the cell suspension with hydrogels to avoid air bubble formation.

Big bubbles covered the pillars	Cold cell growth media used in the 384DeepWellPlate	Warm up the growth media for 1 hour and tap the 384DeepWellPlate to dislodge any air bubbles before pillar plate sandwiching.
	Big air bubbles formed while dispensing the media in the 384-wells	Tap the 384DeepWellPlate containing warm cell growth media to dislodge any trapped bubbles in the 384-wells.
	Insufficient volume of hydrogels or cells suspended in hydrogels used in the loading plate	Load at least 1.5 mL of hydrogels or cells suspended in hydrogels per block in the loading plate and ensure even distribution across the blocks for stamping. After multiple stamping cycles, refill the loading plate with hydrogels or cells suspended in hydrogels.
	Hydrogels not covering the entire surface in the loading plate	Spread the hydrogel or cells suspended in the hydrogel evenly across the entire surface of the blocks in the loading plate.
Unsuccessful spheroid transfer to the pillar plate	Excess or insufficient cell culture media in an ultralow attachment (ULA) 384-well plate	The optimum volume of cell culture media in the ULA 384-well plate for spheroid transfer is 50 µL/well. Excess volume of cell culture media (e.g., 100 µL/well) will lead to overflow after pillar plate stamping, while the pillar plate will not be immersed with insufficient volume of the media, resulting in unsuccessful spheroid transfer.
	Premature gelation of Matrigel before spheroid transfer	When working with Matrigel for pillar plate stamping, do not leave cold Matrigel in the loading plate for longer than 5 minutes, potentially leading to premature gelation of Matrigel on the pillar plate.
Spheroids located out of center on the pillars after transfer	Small spheroids suspended in the ULA 384-well plate during the pillar plate stamping	Leave the sandwiched plates undisturbed for 1 minute to allow spheroids to settle at the bottom of the ULA 384-well plate before inverting for spheroid transfer.
	Tilted pillar plates after sandwiching with the ULA 384-well plate	After sandwiching the pillar plate with the ULA 384-well plate, place an empty pillar plate near the edge of the ULA plate for balancing the pillar plates. While carrying the sandwiched plates to the CO_2 incubator, maintain a level position to prevent disruption of spheroids.
Rapid 2D growth of cell spheroids	Too low concentration of hydrogel used for cell encapsulation	Use a final concentration of 70 - 100% Matrigel or 0.75 - 1.5% alginate for cell encapsulation. Cells can be settled down on the surface quickly with a low concentration of hydrogels.
Overflow of cell culture media from the perfusion plate	Too steep tilting angles used	Before sandwiching the pillar plate onto the perfusion plate, ensure that all perfusion wells are filled with cell culture media and that the flow rates are uniform in each channel on a digital rocker. Should any perfusion wells be empty, manually fill them with 60 µL of media from the reservoirs. Adjust the tilting angles of the digital rocker to range between 5 and 15 degrees to prevent overflow.
	Excess volume of cell culture media added	The volume of cell culture media in the perfusion plate may be increased over time due to improper medium change. It is important to ensure that the old medium is completely drained and removed before adding fresh medium during the medium change process.