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 may precipitate within 1 - 6 minutes, depending on factors such as cell or spheroid size, density, and hydrogel viscosity. Therefore, manual cell loading, pillar plate stamping, and 3D bioprinting should be completed while the cells or spheroids remain well suspended in the hydrogel. Frequent gentle resuspension of the cell-hydrogel mixture helps prevent precipitation and ensures uniform cell distribution.
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, consider the activity of matrix metalloproteinases (MMPs), which can degrade Matrigel and potentially cause premature cell detachment. To optimize cell encapsulation, use a final concentration of either 70% Matrigel (5.6 - 8.4 mg/mL) or undiluted Matrigel (8 - 12 mg/mL) to maintain sufficient matrix stability during culture.
		When working with alginate-encapsulated cells on the pillar plate, consider the effect of excess phosphate ions in cell culture media, which may weaken the alginate matrix and lead to premature cell detachment. For optimal cell encapsulation, use a final alginate concentration of 0.5 - 1.5% (w/v) and gel the alginate using a solution containing 20 mM $CaCl_2$ and 5 mM $BaCl_2$ in 0.9% NaCl. $BaCl_2$ helps stabilize the alginate matrix and reduce degradation. Rinse and remove excess $CaCl_2$ and $BaCl_2$ thoroughly to maintain high cell viability, as residual salts may be toxic to cells.
	Incomplete gelation of hydrogel	To ensure complete gelation of Matrigel, incubate the cell-Matrigel mixture at 37°C for 15 - 20 minutes.
		To achieve complete gelation of 0.5% alginate, incubate the cell-alginate mixture in 0.9% NaCl solution containing 20 mM $CaCl_2$ and 5 mM $BaCl_2$ for 5 minutes to enable strong ionic crosslinking. Avoid adding $CaCl_2$ or $BaCl_2$ directly to cell culture medium, as this may cause salt precipitation.
	Multiple freeze-thaw cycles of Matrigel	Prepare 500 µL aliquots of Matrigel and store them at -20°C. Thaw Matrigel in a 4°C refrigerator 1 day prior to the experiment. After use, discard any remaining Matrigel and do not refreeze.
Small bubbles entrapped on the pillars	Dry pillar surface	Small bubbles may form when the surface of the pillar plate is dry; however, these bubbles typically disappear after 1 - 2 days of culture. To minimize bubble formation, hydrate the surface of the pillar plate by incubating it in a deep petri dish containing 5 mL of sterile, distilled water at 37°C for 30 minutes. Do not immerse the pillars in water during hydration. Use the pillar plate while it remains hydrated to prevent the surface from drying.
	Air bubbles introduced while mixing hydrogels with cell growth media	Gently mix the hydrogel with cell growth medium to avoid bubble formation. After mixing, centrifuge the diluted

		hydrogel at 1,000 rpm for 2 minutes to remove large air bubbles.
	Air bubbles introduced while mixing cells with hydrogels	Centrifuge the hydrogel at 1,000 rpm for 2 minutes before mixing with cells to remove trapped air bubbles. Then gently mix the cell suspension with the hydrogel, taking care to avoid introducing additional air bubbles.
Big bubbles covered the pillars	Cold cell growth media used in the 384DeepWellPlate	Pre-warm the cell growth medium for 1 hour, and gently tap the 384DeepWellPlate to dislodge any trapped air bubbles before sandwiching the pillar plate onto the plate.
	Big air bubbles formed while dispensing the media in the 384-wells	Gently tap the 384DeepWellPlate containing pre-warmed cell growth medium to dislodge any trapped air bubbles from the 384 wells before use.
	Insufficient volume of hydrogels or cells suspended in hydrogels used in the loading plate	Load at least 2 mL of hydrogel, or cells suspended in hydrogel, into each block of the LoadingPlate, ensuring even distribution across the blocks for consistent stamping. After multiple stamping cycles, refill the LoadingPlate with fresh hydrogel or cell-hydrogel suspension as needed.
	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 block in the LoadingPlate to ensure uniform distribution during pillar plate stamping.
Unsuccessful spheroid transfer to the pillar plate	Excess or insufficient cell culture media in an ultralow attachment (ULA) 384-well plate	The optimal volume of cell culture medium in the ULA 384-well plate for spheroid transfer is 35 - 40 µL per well. Excess medium (e.g., > 40 µL per well) may cause overflow after pillar plate stamping, whereas insufficient volume may prevent the pillars from being properly immersed, resulting in unsuccessful spheroid transfer. For the 384DeepWellPlate, the optimal medium volume is 70 µL per well.
	Detachment of spheroids from the pillars after successful spheroid transfer	After 4 hours of transfer, spheroids become attached to the Matrigel on the pillar plate. However, they may detach when exposed to high shear stress during dynamic culture or when non-adherent polymers such as alginate are added to Matrigel at high concentrations. To reduce spheroid detachment, set the digital rocker to a 5° tilt angle with a 5-minute rocking interval to minimize shear stress, or reduce the concentration of alginate in the Matrigel.
Spheroids located out of center on the pillars after transfer	Excess hydrogel loaded on the pillars due to high hydrogel viscosity	When hemispherical domes form on the pillars, it becomes difficult to position spheroids at the center of the pillars. Remove excess hydrogel from the pillars by horizontally sliding a 1 mL pipette tip across the pillar surfaces.
	Small spheroids suspended in the ULA 384-well plate during cell culture medium removal	Leave ULA 384-well plate undisturbed for 1 minute to allow spheroids to settle at the bottom of the wells before stamping the pillar plate 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 to balance the assembly. When transporting the sandwiched plates to a humidified CO_2 incubator, keep

		them in a level position to prevent disturbance or disruption of the 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 0.5 - 1.5% alginate for cell encapsulation. Hydrogels at lower concentrations may allow cells to settle and attach rapidly to the surface of the pillars before gelation occurs, which can lead to undesired 2D cell growth instead of 3D culture.
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 medium and that flow appears uniform across each channel on the digital rocker. If any perfusion wells are empty, manually fill them with 60 µL of medium taken from the reservoirs. To prevent overflow during dynamic culture, adjust the tilt angle of the digital rocker between 5° and 15°.
	Excess volume of cell culture media added	The volume of cell culture medium in the perfusion plate (optimal volumes: 800 µL per channel for the 36PerfusionPlate and 1,600 µL per channel for the 144PerfusionPlate) may increase over time if medium changes are not performed properly. To maintain the correct volume, completely aspirate and remove the old medium from the far end of the lower reservoirs using a vacuum aspiration system. During medium replacement, add the same volume of fresh medium (800 µL or 1,600 µL per channel, respectively). During this process, remove the pillar plate from the perfusion plate and insert it into the PetriLid. Do not place the wet pillar plate directly on a flat surface, as medium may drip from the pillar tip to the bottom of the pillar. This can lead to contamination and overflow when the pillar plate is reinserted into the perfusion plate.