

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.
		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.
Cells in hydrogels on the pillar plate detached during culture	Too low concentration of hydrogel used for cell encapsulation	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 0.75 - 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. If cytotoxic effects are observed, reduce the concentration of BaCl ₂ accordingly.
	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 1% alginate, incubate the cell-alginate mixture in a solution containing 20 mM CaCl ₂ and 5 mM BaCl ₂ in 0.9% NaCl 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, but 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 petri dish containing 500 µL of distilled water at 37°C for 30 minutes, if necessary, followed by chilling at 4°C for 5 minutes. This process generates water condensation on the surface of the pillars. Use the pillar plate while it remains hydrated to prevent the surface from drying.
	Air bubbles introduced while mixing hydrogels	Gently mix the hydrogel with cell growth medium to avoid bubble formation. After mixing, centrifuge the diluted

	with cell growth media	hydrogel at 1,000 rpm for 1 - 2 minutes to remove large air bubbles.
	Air bubbles introduced while mixing cells with hydrogels	Centrifuge the hydrogel at 1,000 rpm for 1 - 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 1.5 mL of hydrogel, or cells suspended in hydrogel, per block in 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 blocks 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 μ L per well. Excess medium (e.g., > 40 μ L 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 μ L per well.
	Premature gelation of Matrigel before spheroid transfer	When working with Matrigel for pillar plate stamping, do not leave cold Matrigel in the LoadingPlate for longer than 5 minutes, as this may lead 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 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 the 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 0.75 - 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 tilting 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.
