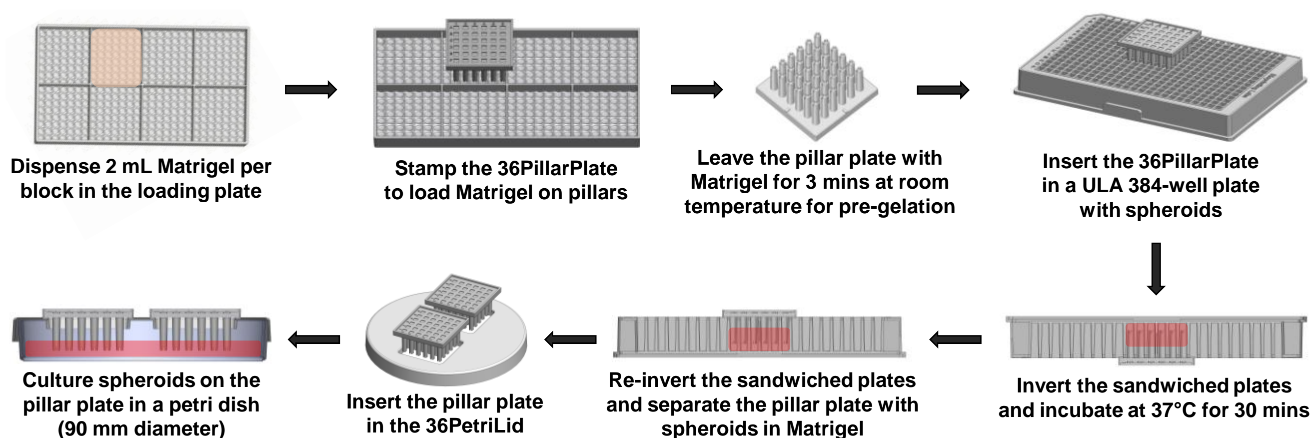
Standard Operating Procedures for Spheroid Culture on a Pillar Plate

This standard operating procedure (SOP) provides step-by-step methods for manual loading of spheroids on a 36PillarPlate and culturing spheroids on the 36PillarPlate with a 384DeepWellPlate or a 36PetriLid. Please read the protocol carefully before performing experiments.

Materials:

- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-03-00)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-02-00)
- Ultralow attachment (ULA) 384-well plate (S-BIO, Cat. no. MS-9384UZ; FaCellitate, Cat. no. F224384)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- Petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- Traditional 384-well plate (Fisher Scientific, Cat. no. 12-565-506)

Methods:



The overall protocol of spheroid encapsulation in Matrigel on the pillar plate using an ultralow attachment (ULA) 384-well plate.

Spheroid transfer from ULA 384-well plate to 36PillarPlate and spheroid culture in petri dish or 384DeepWellPlate

Formation of spheroids in a ultralow attachment (ULA) 384-well plate

1. Prepare spheroids in a ULA 384-well plate by seeding 1,000 - 3,000 cells per well and incubate them for 2 – 4 days.
2. Inspect cell spheroids in the ULA 384-well plate under a brightfield microscope prior to spheroid transfer to the pillar plate. The typical size of cell spheroids transferred is 100 - 400 µm.
   Note: *The typical volume of cell culture media in the ULA 384-well plate required for spheroid transfer is 50 µL per well. Excess volume of cell culture media in the ULA 384-well plate will lead to the overflow of the media after the pillar plate sandwiching onto the ULA 384-well plate. Insufficient volume of cell culture media in the ULA 384-well plate will also lead to unsuccessful spheroid transfer.*

Preparation of Matrigel, cell culture medium, and pillar plate

3. Thaw Matrigel® stock overnight by submerging the unopened bottle in an ice bucket filled with ice in a 4°C refrigerator. Prepare 500 µL aliquots of Matrigel and store at - 20°C for future use.
4. Thaw Matrigel® aliquots overnight in a 4°C refrigerator.
   Note: *It is important to thaw Matrigel aliquots in advance in a 4°C refrigerator and maintain Matrigel chilled on ice during use since Matrigel starts to solidify above 10°C. Do not freeze and thaw Matrigel aliquots.*
5. For cell culture in a 90 x 15 mm petri dish, dispense 20 mL of a cell growth medium in the petri dish, cover with the lid, and place it in a 5% CO_2 incubator at 37°C for at least 1 hour to warm up the growth medium and avoid air bubble formation from the cold growth medium.
   Note: *It is important to use a petri dish with 15 mm height for cell imaging on the pillar plate. The PetriLid fits in with a petri dish with 90 mm diameter.*
   For cell culture in the 384DeepWellPlate, dispense 80 µL/well of a cell growth medium in the 384DeepWellPlate, cover with a well plate lid, and place it in a 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium and avoid air bubble formation.
6. Hydrate the surface of the pillar plate by inserting two 36PillarPlates in the 36PetriLid on a 90 x 15 mm petri dish containing 500 µL of sterile, distilled water and placing it in a 5% CO_2 incubator at 37°C for 20 - 30 minutes (**Fig. 1**).
   Note: *Changing the surface of the pillar plate to hydrophilic by hydration in a humid environment is necessary to minimize air bubble entrapment on the pillars after Matrigel loading.*

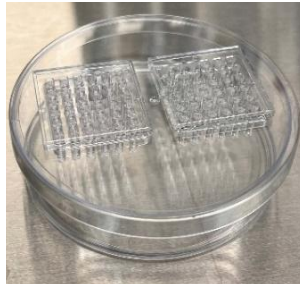


Figure 1. Hydration of the pillar plate surface in a 90 x 15 mm petri dish with 500 µL of sterile, distilled water to minimize air bubble entrapment.

7. Gently mix 0.5 mL of a cold cell growth medium with 1.5 mL of cold Matrigel to obtain a final concentration of 6 - 8 mg/mL Matrigel (i.e., 75% Matrigel).
   Note: *Keep the diluted Matrigel solution on ice during use. Do not reuse thawed/diluted Matrigel. In case of long-term organoid culture necessary or severe cell detachment observed from the pillar plate (typically after 10 days of cancer cell spheroid culture) due to Matrigel degradation by metalloproteases from the cells, either use non-diluted Matrigel or Matrigel supplemented with 0.5% alginate. Alginate cannot be degraded by metalloproteases.*
8. Centrifuge the diluted Matrigel solution at 1,000 rpm for 2 minutes to remove micro-bubbles generated during the process of mixing Matrigel and the cell growth medium.
   Note: *Avoiding micro-bubbles entrapped in Matrigel during the mixing process is critical to prevent air bubble formation on the pillars. This step is unnecessary if non-diluted Matrigel is used.*

Matrigel loading on single pillar plate using a 1 mL pipette tip

9. Aspirate 250 µL of 75% Matrigel (or non-diluted Matrigel) using a 1 mL pipette tip for single 36PillarPlate.
   Note: *It will require 5 µL of Matrigel per pillar (at least 180 µL per 36PillarPlate).*
10. Separate the 1 mL pipette tip from the pipette gently to prevent Matrigel spillage.
11. Using the index finger, block the back of the pipette tip (i.e., the large open area of the tip) to

prevent overflow of Matrigel while tapping the pillar surface.

12. Gently tap the 1 mL pipette tip with Matrigel in the center of the pillar to load Matrigel while blocking the large open area of the tip using the index finger (**Fig. 2**).
__Note:__ Do not touch the bottom of the pillar with the pipette tip so as not to damage the surface coating. Use hydrated pillar plates in the petri dish within 5 minutes before complete water dying.

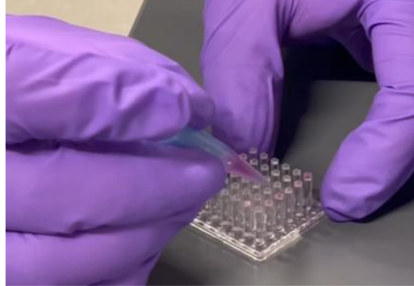


Figure 2. Loading Matrigel on the pillar plate using a 1 mL pipette tip with Matrigel.

13. Repeat **Step 11** for all pillars.
14. After loading Matrigel on all pillars, scrape excess Matrigel off the pillars by sliding the 1 mL pipette tip in a horizontal position on all pillars (**Fig. 3**).
__Note:__ Pre-gelation of Matrigel will be unnecessary as it takes 2 – 3 minutes to load Matrigel on the pillar plate using the 1 mL pipette tip.

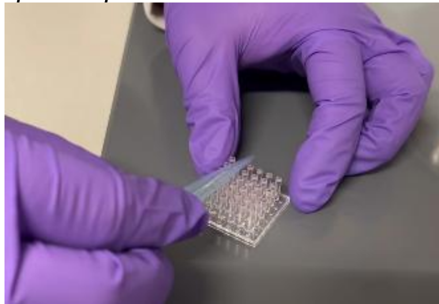


Figure 3. Scrapping excess Matrigel off the pillars using the 1 mL pipette tip.

Matrigel loading on multiple pillar plates using a LoadingPlate

15. Place the LoadingPlate on a flat surface, dispense 1.5 - 2 mL of the cold Matrigel solution per small block without big bubbles, and spread it properly with the pipette tip (**Fig. 4**).

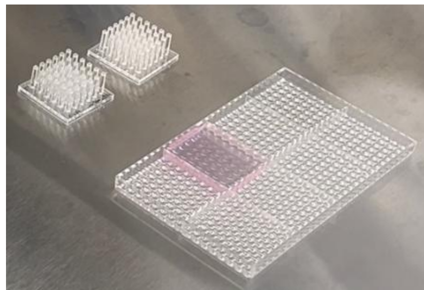
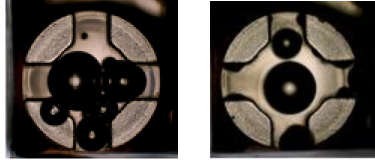


Figure 4. Dispensing 1.5 - 2 mL of cold Matrigel per block in the LoadingPlate for rapid loading of Matrigel on the pillar plate.

__Note:__ It is critical to have a sufficient volume of Matrigel in each block of the LoadingPlate (e.g., minimum 1.5 mL) and spread Matrigel uniformly as all the pillars should be wet with Matrigel. When Matrigel is not loaded properly on the pillars by pillar stamping in Matrigel, macro-bubbles are formed on the pillars after spheroid transfer.



Note: *Do not leave the cold Matrigel solution on the LoadingPlate for longer than 5 minutes to avoid premature gelation during stamping. Since the stamping process is quick, we don't need to place the LoadingPlate with cold Matrigel on ice.*

16. Stamp the 36PillarPlate on the LoadingPlate and press gently to load Matrigel evenly on the entire pillar plate. Repeat this Matrigel loading step for another pillar plate (**Fig. 5**).
    Note: *With 1.5 - 2 mL of the cold Matrigel solution, we can prepare at least three 36PillarPlates (5 μL Matrigel per pillar or 180 μL Matrigel per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform wetting of the pillars and robust Matrigel loading, you can wiggle the pillar plate slightly during stamping. Add extra cold Matrigel solution to the LoadingPlate when necessary.*

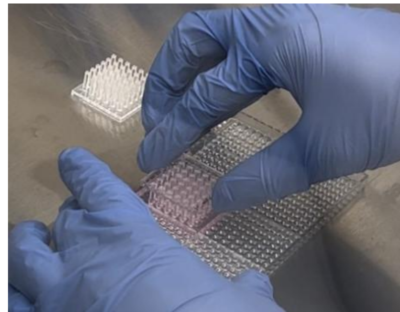


Figure 5. Stamping of the 36PillarPlate on the LoadingPlate to load Matrigel on pillars.

17. Keep the pillar plate with Matrigel at room temperature for 2 - 3 minutes for pre-gelation of 75% Matrigel.
    Note: *Pre-gelation is used to prevent excessive dilution of Matrigel from the pillar plate during spheroid transfer. For 2-fold diluted Matrigel (50% Matrigel), it will require 5 - 7 minutes of pre-gelation at room temperature, whereas pre-gelation is unnecessary for undiluted Matrigel due to quicker gelation.*

Spheroid transfer and encapsulation on the pillar plate

18. Align one pillar located in the corner of the 36PillarPlate with one well in the ULA 384-well plate, insert the pillar plate with Matrigel in the ULA 384-well plate with spheroids, cover the sandwiched plates with a 384-well plate lid, leave the sandwiched plates for 1 minute on a flat surface for letting the spheroids settle down in the center of the 384-well bottom, and then quickly invert the sandwiched plates (with the pillar plate down) for spheroid transfer (**Fig. 6**).
    Note: *To obtain spheroids in the center of the pillars, the pillar plate should be inserted in the ULA 384-well plate gently. Small spheroids can be easily suspended during the sandwiching process. Thus, we may need to leave the sandwiched plates for 1 minute on a flat surface. In addition, carefully carry the inverted and sandwiched plates without tilting to a 5% CO_2 incubator so as not to disturb vertical spheroid precipitation on the pillars (**Fig. 8D**).*

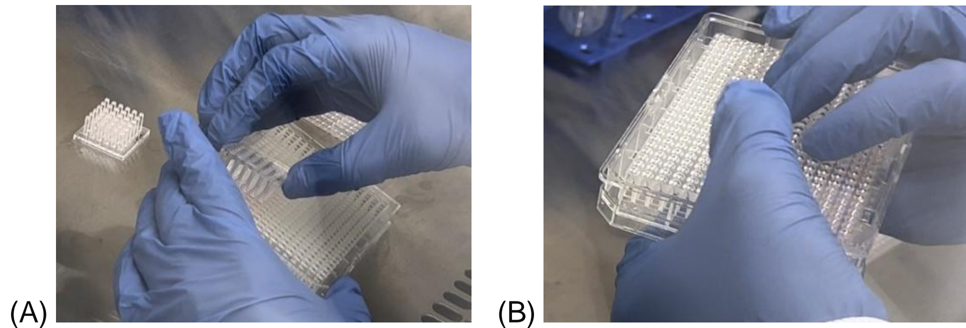


Figure 6. (A) Inserting of the 36PillarPlate in the ULA 384-well plate with spheroids. **(B)** Inverting of the sandwiched plates for spheroid transfer.

19. Incubate the inverted and sandwiched plates for 30 - 40 minutes in a 5% CO_2 incubator at 37°C to transfer spheroids from the ULA 384-well plate to the pillar plate and complete Matrigel gelation.
20. Invert the sandwiched plate again to have the pillar plate up and separate the pillar plate with spheroids encapsulated in Matrigel.
21. Insert the pillar plate with spheroids in the 90 x 15 mm petri dish with the 36PetriLid containing the warm cell growth medium (**Fig. 7**) or in the 384DeepWellPlate with the warm growth medium.
    Note: *It is critical to warm up the growth media for 1 hour and tap the 384DeepWellPlate to dislodge any air bubbles in wells before pillar plate sandwiching. Some micro-bubbles may appear on the edge of the pillars (**Fig. 8C**), which go away in 1 - 2 days with medium change.*

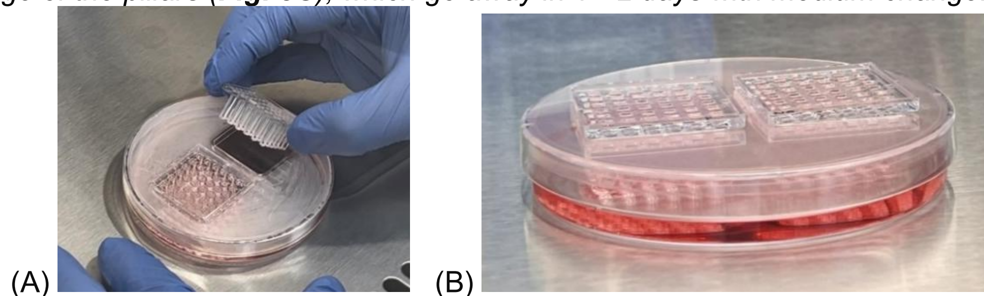


Figure 7. (A) Inserting of the 36PillarPlate with spheroids in the 36PetriLid. **(B)** Spheroids culture on the pillar plate in the petri dish with a cell growth medium.

22. Inspect the pillar plate under the microscope to ensure successful spheroid transfer (**Fig. 8**).
23. Culture the spheroids on the pillar plate in a 5% CO_2 incubator at 37°C with medium change every 3 - 5 days for petri dish culture or every 2 - 3 days for 384DeepWellPlate culture.
    Note: *Cells on the pillar plate in the petri dish can be cultured in a dynamic condition in a 5% CO_2 incubator with a low-speed rocker/digital rocker (See "Dynamic Cell Culture with PetriLid" and "Dynamic Cell Culture in Perfusion Plate").*

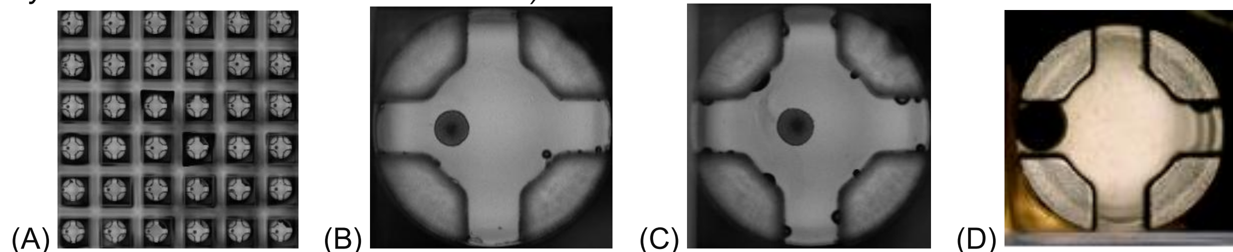


Figure 8. (A) Stitched image of the entire 36PillarPlate with spheroid transfer. **(B)** Single pillar with successful spheroid transfer. **(C)** Single pillar with micro-bubbles on the surface. **(D)** Single pillar with a spheroid in the corner due to unsuccessful spheroid transfer.