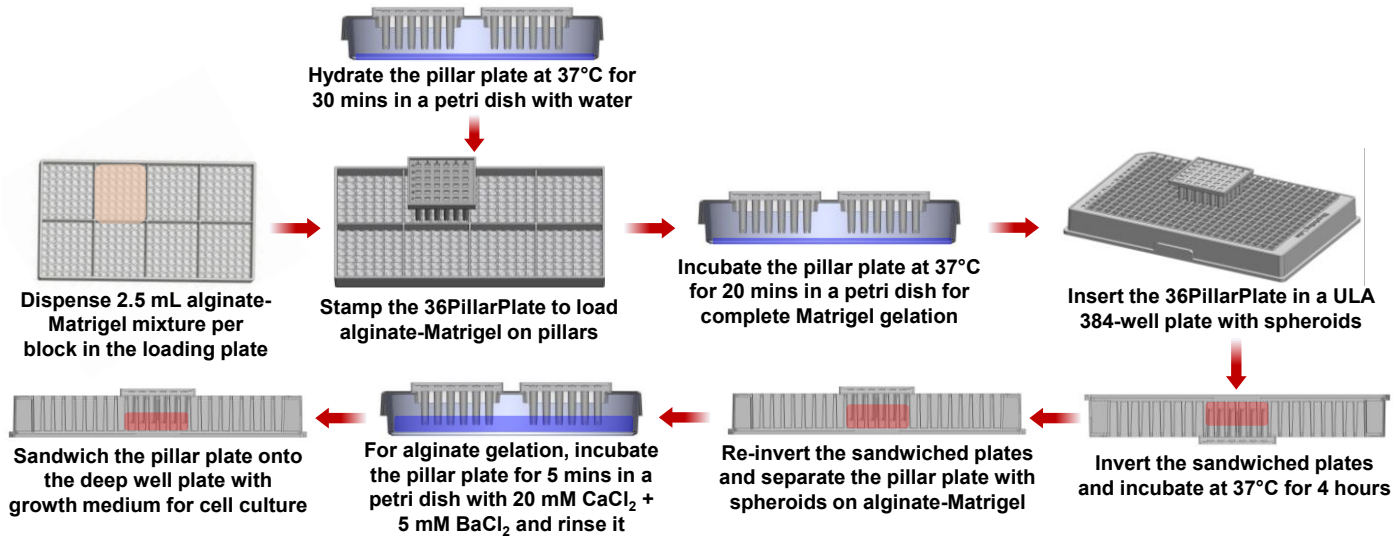
Spheroid Culture in Alginate-Matrigel Mixture on a Pillar Plate

This standard operating procedure (SOP) describes the step-by-step procedure for manually loading spheroids in an alginate-Matrigel mixture onto a 36PillarPlate and culturing the spheroids using either a 384DeepWellPlate or a 36PerfusionPlate. This method is recommended when cells spread aggressively on 2D surfaces during culture. Please read the protocol carefully before performing the 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)
- Alginic acid sodium salt, medium viscosity (Sigma Aldrich, Cat. no. A2033; Fisher Scientific, Cat. no. ICN15472480)
- Millex™ PVDF syringe filter, pore size 0.45 µm, diameter 33 mm, sterile, hydrophilic (Sigma Aldrich, Cat. no. SLHVR33)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- Calcium chloride (Sigma Aldrich, Cat. no. C7902)
- Barium chloride (Sigma Aldrich, Cat. no. B0750)
- Sodium chloride (Sigma Aldrich, Cat. no. S9625)
- Deep petri dish, 100 mm x 20 mm (Corning, Cat. no. 70165-102)

Methods:



The overall protocol of spheroid transfer and attachment to alginate-Matrigel mixture on the pillar plate using an ultralow attachment (ULA) 384-well plate.

Spheroid transfer from ultralow attachment (ULA) 384-well plate to 36PillarPlate and spheroid culture in either 384DeepWellPlate or 36PerfusionPlate

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

1. Prepare spheroids in a ULA 384-well plate by seeding 500 - 3,000 cells per well and incubate them for 2 - 4 days.
   Note: *If spheroids form within 2 days, add 40 µL of cell suspension to each 384-well for spheroid formation, then proceed with pillar plate insertion and spheroid transfer without removing the existing cell culture medium. If spheroid formation takes longer than 2 days, requiring a medium change, add 80 µL of cell suspension to each 384-well. Before pillar plate insertion and spheroid transfer, carefully remove 40 µL of the old medium, leaving 35 - 40 µL in each well. This step is critical to prevent medium overflow during pillar plate insertion and spheroid transfer.*
   Note: *If edge effects are observed, add 80 µL of sterile water to the surrounding wells of the ULA 384-well plate to minimize medium evaporation and promote uniform spheroid formation.*
2. Inspect cell spheroids in the ULA 384-well plate under a bright-field microscope prior to spheroid transfer to the pillar plate. The typical size of cell spheroids transferred is 100 - 400 µm.
   Note: *The optimal volume of cell culture medium in each well of the ULA 384-well plate for successful spheroid transfer is 35 - 40 µL. An excess of medium can cause overflow during the pillar plate sandwiching process, while an insufficient volume may result in unsuccessful spheroid transfer due to bubble formation.*

Preparation of 2% medium-viscosity alginate solution, Matrigel stock, cell culture medium, and pillar plate.

3. Add 200 mg of medium-viscosity alginic acid sodium salt in 10 mL of sterile distilled water in a 20 mL glass vial to prepare 2% (w/v) stock solution.
4. Dissolve the medium-viscosity alginate by continuously stirring for 3 days on a magnetic stirrer.
   Note: *Use a large magnetic bar for stirring to ensure proper dissolution of alginate since the use of a small magnetic bar for stirring will lead to improper dissolution of alginate due to high viscosity.*
5. Sterile the 2% alginate solution by passing it through a Millex™ PVDF syringe filter (0.45 µm pore size) using a sterile syringe inside a biosafety cabinet.
   Note: *Because the 2% alginate solution is highly viscous, apply slow and steady pressure during filtration to avoid filter clogging*.
6. Store the sterile alginate stock solution at 4°C until use.
7. Thaw Matrigel® stock overnight by submerging an unopened bottle in a bucket of ice placed in a 4°C refrigerator. Prepare 500 µL aliquots of Matrigel and store them at - 20°C for future use.
8. Thaw Matrigel® aliquots overnight in a 4°C refrigerator prior to spheroid transfer.
   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.*
9. For cell culture, dispense 70 µL/well of cell growth medium into a 384DeepWellPlate or 800 µL/fluidic channel of cell growth medium into a 36PerfusionPlate. Cover the plate with an appropriate well plate lid and incubate it in a humidified 5% CO_2 incubator at 37°C for at least 1 hour prior to use.
   Note: *Prewarming the medium helps minimize temperature shock and reduces air bubble formation during plate assembly and culture*. *Adding an excessive volume of cell culture medium to the 384DeepWellPlate or 36PerfusionPlate may cause overflow after the pillar plate is sandwiched with the well plate. Avoid wetting the bottom of the pillars with culture medium during this process, as it may result in cross-talk or contamination between wells.*
10. Hydrate the surface of the pillar plate by inserting two 36PillarPlates into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile distilled water. Incubate the assembly in a

humidified 5% CO_2 incubator at 37°C for 30 minutes prior to hydrogel loading (**Fig. 1**).
Note: *Hydrating the surface of the pillar plate in a humid environment is necessary to increase surface hydrophilicity and minimize air bubble entrapment on the pillars after hydrogel loading. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with uniform hydration.*

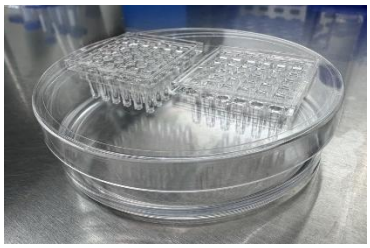


Figure 1. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

Preparation of alginate-Matrigel mixture

11. Gently mix 1 mL of warm cell culture medium with 1 mL of 2% medium-viscosity alginate to obtain a final concentration of 1% alginate.
    Note: *Cut the end of a 1 mL pipette tip to facilitate accurate aspiration of the 2% medium-viscosity alginate solution while minimizing the introduction of large air bubbles.*
12. Mix 1.5 mL of 1% alginate with 1.5 mL of cold Matrigel to obtain a final concentration of 0.5% alginate and 4 - 6 mg/mL Matrigel (i.e., 50% Matrigel).
    Note: *Prepare the alginate-Matrigel mixture at room temperature to minimize bubble formation. Use the mixture immediately to prevent premature gelation of Matrigel.*
13. If bubbles form on the pillar plate after hydrogel loading, centrifuge the mixture of 0.5% alginate and 50% Matrigel at 1,000 rpm for 2 minutes to remove air bubbles generated during the mixing process.
    Note: *Avoiding micro-bubble entrapment during alginate-Matrigel mixing is critical to prevent air bubble formation on the pillars after 1-day culture.*

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

14. Aspirate 250 µL of the alginate-Matrigel mixture using a 1 mL pipette tip for single 36PillarPlate.
    Note: *It will require 5 µL of alginate-Matrigel per pillar (at least 180 µL per 36PillarPlate).*
15. Separate the 1 mL pipette tip from the pipette gently to prevent alginate-Matrigel spillage.
16. Using the index finger, block the back opening of the pipette tip to prevent alginate-Matrigel overflow while tapping the pillar surface.
17. Gently tap the 1 mL pipette tip containing the hydrogel onto the center of the pillar to load alginate-Matrigel while blocking the large back opening of the tip with the index finger (**Fig. 2**).
    Note: *Do not touch the bottom surface of the pillar with the pipette tip to avoid damaging the surface coating. Use the hydrated pillar plates within 5 minutes after removal from the humidified petri dish to prevent complete dying of the surface.*

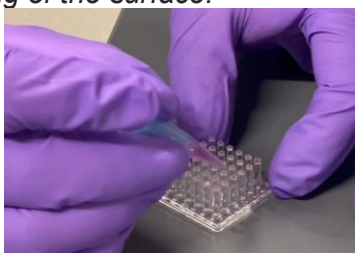


Figure 2. Loading alginate-Matrigel on the pillar plate using a 1 mL pipette tip with the hydrogel.

18. Repeat **Step 17** for all pillars.
19. After loading alginate-Matrigel on all pillars, remove excess alginate-Matrigel by horizontally sliding a 1 mL pipette tip across the pillar surfaces (**Fig. 3**).

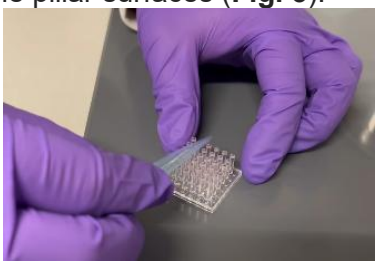


Figure 3. Scraping excess alginate-Matrigel from the pillars using a 1 mL pipette tip.

Alginate-Matrigel loading on multiple pillar plates using a LoadingPlate

20. Place a LoadingPlate on a flat surface. Dispense 2 - 2.5 mL of the 0.5% alginate and 50% Matrigel mixture into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 4**).

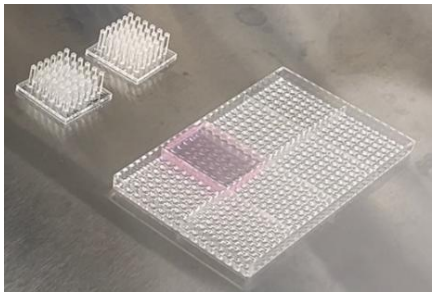


Figure 4. Dispensing 2 - 2.5 mL of the 0.5% alginate and 50% Matrigel mixture per block in the LoadingPlate for rapid loading of alginate-Matrigel on the pillar plate.

Note: *It is critical to maintain a sufficient volume of the alginate-Matrigel mixture in each block of the LoadingPlate; a minimum volume of 2 mL per block is recommended. The hydrogel mixture should be distributed uniformly to ensure complete wetting of all pillars with the alginate-Matrigel solution. Improper loading of the alginate-Matrigel mixture onto the pillars during the stamping process may result in macro-bubble formation on the pillars after spheroid transfer.*

21. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the alginate-Matrigel solution evenly on the entire pillar plate. Repeat this loading step for another pillar plate (**Fig. 5A**).
Note: *Using 2 - 2.5 mL of the alginate-Matrigel solution, it is possible to prepare at least four 36PillarPlates without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust hydrogel loading, gently wiggle the pillar plate during stamping. Add additional alginate-Matrigel solution to the LoadingPlate as needed. When using high-viscosity hydrogels, pillar plate stamping may cause hydrogel to dangle around the pillars, which can result in spheroid transfer failure (**Fig. 5B**).*

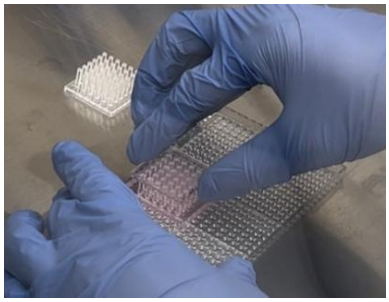
(A)  (B) 

Figure 5. (A) Stamping the 36PillarPlate onto the LoadingPlate to load alginate-Matrigel onto the pillars. **(B)** Hydrogel dangling around the pillars due to high viscosity.

Complete Matrigel gelation in the alginate-Matrigel mixture prior to spheroid transfer

22. For minimizing water evaporation during complete Matrigel gelation and preventing dilution of the alginate-Matrigel mixture during spheroid transfer, insert two 36PillarPlates loaded with alginate-Matrigel into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile, distilled water (**Fig. 1**).
23. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 20 minutes to allow complete gelation of Matrigel in the alginate-Matrigel mixture.
    Note: *It is critical to minimize water evaporation during Matrigel gelation to ensure proper spheroid transfer. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with proper gelation.*

Spheroid transfer and attachment on the pillar plate

24. Align one corner pillar of the 36PillarPlate with the corresponding corner well of the ULA 384-well plate. Carefully sandwich the pillar plate with alginate-Matrigel onto the ULA 384-well plate containing spheroids. Cover the assembled plates with a 384-well plate lid and quickly invert the sandwiched plates so that the pillar plate faces downward to initiate spheroid transfer (**Fig. 6**).
    Note: *Ensure that each well of the ULA 384-well plate contains 35 - 40 µL of cell culture medium for spheroid transfer. Excessive medium volume in the ULA 384-well plate may cause overflow after sandwiching the pillar plate onto the ULA 384-well plate. Avoid wetting the bottom of the pillars during this process, as it may lead to cross-talk or contamination.*
    Note: *To position spheroids at the center of the pillars, ensure that there is no excess hydrogel on the pillars and gently insert the pillar plate into the ULA 384-well plate. Remove excess hydrogel from the pillars by horizontally sliding a 1 mL pipette tip across the pillar surfaces. In addition, carefully transport the inverted and sandwiched plates to a 5% CO_2 incubator without tilting, so as not to disturb vertical spheroid precipitation onto the pillars (**Fig. 8D**).*

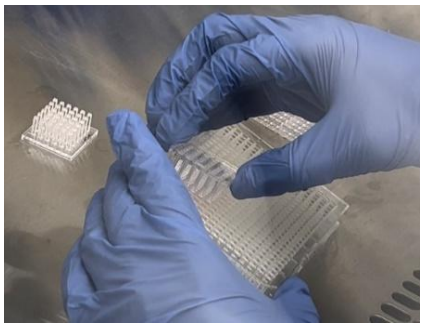
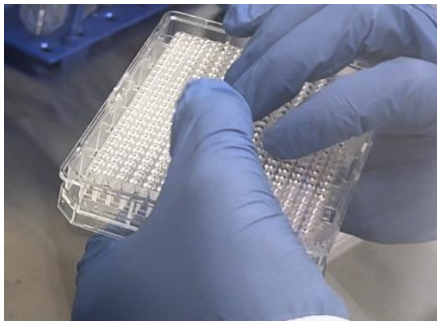
(A)  (B) 

Figure 6. (A) Insertion of the 36PillarPlate into the ULA 384-well plate containing spheroids. **(B)** Inversion of the sandwiched plates for spheroid transfer.

25. Incubate the inverted and sandwiched plates in a humidified 5% CO_2 incubator at 37°C for 4 hours to allow spheroid transfer from the ULA 384-well plate onto the pillar plate and attachment of the spheroids to the alginate-Matrigel.
26. After incubation, carefully re-invert the sandwiched plates to return the 36PillarPlate to the top position, and then separate the pillar plate containing the transferred spheroids attached to the alginate-Matrigel.
27. For alginate gelation, immediately insert two 36PillarPlates into a 36PetriLid placed on a 100 x 20 mm petri dish containing 60 mL of 20 mM $CaCl_2$ and 5 mM $BaCl_2$ prepared in 0.9% NaCl. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 5 minutes.
Note: *Prepare the 20 mM $CaCl_2$ and 5 mM $BaCl_2$ solution in 0.9% NaCl in advance so that alginate gelation can begin immediately after spheroid transfer onto the pillar plate. Ensure that only the tips of the pillars are immersed in the gelation solution during alginate gelation and rinsing procedures.*
28. Remove excess $CaCl_2$ and $BaCl_2$ by separating the 36PetriLid containing the pillar plates from the gelation solution and sandwiching it onto a 100 x 20 mm petri dish containing 60 mL of 0.9% NaCl for 5 minutes.
Note: *This rinsing step is critical for maintaining high cell viability, as residual $CaCl_2$ and $BaCl_2$ can be toxic to cells.*
29. Separate and insert the pillar plate into the 384DeepWellPlate containing 70 µL/well of prewarmed growth medium (**Fig. 7**).
Note: *It is critical to prewarm the growth medium in the 384DeepWellPlate for at least 1 hour and gently tap the plate to dislodge any air bubbles in the wells before sandwiching with the pillar plate. Small micro-bubbles may appear at the edges of the pillars after sandwiching (**Fig. 8C**); however, these bubbles typically disappear within 1 - 2 days during routine medium changes.*

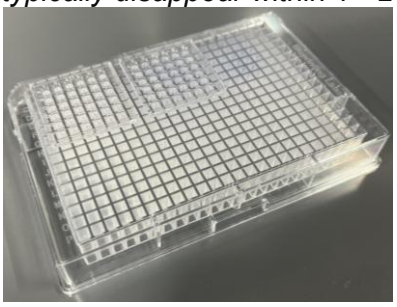
(A)  (B) 

Figure 7. (A) The 36PillarPlates sandwiched onto the 384DeepWellPlate. **(B)** Close-up image of the pillars of the 36PillarPlate inserted into the wells of the 384DeepWellPlate for cell culture.

30. Inspect the pillar plate under a brightfield microscope to confirm successful spheroid transfer onto the pillars (**Fig. 8**)
31. Culture the spheroids on the pillar plate in a humidified 5% CO_2 incubator at 37°C, replacing the culture medium every 1 - 2 days for culture using the 384DeepWellPlate.
Note: *Cells on the pillar plate may also be cultured under dynamic conditions using a 36PerfusionPlate or petri dish combined with a digital rocker or low-speed rocker. However, low shear stress conditions (e.g., 5° tilt angle with 5-minute interval rocking) should be maintained for the first 4 days to prevent spheroid detachment from the pillar plate. Refer to the protocols titled "Dynamic Cell Culture in Perfusion Plate" or "Dynamic Cell Culture with PetriLid" for additional details.*

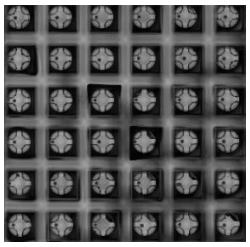
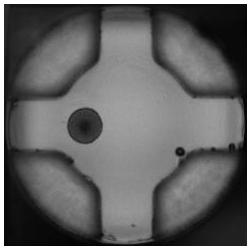
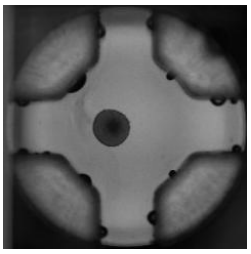
(A)  (B)  (C)  (D) 

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