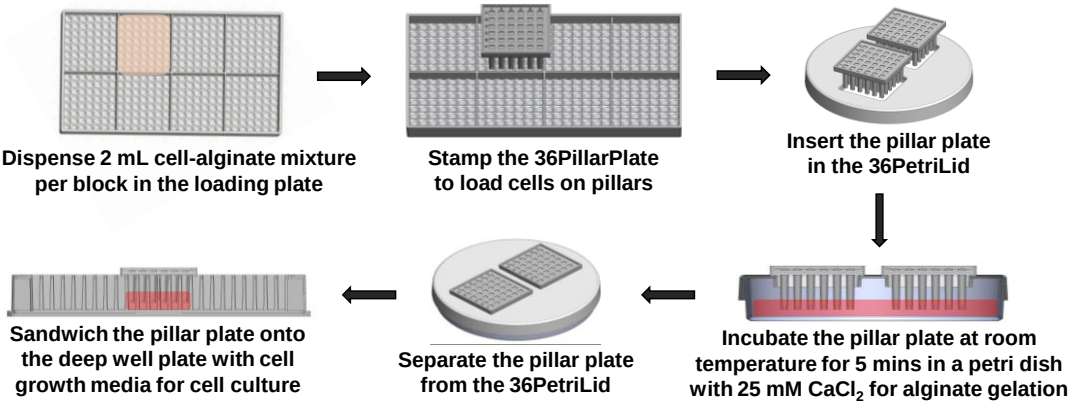
Standard Operating Procedures for Cell Suspension Culture in Alginate on a Pillar Plate

This standard operating procedure (SOP) provides step-by-step methods for manual loading of single cell suspension in alginate on a 36PillarPlate and culturing cells in 3D 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)
- Alginic acid (Sigma Aldrich, Cat. no. A1112)
- Calcium chloride (Sigma Aldrich, Cat. no. C7902)
- 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 cell suspension culture in alginate on the pillar plate.

Preparation of 3% (w/v) alginate stock solution.

1. Add 300 mg of low viscosity alginic acid sodium salt in 10 mL of sterile distilled water in a 20 mL glass vial to prepare 3% (w/v) stock solution.
2. Dissolve the alginic acid sodium salt by continuously stirring for 3 days on a magnetic stirrer.
3. Store the alginate stock solution at 4°C until use.

Cell suspension culture in alginate on 36PillarPlate in 384DeepWellPlate or petri dish

1. 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.
   For cell culture in a 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.

2. 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 cell loading in alginate.*

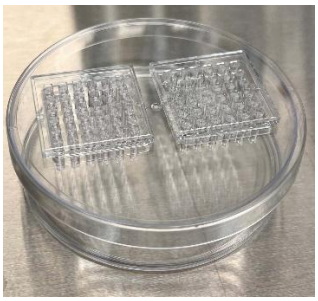


Figure 1. Hydration of the pillar plate surface in a 90 x 15 mm petri dish with 500 µL of sterile, distilled water to minimize air bubble entrapment.

3. Prepare 2 mL of cell suspension by gently mixing a cell pellet of 0.8 – 4 x 10^6 cells/pellet with 2 mL of a warm cell culture medium in a 15 mL centrifuge tube.
   Note: *We use a warm cell culture medium to avoid micro-bubble formation during the mixing with cold alginate, which is critical to prevent air bubble entrapment on the pillars.*
4. Gently mix 1.8 mL of warm cell suspension with 600 µL of cold 3% (w/v) alginate stock solution to generate a homogenous mixture of cells and alginate without air bubbles entrapped.
   Note: *The final cell seeding density will be 0.2 – 1 x 10^6 cells/mL in 0.75% alginate (1,000 – 5,000 cells/pillar). Cell seeding density can be adjusted depending on the doubling time. Gently mix and dispense, making sure to avoid air bubbles entrapped in alginate. This is the most critical step to avoid air bubble formation on the pillars.*
5. Place the LoadingPlate on a flat surface, dispense 1.5 – 2 mL of the cell-alginate mixture per small block, and spread it properly with the pipette tip (**Fig. 2**).
   Note: *Make sure to use the cell-alginate mixture within 4 minutes as cells in alginate could settle down in 4 – 8 minutes, which could lead to non-uniform cell loading on the pillar plate. Keep resuspending the cell-alginate mixture before dispensing on the LoadingPlate.*

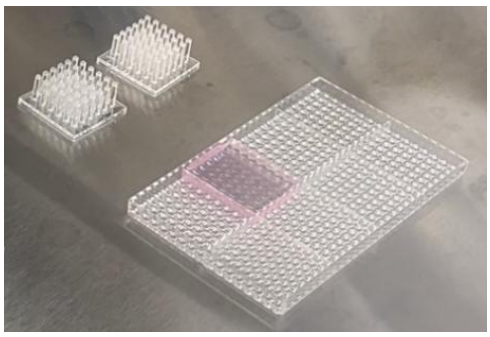


Figure 2. Dispensing 1.5 - 2 mL of the cell-alginate mixture per block in the LoadingPlate for rapid loading of the cells on the pillar plate.

6. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the cell-alginate mixture evenly on the entire pillar plate (**Fig. 3**). Repeat this cell loading step for another pillar plate.
   Note: *With* 1.5 - *2 mL of the cell-alginate mixture, we can prepare at least three 36PillarPlates (5 µL cell-alginate mixture per pillar or 180 µL the cell-alginate mixture per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform wetting of the pillars and robust cell loading,*

you can wiggle the pillar plate slightly during stamping.

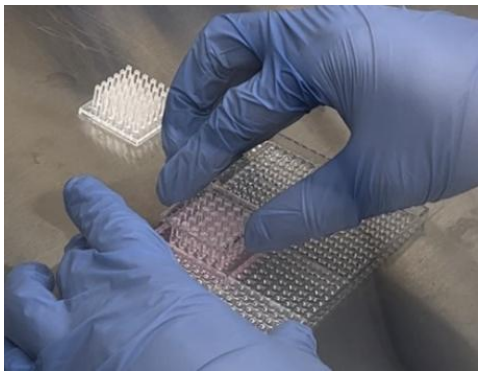


Figure 3. Stamping of the 36PillarPlate on the LoadingPlate to load cells suspended in alginate on pillars.

7. Prepare 25 mM $CaCl_2$ in basal cell culture medium and dispense 20 mL in a 90 x 15 mm petri dish for alginate gelation.
   Note: *Make sure to prepare the petri dish with $CaCl_2$ before preparing the cell suspension to immediately start alginate gelation after loading on the pillar plate.*
8. Insert the pillar plate with cells in alginate in the 36PetriLid on the 90 x 15 mm petri dish containing 20 mL of 25 mM $CaCl_2$ in a basal medium and incubate it for 5 minutes at room temperature for complete gelation of alginate on the pillar plate (**Fig. 4**).

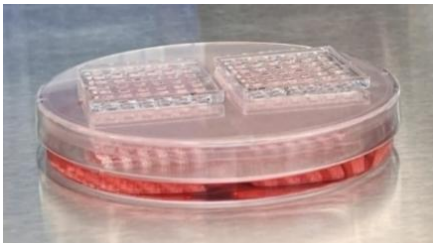


Figure 4. Gelation of alginate on the pillar plate inserted in the 36PetriLid on the 90 x 15 mm petri dish with 20 mL of 25 mM $CaCl_2$ in a basal cell growth medium.

9. Separate the 36PetriLid with the pillar plate and sandwich it onto the 90 x 15 mm petri dish containing 20 mL of the warm cell growth medium (**Fig. 5**) or insert the pillar plate in the 384DeepWellPlate with 80 µL/well of 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. 6C**), which go away in 1 - 2 days with medium change.*

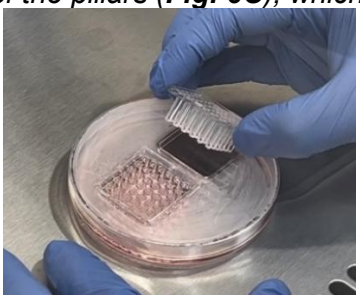
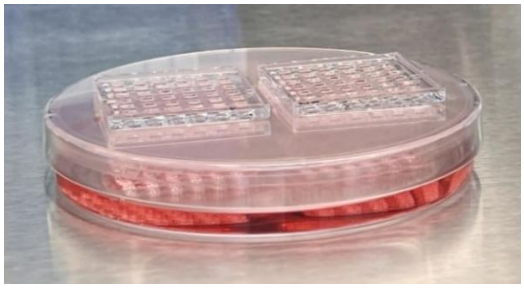
(A)  (B) 

Figure 5. (A) Inserting of the 36PillarPlate with cells in alginate in the 36PetriLid. **(B)** Cell suspension culture on the pillar plate in the petri dish with a cell growth medium.

10. Inspect the pillar plate under the microscope to ensure uniform cell loading throughout the entire pillar plate (**Fig. 6**).

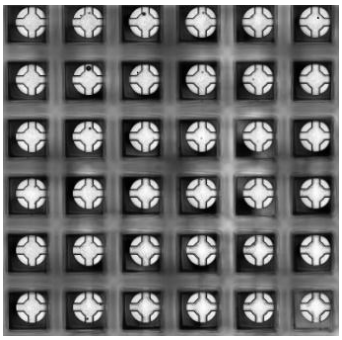
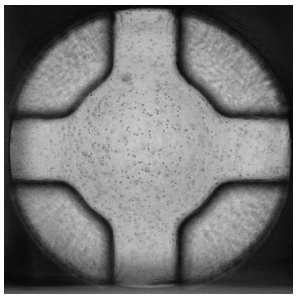
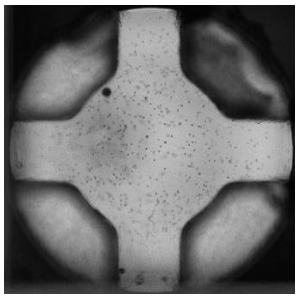
(A)  (B)  (C) 

Figure 6. (A) Stitched image of the entire 36PillarPlate with cells encapsulated in alginate. **(B)** Single pillar with cells in alginate. **(C)** Single pillar with micro-bubbles on the surface.

11. Culture the cells 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 could be cultured in a dynamic condition in a 5% CO_2 incubator with a low-speed rocker/digital rocker (For dynamic 3D cell culture, refer to "Dynamic Cell Culture with PetriLid" and "Dynamic Cell Culture in Perfusion Plate").*