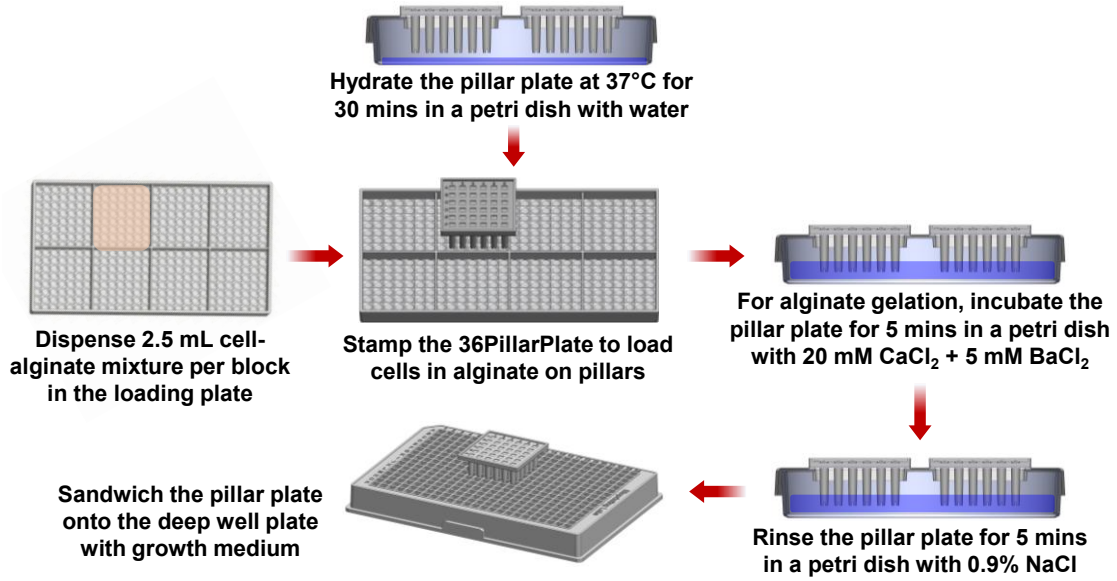
Cell Suspension Culture in Alginate on a Pillar Plate

This standard operating procedure (SOP) describes step-by-step procedures for manually loading a single-cell suspension in alginate onto a 36PillarPlate and culturing cells in three-dimensional (3D) conditions using either a 384DeepWellPlate or a 36PerfusionPlate. Please read this protocol carefully before conducting 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 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)
- 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 cell suspension culture in alginate on the pillar plate.

Preparation of 2% (w/v) alginate stock solution.

1. 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.
2. 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.*

3. 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*.
4. Store the sterile alginate stock solution at 4°C until use.

Cell suspension culture in alginate on 36PillarPlate in 384DeepWellPlate or 36PerfusionPlate

1. 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.*
2. 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 cell 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*.

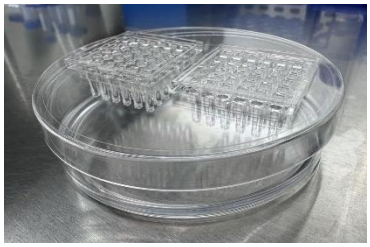


Figure 1. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

3. Prepare 2.0 mL of cell suspension by gently mixing a cell pellet of 0.4 - 4 x 10^6 cells/pellet with 2.0 mL of warm culture medium in a 15 mL centrifuge tube.
4. Prepare 2.0 mL of 1% (w/v) alginate solution by gently mixing 1.0 mL of warm culture medium with 1.0 mL of 2% (w/v) alginate stock solution in a 15 mL centrifuge tube.
   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. 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.*
5. Gently mix 1.5 mL of warm cell suspension with 1.5 mL of 1% (w/v) alginate solution to generate a homogenous mixture of cells and alginate without air bubbles entrapped.
   Note: *If cells require ECM components in culture, prepare 0.5% alginate containing 4 - 6 mg/mL Matrigel. The final cell seeding density should be 0.1 - 1 x 10^6 cells/mL in 0.5% alginate (500 - 5,000 cells/pillar), which can be adjusted depending on the cell doubling time. Mix gently and dispense carefully, ensuring no air bubbles are entrapped in the cell-alginate mixture*.

6. Place a LoadingPlate on a flat surface. Dispense 2 - 2.5 mL of the cell-alginate mixture into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 2**).
   Note: *Ensure to use the cell-alginate mixture immediately as cells in alginate could settle down in 5 minutes, which could lead to non-uniform cell loading on the pillar plate. Keep resuspending the cell-alginate mixture before dispensing on the LoadingPlate.*

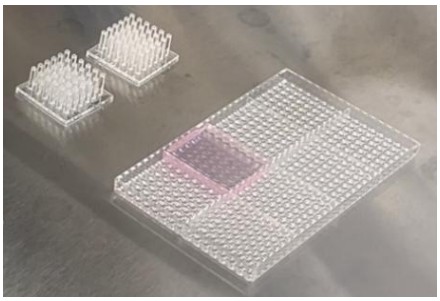
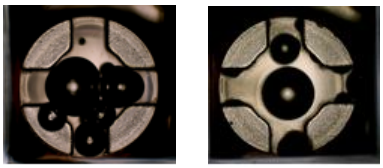


Figure 2. Dispensing 2 - 2.5 mL of the cell-alginate mixture per block in the LoadingPlate for rapid loading of the cells on the pillar plate.

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



7. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the cell-alginate mixture evenly on the entire pillar plate. Repeat this cell loading step for another pillar plate (**Fig. 3**).
8. ***Note:*** *With 2 - 2.5 mL of the cell-alginate mixture, it is possible to prepare at least four 36PillarPlates (5 µL cell-alginate mixture per pillar or 180 µL per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust cell loading, gently wiggle the pillar plate during stamping. Add additional cell-hydrogel solution to the LoadingPlate as needed.*

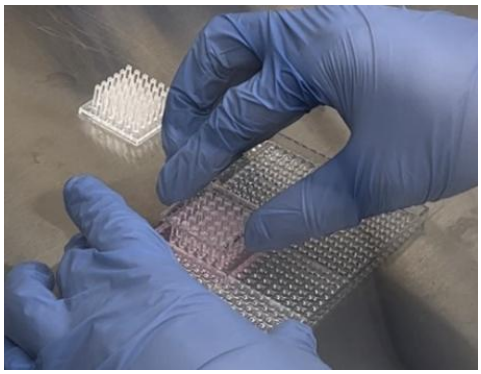


Figure 3. Stamping of the 36PillarPlate onto the LoadingPlate to load cells suspended in alginate on pillars.

9. 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 pillar plate stamping. Use cell culture medium containing 20 mM $CaCl_2$ and 5 mM $BaCl_2$ cautiously for alginate gelation, as salt precipitation may occur. Ensure that only the tips of the pillars are immersed in the gelation solution during alginate gelation and rinsing procedures.*

10. 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.*
11. Separate and insert the pillar plate into the 384DeepWellPlate containing 70 µL/well of prewarmed growth medium (**Fig. 4**).
    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. 5C**); however, these bubbles typically disappear within 1 - 2 days during routine medium changes.*

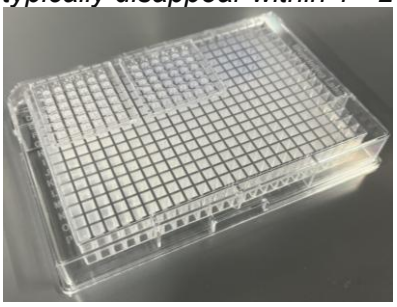
(A)  (B) 

Figure 4. (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.

12. Inspect the pillar plate under a brightfield microscope to confirm uniform cell loading throughout the entire pillar plate (**Fig. 5**).
13. Culture single cells encapsulated in alginate 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. Refer to the protocols titled "Dynamic Cell Culture in Perfusion Plate" or "Dynamic Cell Culture with PetriLid" for additional details.*

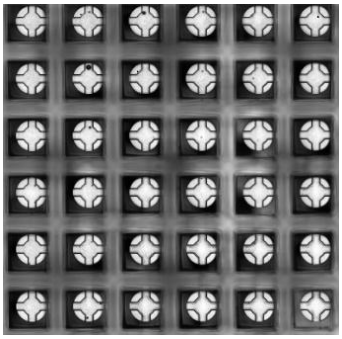
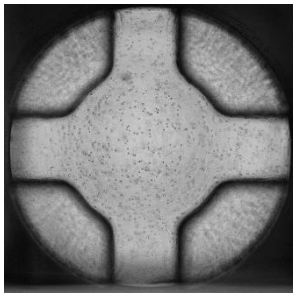
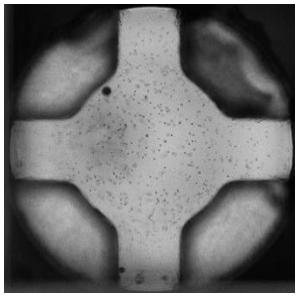
(A)  (B)  (C) 

Figure 5. (A) Stitched image of the entire 36PillarPlate with single cells encapsulated in alginate. **(B)** Single pillar showing single cells in alginate. **(C)** Single pillar with micro-bubbles on the surface.