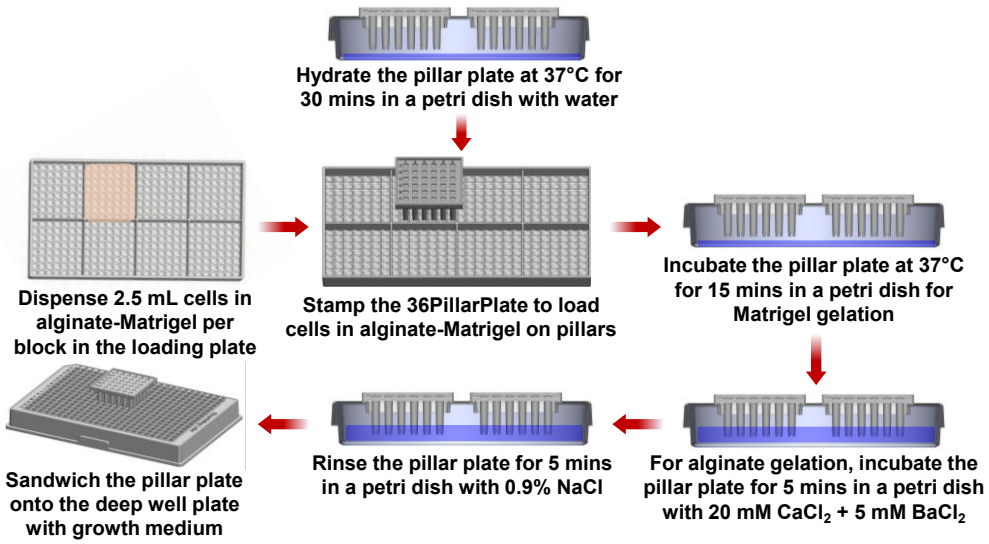
Cell Suspension Culture in Alginate-Matrigel on a Pillar Plate

This standard operating procedure (SOP) describes step-by-step procedures for manually loading a single-cell suspension in alginate-Matrigel mixture onto a 36PillarPlate and culturing cells in 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)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- 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-Matrigel on the pillar plate.

Cell suspension culture in alginate-Matrigel on 36PillarPlate in 384DeepWellPlate

Preparation of alginate stock, Matrigel stock, cell culture medium, and pillar plate

1. To prepare 2% (w/v) alginate stock solution, add 200 mg of medium-viscosity alginic acid sodium salt in 10 mL of sterile distilled water in a 20 mL glass vial.

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.
5. 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.
6. Thaw Matrigel® aliquots overnight in a 4°C refrigerator prior to mixing with cell suspension.
   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.*
7. 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.*
8. 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.*

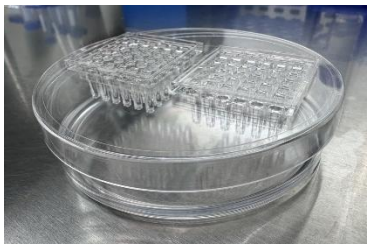


Figure 1. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

Preparation of single-cell suspension in alginate-Matrigel

9. Prepare 1.0 mL of cell suspension by gently mixing a cell pellet of 0.4 - 4 x 10^6 cells/pellet with 1.0 mL of warm culture medium in a 15 mL centrifuge tube.
10. Prepare 2.0 mL of cell suspension in 1% (w/v) alginate by gently mixing 1.0 mL of cell suspension with 1.0 mL of 2% (w/v) alginate stock solution in the 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 warm alginate stock solution to avoid micro-bubble formation during the mixing with cell suspension, which is critical to prevent air bubble entrapment on the pillars.*

11. Gently mix 1.5 mL of warm cell suspension in 1% (w/v) alginate with 1.5 mL of cold Matrigel to generate a homogenous mixture of cells, alginate, and Matrigel without air bubbles entrapped.
Note: *The final cell seeding density should be 0.1 - 1 x 10^6 cells/mL in 0.5% (w/v) alginate and 4 - 6 mg/mL Matrigel (500 - 5,000 cells/pillar), which may be adjusted depending on the cell doubling time. Use the cell-alginate-Matrigel mixture immediately, as cells can settle down within 5 minutes in hydrogel, leading to non-uniform cell loading on the pillar plate. Gently resuspend the cell-alginate-Matrigel mixture frequently before dispensing onto the LoadingPlate.*

Loading cell suspension in alginate-Matrigel on single pillar plate using a 1 mL pipette tip

12. Aspirate 250 µL of the cell-alginate-Matrigel mixture using a 1 mL pipette tip for single 36PillarPlate.
Note: *It will require 5 µL of the cell-alginate-Matrigel mixture per pillar (at least 180 µL per 36PillarPlate).*
13. Separate the 1 mL pipette tip from the pipette gently to prevent cell-alginate-Matrigel spillage.
14. Using the index finger, block the back opening of the pipette tip to prevent cell-alginate-Matrigel overflow while tapping the pillar surface.
15. Gently tap the 1 mL pipette tip containing the cell-alginate-Matrigel mixture onto the center of the pillar to load single-cell suspension in 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.*

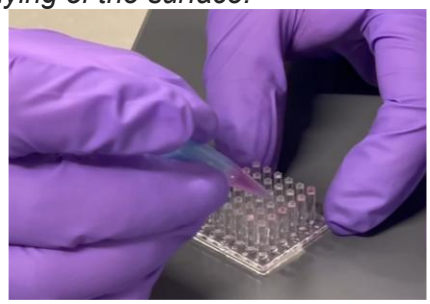


Figure 2. Loading the cell-alginate-Matrigel mixture on the pillar plate using a 1 mL pipette tip.

16. Repeat **Step 15** for all pillars.
17. After loading the cell-alginate-Matrigel mixture on all pillars, remove excess cell-alginate-Matrigel by horizontally sliding a 1 mL pipette tip across the pillar surfaces (**Fig. 3**).

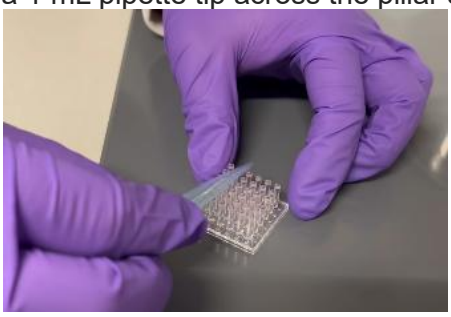


Figure 3. Scraping excess cell-alginate-Matrigel mixture from the pillars using a 1 mL pipette tip.

Loading cell suspension in alginate-Matrigel on multiple pillar plates using a LoadingPlate

18. Place a LoadingPlate on a flat surface. Dispense 2 - 2.5 mL of the cell-alginate-Matrigel mixture into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 4**).
Note: *Ensure to use the cell-alginate-Matrigel mixture immediately as cells in alginate-Matrigel*

could settle down in 5 minutes, which could lead to non-uniform cell loading on the pillar plate. Keep resuspending the cell-alginate-Matrigel mixture before dispensing on the LoadingPlate.

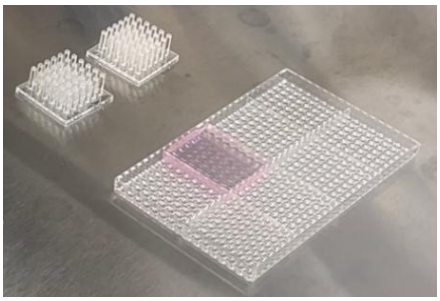


Figure 4. Dispensing 2 - 2.5 mL of the cell-alginate-Matrigel mixture per block in the LoadingPlate for rapid loading of the cells on the pillar plate.

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

Note: *Do not leave the cell-alginate-Matrigel mixture on the LoadingPlate for longer than 5 minutes to avoid premature Matrigel gelation during the stamping process. Because pillar stamping is performed rapidly, it is generally not necessary to place the LoadingPlate containing the cell-alginate-Matrigel mixture on ice during this step.*

19. Stamp the 36PillarPlate on the LoadingPlate and press gently to load the cell-alginate-Matrigel mixture evenly on the entire pillar plate. Repeat this cell loading step for another pillar plate (**Fig. 5**).
Note: *With 2 - 2.5 mL of the cell-alginate-Matrigel mixture, it is possible to prepare at least four 36PillarPlates (5 µL cell-alginate-Matrigel 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-alginate-Matrigel solution to the LoadingPlate as needed.*

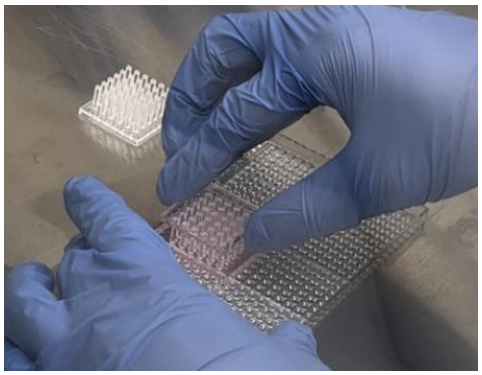


Figure 5. Stamping of the 36PillarPlate onto the LoadingPlate to load cells suspended in alginate-Matrigel on pillars.

Hydrogel gelation and cell culture on the pillar plate

20. For minimizing water evaporation during Matrigel gelation, insert two 36PillarPlates loaded with cells in alginate-Matrigel into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile, distilled water (**Fig. 1**).
21. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 15 minutes to allow complete gelation of Matrigel.
    Note: *It is critical to minimize water evaporation during Matrigel gelation to maintain high cell viability. 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.*
22. 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.*
23. 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.*
24. Separate the pillar plate with single cells encapsulated in alginate-Matrigel from the 36PetriLid and insert it into the 384DeepWellPlate containing 70 µL/well of prewarmed growth medium (**Fig. 6**).
    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. 7C**); however, these bubbles typically disappear within 1 - 2 days during routine medium changes.*

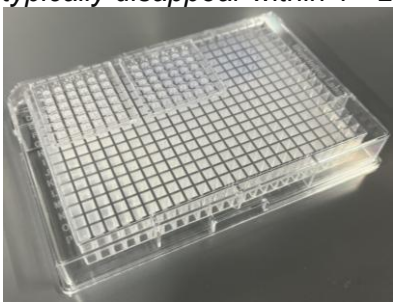
(A)  (B) 

Figure 6. (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.

25. Inspect the pillar plate under a brightfield microscope to confirm uniform cell loading throughout the entire pillar plate (**Fig. 7**).
26. Culture single cells encapsulated in alginate-Matrigel 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.*

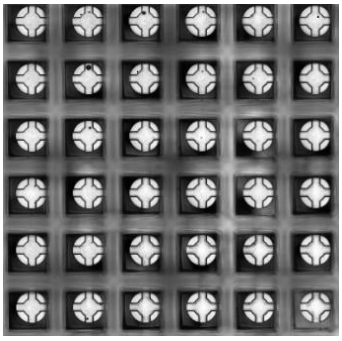
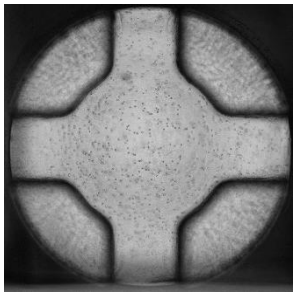
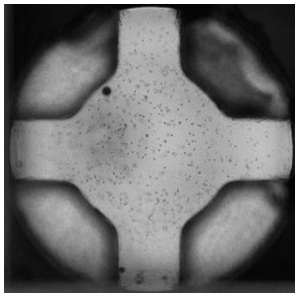
(A)  (B)  (C) 

Figure 7. (A) Stitched image of the entire 36PillarPlate with single cells encapsulated in alginate-Matrigel. **(B)** Single pillar showing single cells in alginate-Matrigel. **(C)** Single pillar with micro-bubbles on the surface.