Bioprinting® Laboratories

Immune-Tumor Cell Co-Culture in a Pillar/Perfusion Plate

This standard operating procedure (SOP) provides step-by-step methods for co-culturing tumor spheroids and fibroblasts encapsulated in Matrigel on the pillar plate with immune cells bi-directionally flowing in the perfusion plate. 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)
- 36PerfusionPlate (Bioprinting Laboratories Inc., Cat. no. 36-02-00)
- 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)
- Lung tumor cell line (e.g., A549)
- Patient-derived fibroblasts
- Peripheral blood mononuclear cells (PBMCs)
- OrganoFlow L Digital Rocker (MIMETAS) or Infinity Rocker™ Pro (Next Advance)
- Vacusafe vacuum aspiration system (Integra Biosciences, Part no. 158320)

Tumor spheroid transfer from ULA 384-well plate to 36PillarPlate

Formation of tumor 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 40 µL in each well. This step is critical to prevent medium overflow during pillar plate insertion and spheroid transfer.*
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 optimal volume of cell culture medium in each well of the ULA 384-well plate for successful spheroid transfer is 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.*

Preparation of Matrigel, cell culture medium, and pillar plate

3. Thaw Matrigel® stock overnight by submerging the 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.
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 70 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.
   Note: *Adding an excessive volume of cell culture media to the petri dish or the 384DeepWellPlate may cause overflow after the pillar plate is sandwiched. Avoid wetting the bottom of the pillars with cell culture media during this process, as it can lead to cross-talk or contamination.*
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: *Hydrating the surface of the pillar plate in a humid environment is necessary to make it hydrophilic and minimize air bubble entrapment on the pillars after Matrigel loading.*

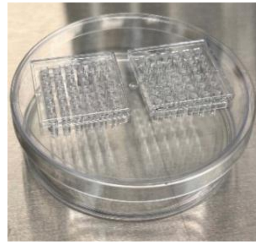


Figure 1. Hydration of the pillar plate surface in a 90 x 15 mm petri dish with 500 µL of sterile, distilled water to minimize air bubble entrapment.

7. Prepare 1.0 mL of fibroblast cell suspension by gently mixing a cell pellet of 0.8 – 4 x 10^6 cells/pellet with 1.0 mL of warm culture medium in a 15 mL centrifuge tube.
8. Gently mix 0.5 mL of warm fibroblast cell suspension with 1.5 mL of cold Matrigel to obtain a final concentration of 6 - 8 mg/mL Matrigel (i.e., 75% Matrigel).
   Note: *The typical density of fibroblasts ranges from 0.2 to 1 x 10^6 cells/mL in 6 – 8 mg/mL Matrigel, corresponding to 1,000 to 5,000 cells per pillar, which can be adjusted as needed. Use diluted Matrigel immediately. If not used right away, keep it on ice. Do not reuse thawed or diluted Matrigel. Cells in Matrigel could settle down in 5 – 10 minutes, leading to non-uniform cell loading on the pillar plate. Keep resuspending the cell-Matrigel mixture before loading in the LoadingPlate.*

Matrigel loading on multiple pillar plates using a LoadingPlate

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

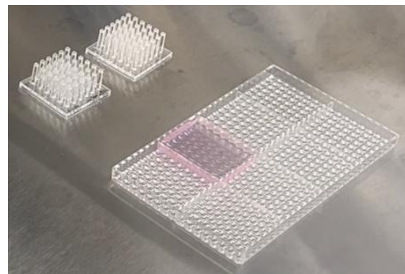
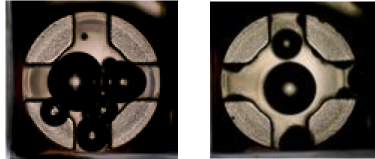


Figure 2. Dispensing 1.5 - 2 mL of single cell suspension in Matrigel per block in the LoadingPlate for rapid loading of cells in Matrigel on the pillar plate.

Note: *It is critical to ensure a sufficient volume of Matrigel in each block of the LoadingPlate (a minimum of 1.5 - 2 mL is recommended). Matrigel should be spread uniformly to ensure that all pillars are fully wetted. Improper loading of Matrigel onto the pillars during pillar stamping can result in macro-bubble formation 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.*

10. Stamp the 36PillarPlate on the LoadingPlate and press gently to load single cell suspension in Matrigel evenly on the entire pillar plate. Repeat this cell-Matrigel loading step for another pillar plate (**Fig. 3**).
Note: *Using 1.5 - 2 mL of cold Matrigel solution, it is possible to prepare at least three 36PillarPlates (5 µL Matrigel per pillar or 180 µL per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust Matrigel loading, gently wiggle the pillar plate during stamping. Add additional cold Matrigel solution to the LoadingPlate as needed.*

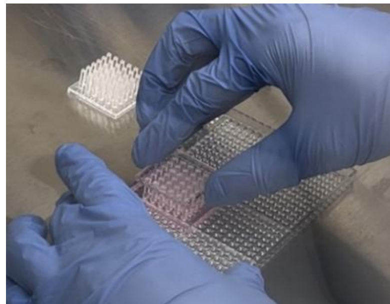


Figure 3. Stamping of the 36PillarPlate on the LoadingPlate to load single cell suspension in Matrigel on pillars.

11. <u>If necessary</u>, keep the pillar plate with Matrigel at room temperature for 2 - 3 minutes for pre-gelation of 75% Matrigel.
Note: *Pre-gelation can be used to prevent excessive dilution of Matrigel in the ULA 384-well plate during spheroid transfer to the pillar plate. For 2-fold diluted Matrigel (i.e., 50% Matrigel), allow 4 - 6 minutes of pre-gelation at room temperature. Pre-gelation is not necessary for undiluted Matrigel, as it gels more rapidly.*

Spheroid transfer and encapsulation on the pillar plate

12. <u>Align one pillar located in the corner of the 36PillarPlate with one well in the ULA 384-well plate</u>, insert the pillar plate with fibroblasts in Matrigel in the ULA 384-well plate with tumor spheroids, <u>cover the sandwiched plates with a 384-well plate lid</u>, and then quickly invert the sandwiched plates (with the pillar plate down) for spheroid transfer (**Fig. 4**).
Note: *Ensure that each well of the ULA 384-well plate contains <u>40 µL of cell culture medium</u> for spheroid transfer. Using a large volume of cell culture medium in the ULA 384-well plate causes overflow or excessive hydrogel dilution after the pillar plate is sandwiched onto the ULA 384-well plate. Avoid wetting the bottom of the pillars during this process, as it can lead to cross-talk or contamination.*
Note: *To obtain spheroids in the center of the pillars, the pillar plate should be inserted in the ULA 384-well plate gently. In addition, carefully carry the inverted and sandwiched plates <u>without tilting</u> to a 5% CO_2 incubator so as not to disturb vertical spheroid precipitation on the pillars (**Fig. 5D**).*

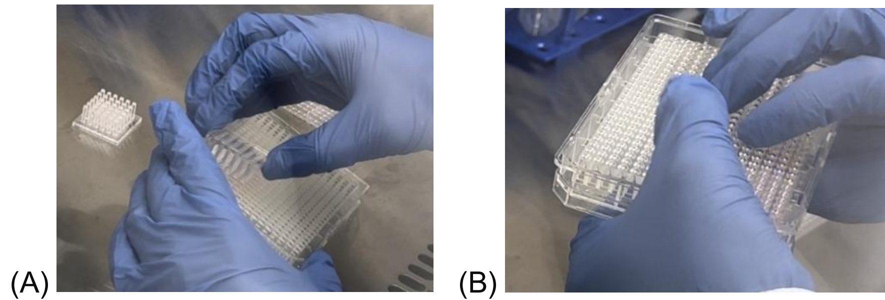


Figure 4. (A) Insertion of the 36PillarPlate in the ULA 384-well plate with spheroids. **(B)** Inverting of the sandwiched plates for spheroid transfer.

13. Incubate the inverted and sandwiched plates for 10 minutes in a 5% CO_2 incubator at 37°C to transfer spheroids from the ULA 384-well plate to the pillar plate.
14. After incubation, re-invert the sandwiched plate to bring the 36PillarPlate top and separate the pillar plate with transferred spheroids in Matrigel.
15. For complete Matrigel gelation, insert the 36PillarPlate in the 36PetriLid on a 90 x 15 mm petri dish containing 500 µL of sterile, distilled water to prevent water evaporation and incubate the petri dish with the pillar plate in a 5% CO_2 incubator for 15 minutes at 37°C.
16. Separate the pillar plate and sandwich it onto the 384DeepWellPlate with 70 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. 5C**), which go away in 1 - 2 days with medium change.*
17. Inspect the pillar plate under the microscope to ensure successful spheroid transfer (**Fig. 5**).

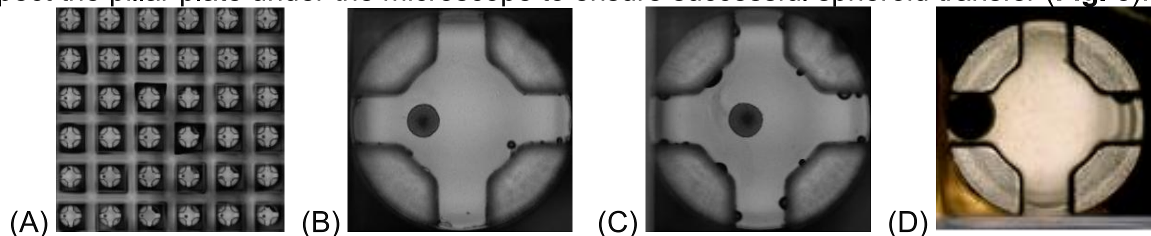


Figure 5. (A) Stitched image of the entire 36PillarPlate with spheroids transferred. **(B)** Single pillar with successful spheroid transfer. **(C)** Single pillar with micro-bubbles on the surface. **(D)** Single pillar with a spheroid in the corner due to unsuccessful spheroid transfer.

Tumor spheroid co-culture with immune cells in 36PerfusionPlate

1. Prepare 1.0 mL of immune cell suspension by gently mixing a cell pellet of 0.8 - 4 x 10^6 cells/pellet with 1.0 mL of culture medium in a 15 mL centrifuge tube.
2. Resuspend the cells in 5 mL of culture medium.
   Note: *Users are recommended to adjust the immune cell concentration based on their desired E: T (effector-to-target) ratio.*
3. Set the digital rocker to a 10° tilting angle (5° per second) with a 30-second interval between tilting angle changes to generate bidirectional flow in the perfusion plate (**Fig. 1**).

   Note: *The tilting angle influences the flow rate in the perfusion plate, whereas the interval is determined by the time necessary to drain the cell growth medium in the upper reservoirs in the perfusion plate. With a higher tilting angle, a shorter interval is required.*

Figure 1. OrganoFlow L digital rocker for loading multiple perfusion plates.

4. Dispense 400 µL of immune cell suspension in each reservoir of a 36PerfusionPlate (800 µL cell growth medium per fluidic channel) and place it on a flat surface for 1 - 2 minutes to fill all perfusion wells with the cell growth medium (**Fig. 2**).
   Note: *Adding an excessive volume of cell culture medium to the perfusion plate can cause overflow after the pillar plate is sandwiched and the assembly is placed on the digital rocker. Avoid wetting the bottom of the pillars with cell culture medium, as it can lead to cross-talk or contamination during dynamic cell culture. If any perfusion wells are not filled with cell growth medium, manually dispense 60 µL of the medium in the empty perfusion wells.*

Figure 2. Manual dispensing of a cell growth medium in reservoirs of the 36PerfusionPlate.

5. Cover the 36PerfusionPlate with a lid and place it on the digital rocker in a 5% CO_2 incubator at 37°C for 30 minutes.
   Note: *Ensure uniform flow of the cell growth medium in the perfusion plate by verifying the levels in the upper and lower reservoirs and the perfusion wells. Pre-warming the cell growth medium ensures minimal bubble formation on the 36PillarPlate after loading cells/spheroids in hydrogel.*
6. Take the perfusion plate out of the incubator and check the level of the cell growth medium in upper and lower reservoirs as well as perfusion wells (**Fig. 3**).

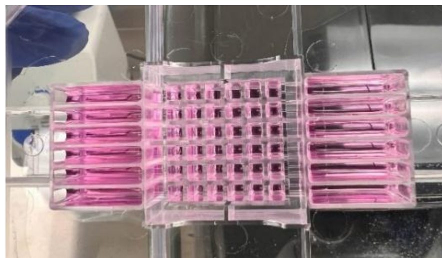


Figure 3. Uniform level of the cell growth medium in the 36PerfusionPlate.

7. Sandwich the pillar plate with spheroids onto the perfusion plate and cover the sandwiched plates with a lid (**Fig. 4**).

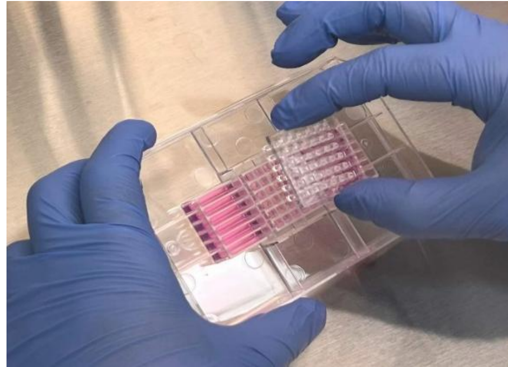


Figure 4. Sandwiching the 36PillarPlate with tumor spheroids onto the perfusion wells of the 36PerfusionPlate for dynamic cell culture.

8. Inspect the sandwiched plates under the microscope to ensure uniform cell loading on the entire pillar plate.
9. Place the sandwiched plates on the digital rocker in the 5% CO_2 incubator at 37°C and perform dynamic cell culture with medium changes every 2 - 3 days (**Fig. 5**).
   Note: *Ensure uniform flow of the cell growth medium in the perfusion plate from the sideview.*

Figure 5. Dynamic cell culture on the 144PillarPlate with the 144PerfusionPlate on the digital rocker at a 10° tilting angle with a 30-second interval.

10. To replace the old cell culture medium, carefully separate the pillar plate containing cells from the perfusion plate, and immediately insert the pillar plate into the 36PetriLid (or 144PetriLid) positioned on a 90 x 15 mm petri dish (**Fig. 6**).
    Note: *Do not place the wet pillar plate directly on a flat surface. This causes cell culture medium to drip from the pillar tip to the bottom of the pillar. If the bottom of the pillar becomes wet, it can lead to "overflow" when the pillar plate is reinserted into the perfusion plate. Therefore, always keep the pillar plate inserted in the PetriLid during medium replacement to prevent pillar bottom wetting and minimize evaporation of residual moisture.*

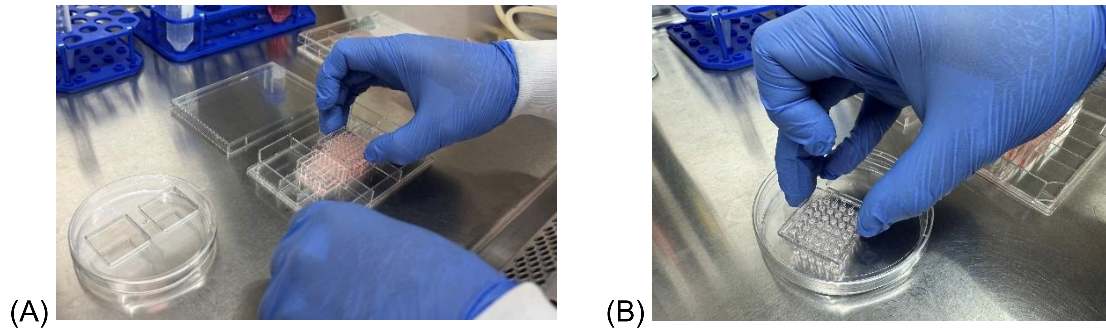
(A) (B)


Figure 6. (A) Separation of the 36PillarPlate from the 36PerfusionPlate. **(B)** Insertion of the 36PillarPlate into the 36PetriLid placed on a 90 x 15 mm petri dish to maintain the pillars in a downward orientation and prevent wetting of the pillar bottoms during medium replacement.

11. While holding the perfusion plate at approximately a 45° angle, allow the old cell culture medium to drain toward the lower reservoirs. Aspirate and remove the medium completely from the far end of the lower reservoirs using a Vacusafe vacuum aspiration system (Integra Biosciences) (**Fig. 7**).

Note: *Ensure complete removal of the old medium from all perfusion wells and reservoirs to prevent dilution of freshly added medium and accumulation of waste metabolites.*

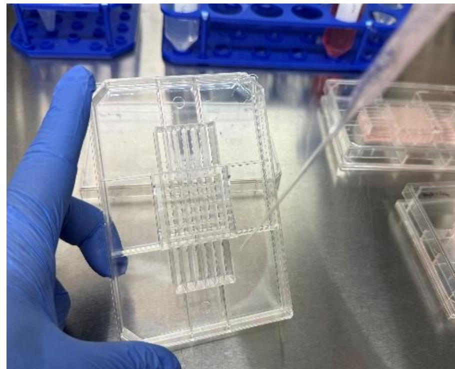


Figure 7. Vacuum aspiration of the old medium from the far end of the lower reservoirs.

12. Carefully remove the pillar plate from the 36PetriLid (or 144PetriLid) and reinsert it onto the corresponding perfusion plate (**Fig. 8**).

Note: *Before insertion, ensure that all pillars are properly aligned with the perfusion wells. Misalignment may cause mechanical disturbance of the cultured cells on the pillars.*

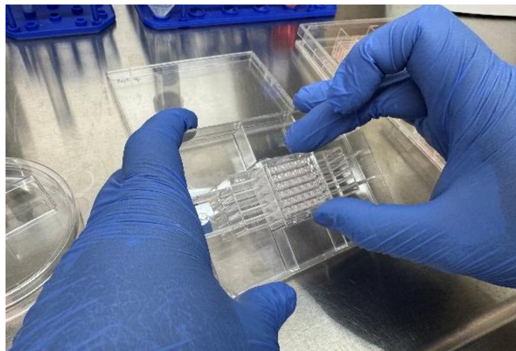


Figure 8. Reinserting the pillar plate onto the perfusion plate after old medium removal.

13. Slowly dispense 400 µL of fresh, warm immune cell suspension into each reservoir of the 36PerfusionPlate (for a total of 800 µL per fluidic channel) (**Fig. 9**).

Note: *Dispense the fresh cell suspension from the far end of the reservoirs (away from the microchannels) to prevent overflow in the perfusion wells caused by capillary action.*

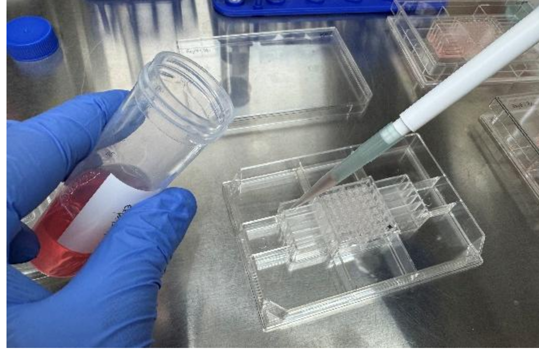


Figure 9. Dispensing fresh, cell suspension from the far end of each reservoir in the 36PerfusionPlate to prevent capillary-driven overflow into the perfusion wells.

14. Leave the sandwiched plates on a flat surface for approximately 1 minute to allow the medium to equilibrate and distribute evenly across all perfusion wells and channels.
15. Place the sandwiched plates with fresh immune cell suspension on the digital rocker and resume dynamic cell culture inside the CO_2 incubator.