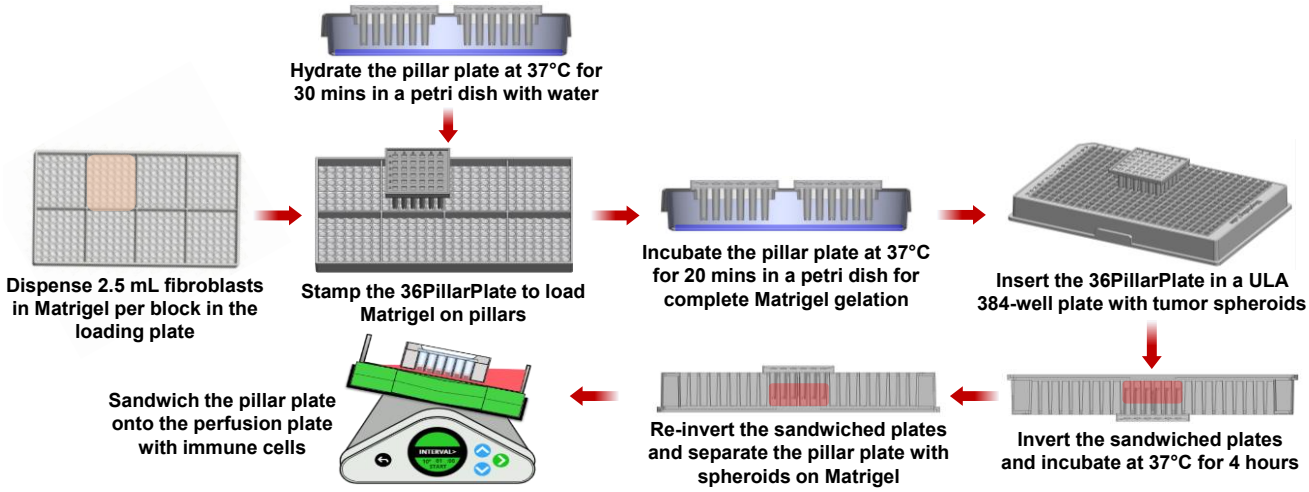
Immune-Tumor Cell Co-Culture in a Pillar/Perfusion Plate

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)
- 36PerfusionPlate (Bioprinting Laboratories Inc., Cat. no. 36-02-00)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-03-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- Ultralow attachment (ULA) 384-well plate (S-BIO, Cat. no. MS-9384UZ; FaCellitate, Cat. no. F224384)
- Growth factor reduced Matrigel (Corning, Cat. no. 354230)
- Deep petri dish, 100 mm x 20 mm (Corning, Cat. no. 70165-102)
- 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)

Methods:



The overall protocol of tumor spheroid transfer and attachment to Matrigel containing fibroblasts on the pillar plate using an ultralow attachment (ULA) 384-well plate and dynamic immune cell perfusion.

Tumor spheroid transfer from ultralow attachment (ULA) 384-well plate to 36PillarPlate and dynamic culture in 36PerfusionPlate

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 35 - 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 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 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 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.*
5. 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.*
6. 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 Matrigel 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.*

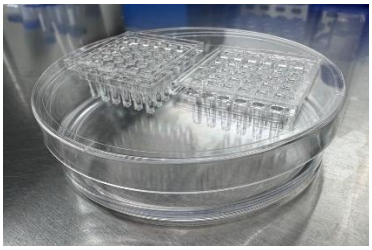


Figure 1. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

Preparation of Matrigel containing fibroblast

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 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 2 - 2.5 mL of the fibroblast suspension in Matrigel into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 2**).

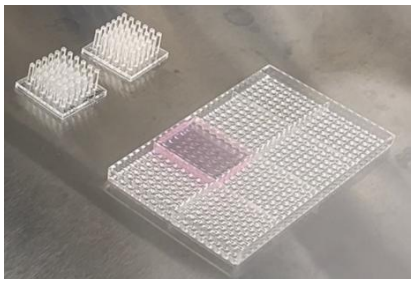


Figure 2. Dispensing 2 - 2.5 mL of single cell suspension in Matrigel per block in the LoadingPlate for rapid loading of cells in Matrigel on the pillar plate.

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

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

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 2 - 2.5 mL of the cell-Matrigel solution, it is possible to prepare at least four 36PillarPlates (5 µL Matrigel per pillar or 180 µL per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust cell-Matrigel loading, gently wiggle the pillar plate during stamping. Add additional cell-Matrigel solution to the LoadingPlate as needed.*

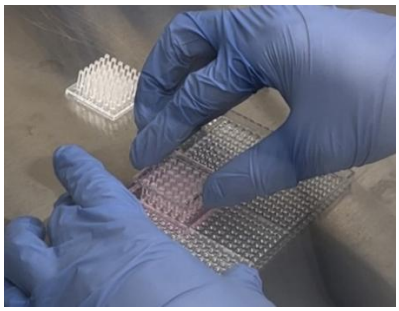


Figure 3. Stamping of the 36PillarPlate on the LoadingPlate to load single cell suspension in Matrigel on pillars.

Complete Matrigel gelation prior to spheroid transfer

11. For minimizing water evaporation during complete Matrigel gelation, insert two 36PillarPlates loaded with the cell-Matrigel mixture into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile, distilled water (**Fig. 1**).
12. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 20 minutes to allow complete gelation of the Matrigel.
    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

13. Align one corner pillar of the 36PillarPlate with the corresponding corner well of the ULA 384-well plate. Carefully sandwich the pillar plate with fibroblasts in Matrigel onto the ULA 384-well plate containing tumor 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. 4**).
    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.*

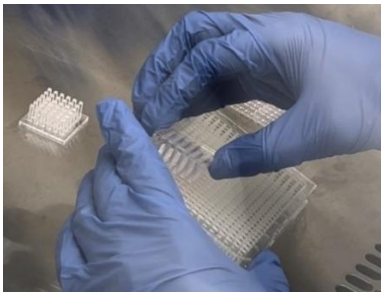
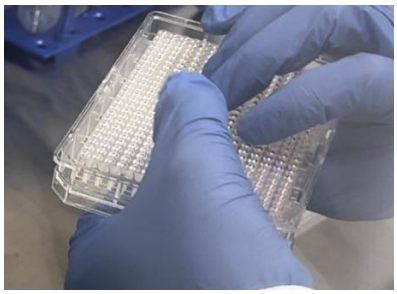
(A)  (B) 

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

14. 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 Matrigel.

15. 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 Matrigel.
16. Immediately insert the pillar plate into the 36PerfusionPlate containing 800 µL/channel of prewarmed growth medium.
17. Inspect the pillar plate under a brightfield microscope to confirm successful spheroid transfer onto the pillars.
18. Culture the spheroids on the pillar plate in a humidified 5% CO_2 incubator at 37°C, replacing the culture medium every 2 - 3 days for culture using the 36PerfusionPlate.
    Note: *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 protocol titled "Dynamic Cell Culture in Perfusion Plate" for additional details.*

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. Immune cells may aggregate during dynamic culture, which can be minimized by using FCeM Advance-CR from Nissan Chemical America Corp.*
3. Set the digital rocker to a 10° tilt angle (5° per second) with a 30-second rocking interval to generate bidirectional flow in the perfusion plate (**Fig. 1**).

   Note: *The tilt 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 tilt 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.*

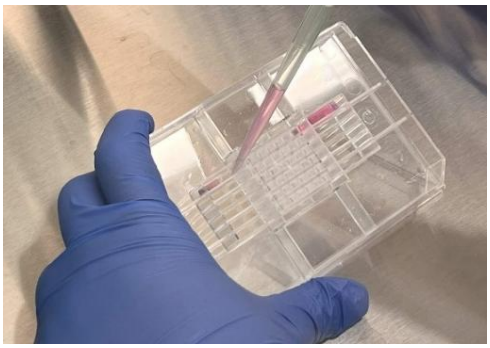


Figure 2. Manual dispensing of a cell growth medium in reservoirs of the 36PerfusionPlate.

5. Cover the 36PerfusionPlate with a lid and place it on the digital rocker in a humidified 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 medium levels in the upper and lower reservoirs as well as the perfusion wells. Pre-warming the cell growth medium helps minimize bubble formation on the 36/144PillarPlate after loading cells or 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**).

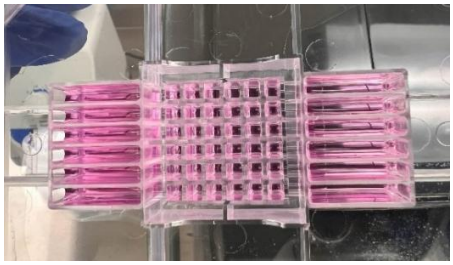


Figure 3. Uniform level of the cell growth medium in the 36PerfusionPlate.

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

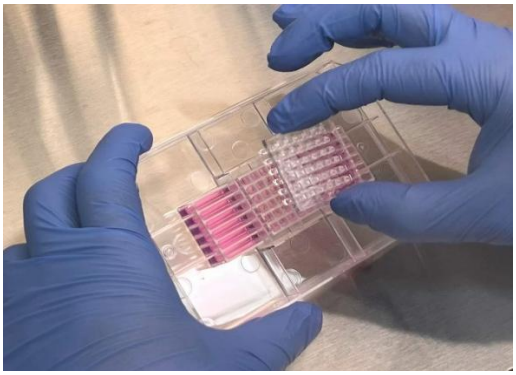


Figure 4. Sandwiching the 36PillarPlate with tumor spheroids onto the perfusion wells of the 36PerfusionPlate for dynamic cell culture.

8. Inspect the sandwiched plates under a brightfield microscope to ensure uniform cell loading on the entire pillar plate.
9. Place the sandwiched plates on the digital rocker in a humidified 5% CO_2 incubator at 37°C and perform dynamic cell culture with medium changes every 2 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° tilt 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 positioned on a 100 x 20 mm petri dish containing 5 mL of sterile distilled water (**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.*

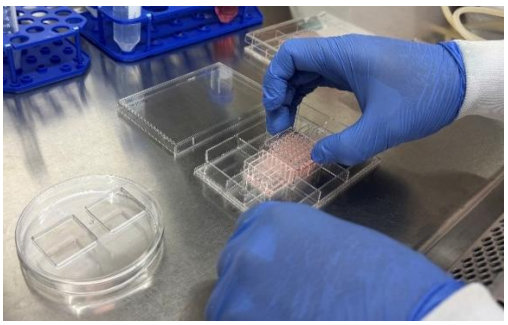
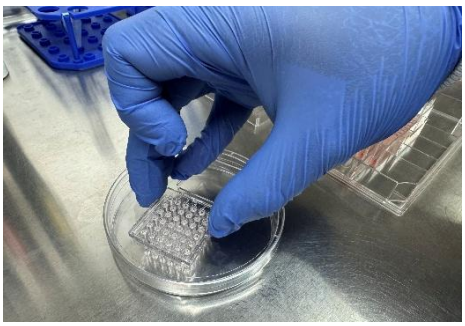
(A)  (B) 

Figure 6. (A) Separation of the 36PillarPlate from the 36PerfusionPlate. **(B)** Insertion of the 36PillarPlate into the 36PetriLid placed on a 100 x 20 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.*

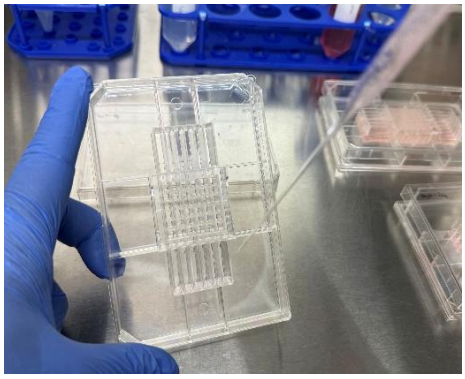


Figure 7. Vacuum aspiration of the old medium from the far end of the lower reservoirs.

12. Carefully remove the pillar plate from the 36PetriLid 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.*

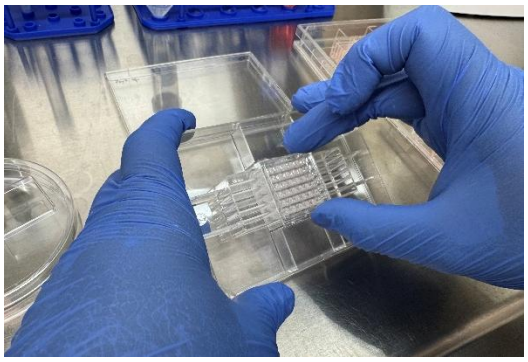


Figure 8. Reinserting the pillar plate onto the perfusion plate after old medium removal.

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.*

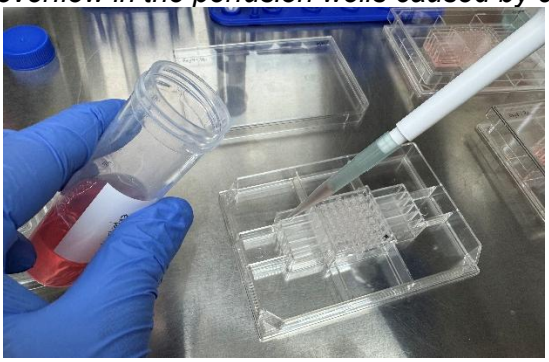


Figure 9. Dispensing fresh, cell suspension from the far end of each reservoir in the 36PerfusionPlate to prevent capillary-driven overflow into the perfusion wells.

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.