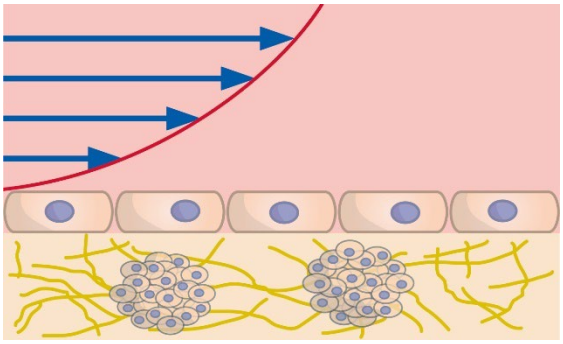
Co-Culture of Tumor Spheroids and HUVEC Under Flow Using the µ-Slide I Luer 3D

To mimic the complex 3D structures of tumors and their microenvironments, 3D tumor spheroid models have become important in vitro model systems. This protocol describes the co-culture of tumor spheroids embedded in a Collagen Type I with endothelial cells (HUVECs) using the µ-Slide I Luer 3D. This model also allows for imitating physiological conditions by applying flow and is particularly useful for studying tumor angiogenesis and metastasis.



ibidi offers various solutions for spheroid and organoid cultivation with perfusion:

- µ-Slide I Luer 3D
- µ-Slide Spheroid Perfusion
- µ-Slide III 3D Perfusion
- µ-Slide With Multi-Cell µ-Pattern ibiTreat
- ibidi Collagen Type I
- ibidi Pump Systems and Accessories

Related Documents

- Application Note 13: Endothelial Cells Under Perfusion (PDF)
- Application Note 16 Immunofluorescence Staining Using the µ-Slide 8 Well high (PDF)
- Application Note 26: Collagen I Gel for 3D cell culture (PDF)
- Application Guide 34: Cell Culture Under Flow (PDF)

Related Software

- ibidi Collagen Calculator

Keywords:

3D culture models, Tumor microenvironment, 3D Co-culture; Endothelial cells, Tumor spheroids, Tumor angiogenesis

1 Material

Important Note: This protocol is optimized for MCF tumor spheroids and HUVEC cells; please adapt the reagents and buffers when using other spheroids or endothelial cells.

1.1 Reagents and Buffers

- Human umbilical vein endothelial cells (HUVEC, 12203, Promocell)
- Tumor-Spheroids (here we used MCF-7, CLS order number: Cryovial 300273, Vital: 330273)
- Endothelial Cell Growth Medium (ECGM, Promocell, C-22010)
- Endothelial Cell Growth Medium 2 (ECGM 2, Promocell, C-22011)
- PBS (14190144, Gibco)
- Accutase (A1110501, Gibco)
- Collagen Type I, Rat Tail, non-pepsinized, 5 mg/ml (ibidi, 50301), diluted to 4 mg/ml in 17.5 mM acetic acid17.5 mM acetic acid
- 10x RPMI 1640 (Sigma, R1145)
- 1x RPMI 1640 (Sigma, R8758)
- Media supplements (e.g., L-glutamine, depending on your cell type)
- NaOH in ultrapure H_2O, 1 M
- $NaHCO_3$ 7.5% (Sigma, S8761)
- Sterile, ultrapure water

1.2 Equipment

- µ-Slide I Luer 3D ibiTreat (87176)
- ibidi Pump System (10902) including a Perfusion Set, grey (10968)
- Standard cell culture equipment (sterile working bench, cell detachment kit, culture flasks, pipets, tips, petri dish etc.)
- Inverted microscope
- Ice and cooling rack

Important Note: To avoid air bubbles, the degassing of the Perfusion Set and the medium is critical. Place the following parts inside the incubator **one day before starting** the experiment. Sterility is maintained as long as the packaging is not opened.

- Perfusion Set(s) (within the packaging)
- Cell culture medium for cell seeding (add the volume needed to a small vessel, and loosen the cap slightly)

This procedure is necessary because of the temperature dependency of gas solubility in water and plastic. At higher temperatures, water and plastic can absorb less gas than at lower temperatures.

2 Preparation of the 3D Gel Containing Spheroids

Perform all the following steps under sterile conditions.

Important Note: Previously generated spheroids are required for the next steps of this co-culture experiment. Some ibidi solutions for generating spheroids are the µ-Slide Spheroid Perfusion (ibidi, 80350) described in Application Note 63, or Bioinert ULA Labware (ibidi 81150, 80800 and 80420).

1. Retrieve previously generated spheroids (e.g., using the µ-Slide Spheroid Perfusion according to the Instructions) and collect them in a tube under the flow hood at room temperature.
2. If supplements are required in the gel matrix, add them to the 1x cell culture medium and put them on ice in the flow hood (Application Note 26).
3. Place all remaining ingredients and a sterile tube with sufficient capacity for the total gel volume on ice in the flow hood.

Important Note for Pipetting the Collagen Gel

Always use precooled pipet tips (4°C) for pipetting the gel.

Reverse pipetting is recommended for all steps of preparing the Collagen I gel matrix due to the high viscosity. Press the pipette to the second pressure point and fill the complete pipette tip with gel. Dispense the gel only until the first pressure point is reached. This leaves a residual amount of gel in the pipette tip to be discarded, but the volume is much more accurate. Alternatively, you can use pipettes designed for high viscosity solutions. Among others, we recommend Eppendorf Visco Tips or Gilson Microman E.

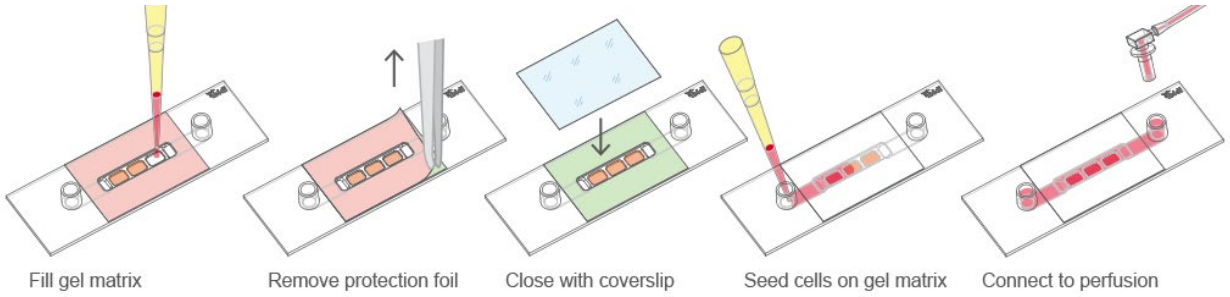
Note: Even at 4°C, the gel mixture can be used for a maximum of 5 minutes before partial gelation occurs.

4. Pipet all ingredients except the collagen and the cell suspension in the order listed in Table 1 to the tube, keeping it on ice. Mix by pipetting up and down and put back on ice.

   Note: The following steps refer to Table 1. Use the ibidi Collagen Calculator for variations of the protocols below, i.e. if different concentrations are needed of the collagen stock solution, the NaOH or the collagen gel.
5. Make sure the Collagen Type I, Rat Tail is diluted to 4.0 mg/ml in 17.5 mM acetic acid. Check the Certificate of Analysis (CoA) for the lot-specific collagen concentration, described in more detail in Application Note 26: Preparation of Collagen I Gels.

   Note: Before diluting the collagen, it must be thoroughly mixed by pipetting up and down several times to create a homogeneous solution.
6. Add the collagen to the mixture prepared in step 5. Mix well by pipetting while always keeping the tube on ice.
7. Add the prepared spheroid suspension to the mixture. Try to pick up as many spheroids as possible in the 50 µl volume. In this final step, mix the sample by short vortexing.
8. The mixture is now ready to be pipetted into the µ-Slide I Luer 3D. Keep the slide on ice during pipetting.

Tip: To avoid scratched originating from the ice, put the µ-Slide in a petri dish and put the petri dish with the slide on ice.



Step-by-step graphical protocol using the µ-Slide I Luer 3D.

9. Remove the protection foil on the upper side of the slide and fill each well with 16µl of the collagen gel with the spheroids. Avoid air bubbles.
10. Next, place the coverslip on the sticky part of the slide. Make sure the adhesive area is tightly sealed by pressing to tighten the connection.
11. Cover the Luer adapters with the supplied caps to maintain sterility and put the slide with the gel into a cell culture incubator (37°C, 5% CO_2) for 45 minutes for gelation.
12. After gelation, collagen fibrils will be visible using phase contrast microscopy with a 10x objective lens.

Table 1 Pipetting scheme for making gels using Collagen I, Rat Tail with RPMI. Volumes in µl. All ingredients are listed in the order of pipetting.

RPMI 1640 Final Collagen I, Rat Tail Concentration 1.5 mg/ml)	
10x RPMI 1640	20
NaOH 1M	2.0
H_2O	60.2
$NaHCO_3$ 7.5%	5.3
1x RPMI 1640 (optionally with supplements)	50
Collagen I 4 mg/ml	112.5
Cell suspension in 1x Medium	50
Total volume	300

3 Cell Seeding of the Endothelial Cells

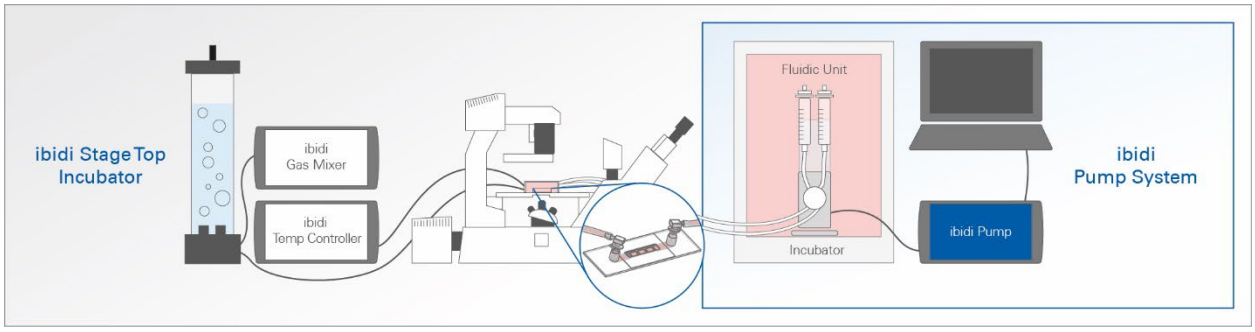
Perform all the following steps under sterile conditions.

1. For perfusion with the ibidi Pump System prewarm the Perfusion Set and the Endothelial Growth Medium (ECGM and ECGM 2) by putting both one day before the experiment into the incubator at 37°C, as described in 1 Material.
2. Treat the HUVECs with Accutase for 1 –2 min for detachment.
3. Harvest the cell suspension, centrifuge, and dilute it in a low amount of culture medium (ECGM) for counting.
4. Count the cells and adjust them to the final concentration of 1.5 × 10^6 cells/ml in culture medium.
5. Apply ca. 250 µl cell suspension into the channel using a biocompatible 1 ml syringe.
6. Remove the leftover cell suspension from the Luer adapters with a standard pipette tip, cover the Luer adapters with the supplied caps to maintain sterility.
7. Put the slide and a wet tissue in a Petri Dish and place in a cell culture incubator (37°C, 5% CO_2)

4 Connecting to the ibidi Pump System for Perfusion

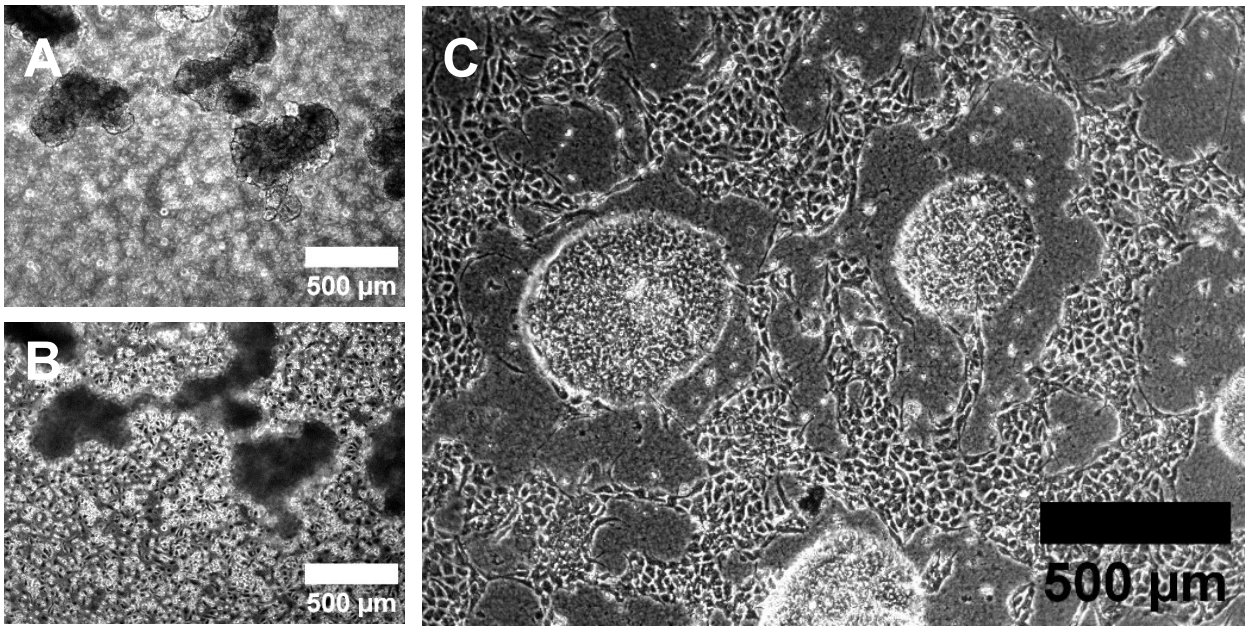
Important Note: Endothelial Growth Medium 2 contain growth factors like VEGF and EGF inducing the migration and sprouting of the cells.

1. During cell incubation, prepare the ibidi Pump System as described in the ibidi Pump System Instructions. Fill the ECGM2 medium into the reservoirs.
2. After incubating for 2 hours, connect the slide to the perfusion system filled with the ECGM 2. (AN 13: Endothelial Cells Under Perfusion, AN 34: Cell Culture Under Flow)
3. For time-lapse series imaging, place the slide into the stage top incubator (e.g., ibidi Stage Top Incubator) on the microscope and start the time-lapse measurement. For single-frame imaging, prepare the microscope settings in advance to keep the imaging period as short as possible. Put the slide back into the incubator when it is not being imaged. For an endpoint analysis, please fix the cells at the end of the experiment and continue with your staining (see 5) or downstream protocol.



Experimental setup of the timelapse measurement under perfusion

5 Example of Live-Cell Phase Contrast Imaging



Phase contrast images with the spheroids (A) in focus and the HUVEC 2h after seeding in focus (B), grown on top of the gel. C) Spheroids and HUVEC after 3 days in co-culture under perfusion (~0.5 ml/min flow rate)

6 Staining Protocol

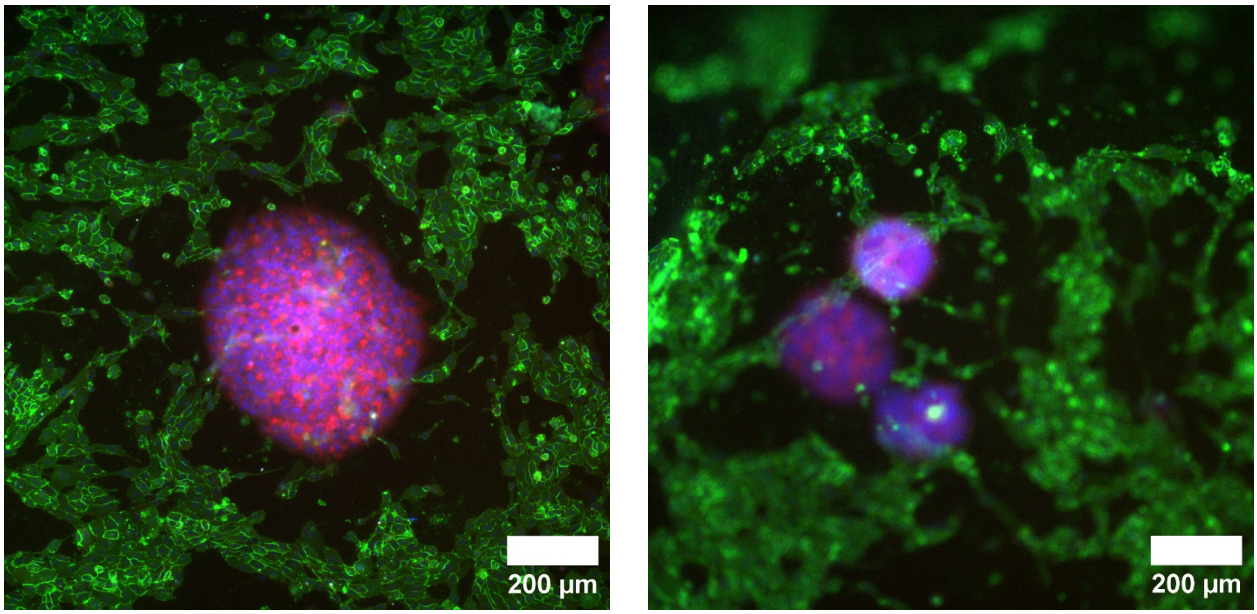
6.1 Material

- PBS (14190144, Gibco)
- Formalin, 10%, ready to use (HT5011, Sigma Aldrich)
- Alexa Fluor 488 labelled Anti-CD31 Antibody, MA5-18135 Invitrogen).
- Phalloidin-iFluor 647 Reagent (ab176758, Abcam)
- 4′,6-diamidino-2-phenyl-indole (DAPI) (D9542 Sigma Aldrich)
- Triton-X-100 (A16046, Thermo Fisher Scientific)
- Perforation Buffer (0.5% Triton X-100 in PBS)
- Blocking Buffer (1% BSA + 0.2% Triton X-100 in PBS)
- Antibody Dilution Buffer (1% BSA + 0.05% Triton X-100 in PBS)
- Bovine Serum Albumin (BSA) (A1470-10G, Sigma Aldrich)

6.2 Method

1. Prepare enough Perforation Buffer and Blocking Buffer for your experiment.
2. Disconnect the slide from the pump.
3. Aspirate the cell culture medium from the Luer port using a pipette.
4. Gently wash the cells twice with 200 µl PBS by filling PBS in one Luer port until you see the fluid coming out at the other Luer port.

5. Replace the PBS with 10% Formalin to fix the cells. First, remove the PBS from the Luer port, then add 200 µl of Formalin to the Luer port. Let it flow through the channels, then remove it from the Luer port and add another 200 µl of Formalin to the Luer port. Incubate the cells for 15 minutes.
6. Remove the Formalin and wash the cells four times with 200 µl PBS.
7. Incubate the cells in 200 µl Perforation Buffer for 10 minutes (exchange the PBS with the Perforation Buffer like in Step 5).
8. Remove the Perforation Buffer and wash the cells twice with 200 µl PBS.
9. Block with 200 µl Blocking Buffer for 30 minutes (exchange the PBS with the Blocking Buffer like done with Formalin).
10. During the blocking step, prepare enough Antibody Dilution Buffer to dilute the primary and secondary antibodies.
11. Dilute the labeled Antibody (or the primary antibody) in Antibody Dilution Buffer (1:100 dilution)
12. Exchange the Blocking Buffer with 200 µl of the primary antibody solution and incubate the cells overnight at 4°C
13. In all the following steps, the samples should be kept in the dark whenever possible to avoid photobleaching effects.
14. Wash three times with 200 µl Blocking Buffer.
15. Dilute (the secondary antibody and) the phalloidin and DAPI in the same Antibody Dilution Buffer (Phalloidin 647 Conjugate 1:1000 dilution, DAPI 10 µg/ml).
16. Exchange the Blocking Buffer with 200 µl of the secondary staining solution and incubate for 2 h in the dark.
17. Wash three times with 200 µl Blocking Buffer.
18. Exchange the Blocking Buffer with PBS (200 µl per channel)
19. Store the slide at 4°C in the dark until imaging. Ideally, proceed immediately with imaging since extended storage periods could reduce image quality.



Staining of the spheroids and HUVEC cells after 3 days in co-culture under perfusion; Nuclei: DAPI (blue), Actin Filaments: Phalloidin-iFluor 647 (red), CD31 (specific for the HUVEC): Alexa Fluor 488 labelled Anti-CD31 Antibody (green)