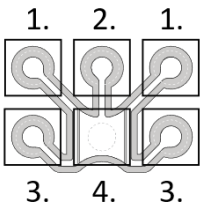
1. Objective

Loading extracellular matrix (ECM) gel and seeding of endothelial cells in the OrganoPlate® Graft for preparation of a vascular network.



1. Perfusion inlets
2. Gel inlet
3. Perfusion outlets
4. Graft chamber with inlet

***Figure 1**: Schematic representation of an OrganoPlate® Graft tissue chip.*

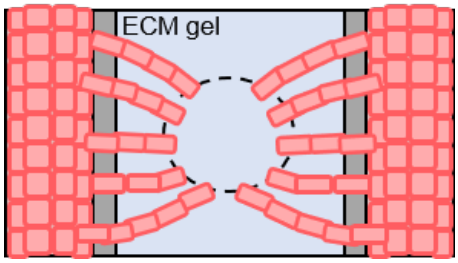


***Figure 2**: Cells are seeded in the perfusion channels of an OrganoPlate® Graft, against an ECM gel. A gradient of angiogenic factors induces the formation of a vascular network.*

2. Background

The OrganoPlate® Graft is a 3D cell culture platform that can be used to vascularize tissues such as organoids, spheroids, and explants. This protocol describes the formation of a vascular network in the OrganoPlate® Graft (see figure 1 and 2). Endothelial cells are seeded in the perfusion channels against an ECM gel. A gradient of angiogenic factors is added to the graft chamber and induces the formation of angiogenic sprouts, resulting in a vascular network. Morphology and function of the vessels can be assessed by microscopy, a perfusion assay, or other functional assays.

This protocol describes the formation of a vascular network using primary human umbilical vein endothelial cells (HUVECs) from Lonza. Different cell types may require optimization of the culture medium and angiogenic cocktail composition.

3. Materials

- OrganoPlate® Graft (MIMETAS, 6401-400-B)
- Collagen-I 5 mg/mL (AMSbio Cultrex® 3D collagen I rat tail, 5 mg/mL, #3447-020-01)
- 1 M HEPES (ThermoFisher 15630-122, pH 7.2-7.5)
- 37 g/L $NaHCO_3$ (Sigma S5761-500G, dissolve in sterile MilliQ water, adjust pH to 9.5 using NaOH)
- Human umbilical vein endothelial cells (HUVECs, Lonza, C2519AS)
- EGM-2 medium (Lonza, CC-3162)
- Components for angiogenic cocktail (see table 1)
- Repeating pipette for gel loading and cell seeding. We recommend the eLINE® electronic pipette (Sartorius, #735021 (previously #730021)) and corresponding pipette tips or the Eppendorf® ep Dualfilter tips (Eppendorf, 022491211 / 0030077512)
- Multichannel pipette (1200 µL and 300 µl)
- Multichannel tips
- Crushed ice

4. Procedure

An ECM gel is loaded in the gel inlet of the OrganoPlate® Graft and fills the graft chamber. After gel polymerization, endothelial cells are seeded in the perfusion channels and perfusion is started to aid the formation of endothelial vessels. A gradient of pro-angiogenic factors is then added to the graft chamber to induce formation of a vascular network (see figure 3).

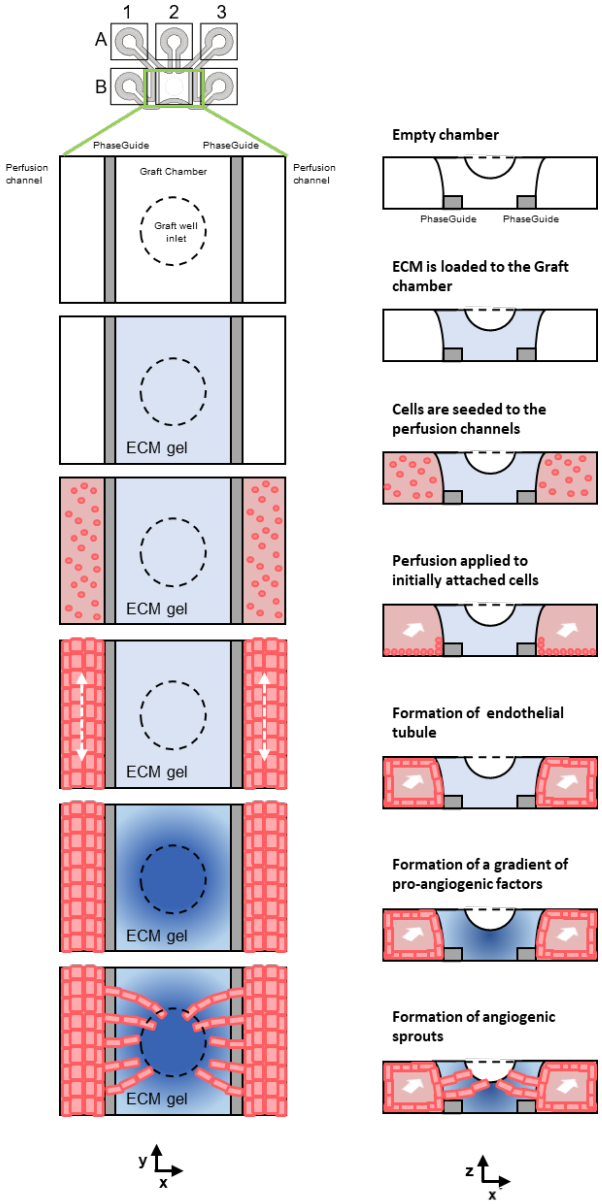


***Figure 3**: Schematic representation of formation of a vascular network in the OrganoPlate® Graft*

Load ECM gel in the OrganoPlate® Graft

Note: avoid touching the bottom glass plate of the OrganoPlate® Graft

1. Take the OrganoPlate® Graft from the packaging
2. Prepare the required amount of ECM gel (e.g. 2.5 µL gel per chip + 40% extra)
    a. Collagen-I 4 mg/mL preparation
        i. Place an Eppendorf tube on ice
        ii. The collagen-I 4 mg/mL gel is prepared by mixing 1 M HEPES, 37 g/L $NaHCO_3$, and 5 mg/mL collagen-I in a 1:1:8 ratio. For example, to prepare 100 µL of gel:
            - Place an Eppendorf tube on ice
            - Mix 10 µL of 1 M HEPES with 10 µL of 37 g/L $NaHCO_3$
            - Add 80 µL of collagen-I 5 mg/mL to the HEPES/$NaHCO_3$ mixture

        iii. Prepare at least 100 µL of total gel volume to ensure proper mixing of all components
        iv. Mix well by pipetting the mixture up and down >20 times, while keeping it on ice
        v. If bubbles are formed, briefly spin the tube down (~5 seconds)
        vi. Use gel immediately after preparation (within 10 minutes)

3. Dispense the gel into the gel inlet (columns 2, 5, 8, 11, 14, 17, 20, 23; rows A, C, E, G, I, K, M, O) using the Sartorius eLINE® electronic pipette
    a. Gently place your pipette tip on top of the hole in the bottom of the well and dispense the gel. Contact between the pipette tip and the hole is essential for gel loading. Correct positioning of the gel on top of hole allows capillary forces to pull the gel into the microfluidic gel channel (see figure 4)
    b. The optimal loading volume depends on several factors, such as the viscosity of the gel and the temperature in the lab
    c. Start by loading 2.5 µL gel per gel inlet for the OrganoPlate® Graft (6401-400-B)
    d. In case of incomplete gel filling, increase the loading volume (i.e. to 3 µL)
    e. In case the gel overflows from the gel chamber into the adjacent perfusion channels, reduce the loading volume (i.e. to 2 µL)
    f. For examples of correct gel filling in the OrganoPlate® Graft, see figure 5

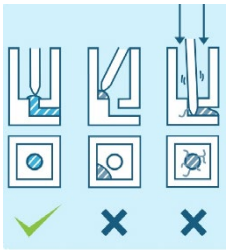


***Figure 4**: Gel loading*

4. Place the OrganoPlate® in a humidified incubator (i.e. 37°C, 5% CO_2) for 15 minutes to allow polymerization of the collagen-I gel
5. Add 50 µL of HBSS to the gel inlet (columns 2, 5, 8, 11, 14, 17, 20, 23; rows A, C, E, G, I, K, M, O) to prevent the gel from drying out
    a. For examples of dried out gel, see section *Trouble Shooting, figure 8.*
6. Place the OrganoPlate® Graft back in the incubator and proceed to cell seeding
    a. You can choose to proceed to cell seeding immediately or to wait until the next day. While cells generally form tubules with either option, some cells show optimal results when seeded one day after gel loading

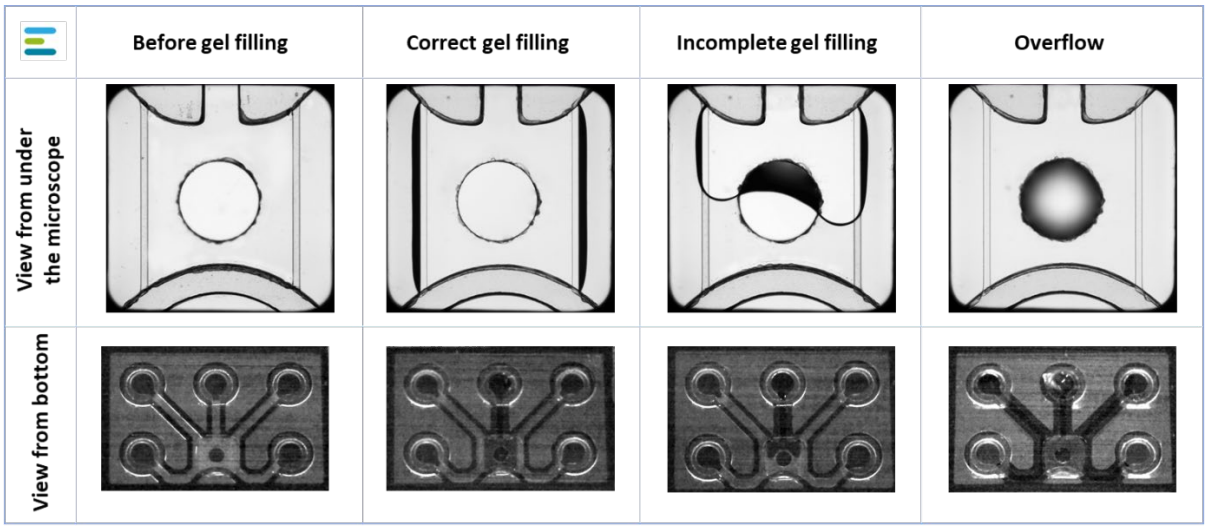


***Figure 5**: Overview of an empty chip, correct gel filling, incomplete gel filling, and overflow in the OrganoPlate® Graft*

Endothelial cell seeding

1. Harvest cells according to their dissociation protocol
2. Count the number of live cells in the cell suspension
3. Calculate the required number of cells for seeding in the OrganoPlate® Graft and pellet them
    a. The optimal cell density for seeding against ECM in the OrganoPlate® is cell type dependent (10,000 cells/µL for Lonza HUVECs)
    b. For example:
        i. Number of chips to seed: 64
        ii. Volume of cell solution to seed per perfusion channel: 2 µL → per chip: 4 µL
        iii. Seeding density: 10,000 cells/µL
        iv. You need: 64 x 4 x 10,000 = $2.56*10^6$ cells
        v. Prepare up to 25% extra: pellet $3.2*10^6$ cells
4. Resuspend pellet in [$3.2*10^6$ / 10,000 =] 320 µL medium to obtain a 10,000 cells/µL cell suspension
5. Seed 2 µL of cell suspension in the perfusion medium inlet (columns 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24; rows A, C, E, G, I, K, M, O, **see plate layout on pg.8**) using the same pipetting procedure as previously used for gel loading (see figure 4)
    a. Regularly resuspend the cell suspension during seeding to ensure homogenous cell density
    b. In case you want to include cell-free controls, introduce 2 µL of medium without cells in the top medium inlet of these chips (instead of the cell suspension)
6. Add 50 µL of medium to the perfusion medium inlet (columns 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24; rows A, C, E, G, I, K, M, O)
7. Place the OrganoPlate® Graft static in the incubator to allow cells to attach
    a. The time cells need to attach is cell type dependent and generally varies between 0.5-3 hours
8. After cells have attached, add 50 µL of medium to the perfusion medium outlet (columns 1, 3, 4, 6, 7, 8, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24; rows B, D, F, H, J, K, L, N, P).
9. Add 50 µl of media to the Graft chamber (columns 2, 5, 8, 11, 14, 17, 20, 23; rows B, D, F, H, J, K, L, N, P)
10. Remove HBSS from the gel inlet (columns 2, 5, 8, 11, 14, 17, 20, 23; rows A, C, E, G, I, K, M, O)

11. Place the plate on the MIMETAS Rocker in a humidified incubator to start cell culture (see figure 6)
    a. An inclination of 14° and an interval of 8 minutes is optimal for most vessels

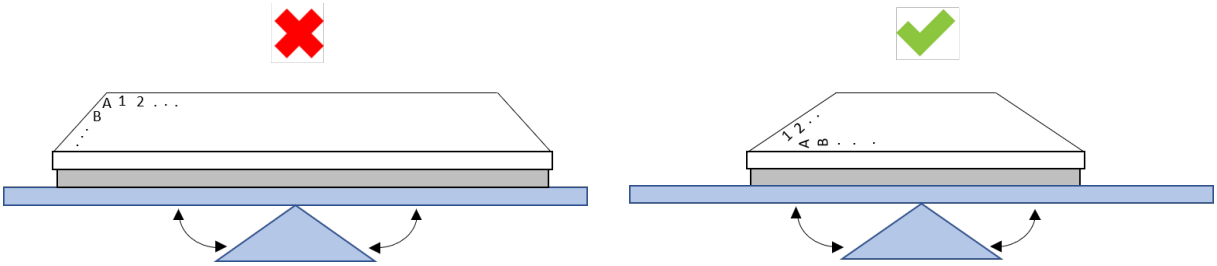


***Figure 6**: Place the OrganoPlate® on the MIMETAS rocker in the correct orientation*

12. Refresh medium every 2-3 days by aspirating and replacing the medium from medium inlets and outlets (50 µL in each) using a repeating multichannel pipette
    a. Note: to avoid disruption of the culture, insert the aspiration tip in the corner or side of each well. Avoid placing the aspiration tip directly on the holes connected to the channels/chambers.
13. An example of a tubule culture against ECM gel in the OrganoPlate® Graft is shown in figure 7.

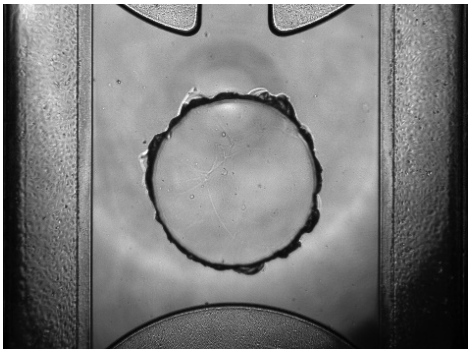


***Figure 7**: Endothelial vessels grown against an ECM gel in the OrganoPlate® Graft*

Induction of angiogenic sprouts

Angiogenic sprouting can be induced after endothelial vessels have formed in the perfusion channels. The time it takes for vessels to form is dependent on the cell source. For Lonza HUVEC, this takes approximately 3 days.

1. Prepare the stock solutions of the six angiogenic compounds as described in column 4 of table 1.
2. Prepare the final angiogenic cocktail (you need 50 µL per chip) by diluting the six angiogenic factors in EGM-2 medium according to the final concentration listed in the last column of table 1.
    a. Prepare 50 µL per chip plus 20% extra volume (for complete OrganoPlate® Graft use ~4 mL)
    b. If stored at 4°C, the cocktail can be used for up to a week after preparation
    c. When using other cell types than Lonza HUVECs, optimization of medium and angiogenic cocktail composition may be required

Table 1: preparation of angiogenic cocktail

Compound	Supplier	Cat. No.	Stock	Stock storage	Final conc.
rhVEGF-165	Peprotech	100-20	100 µg/mL in 0.1% BSA in PBS	-20°C	37.5 ng/mL
S1P	Sigma-Aldrich	S9666	1 mM in 95% DMSO/5% HCl 1M	-80°C	250 nM
PMA	Sigma-Aldrich	P1585	10 µg/mL 0.1% DMSO in MiliQ	-80°C	37.5 ng/mL
rhFGFb	Peprotech	100-18B	50 µg/mL in 0.1% BSA in PBS	-20°C	37.5 ng/mL
rhMCP-1	ImmunoTools	11343384	100 µg/mL in 0.1% BSA in PBS	-20°C	37.5 ng/mL
rhHGF	ImmunoTools	11343413	100 µg/mL 0.1% BSA in PBS	-20°C	37.5 ng/mL

3. Aspirate media from all wells of the OrganoPlate® Graft
4. Add 50 µL of angiogenic cocktail in all Graft chambers (columns 2, 5, 8, 11, 14, 17, 20, 23; rows B, D, F, H, J, K, L, N, P)
5. Add 50 µL of medium without angiogenic factors in all perfusion inlets and outlets
6. Place the plate on the MIMETAS Rocker in a humidified incubator to continue cell culture (see figure 6)
    a. An inclination of 14° and an interval of 8 minutes works well for vascular network formation

The angiogenic potential of endothelial vessels grown in OrganoPlate® Graft can vary between endothelial cell sources and media compositions. Usually angiogenic sprouts can reach the glass Graft chamber opening within 3-7 days after sprouting induction. Observe cultures daily to monitor sprouting. An example of a vascular network in the OrganoPlate® Graft is shown in figure 8.

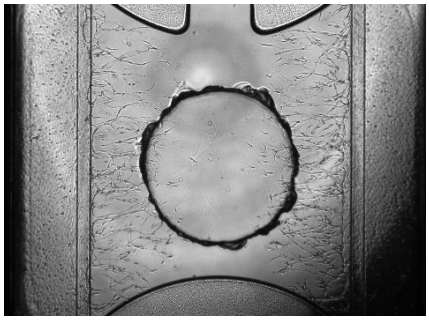


***Figure 8**: Example of vascular network formation after 3 days of sprouting induction in the OrganoPlate® Graft*

5. Troubleshooting

In some cases, the ECM gel can dry out during the gel loading and polymerization process (see figure 9). This generally happens when the loading process takes longer than expected and the gel in the chips that were loaded first has been incubated much longer than the gel in the chips that were loaded last. When gel loading goes smoothly, this problem doesn't occur. However, if loading takes longer than expected (> 10 min), check regularly under the microscope to see if the gel starts to dry out and if you observe that it is, quickly add HBSS to the gel inlet of those chips to prevent further drying.

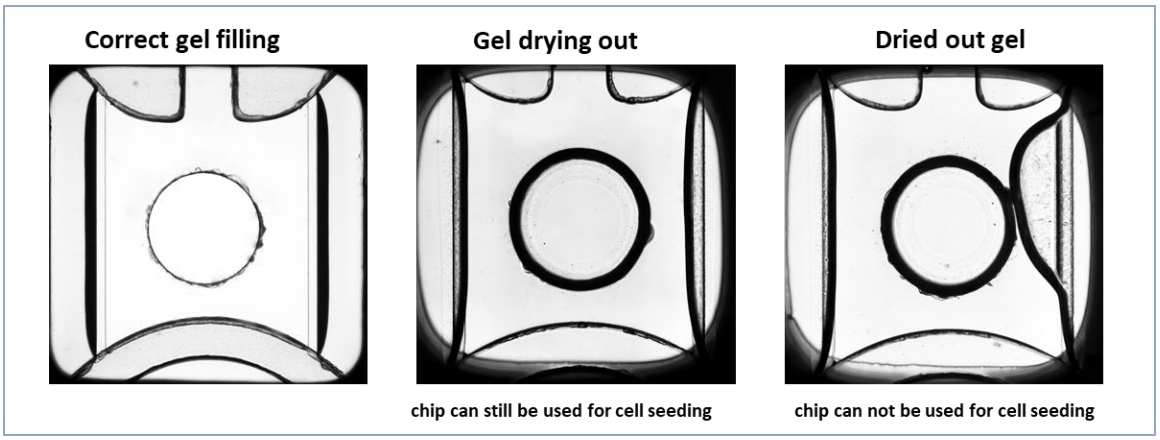
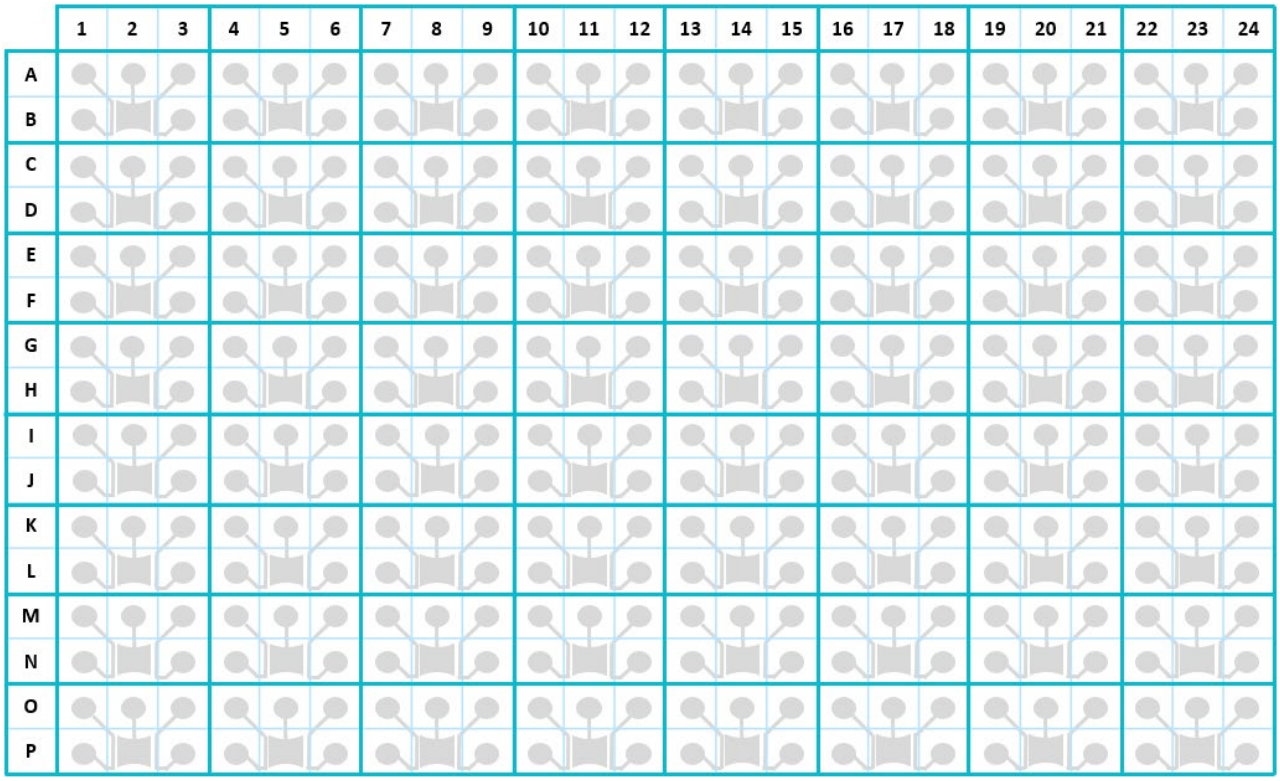


***Figure 9**: ECM gel drying out due to prolonged gel loading or polymerization*

Plate layout



MIMETAS product list

Cat. No.	Product Name
MI-AR-CC-01	OrganoReady® Caco-2
9605-400-B	OrganoPlate® 2-lane
4004-400-B	OrganoPlate® 3-lane 40
6405-400-B	OrganoPlate® 3-lane 64
6401-400-B	OrganoPlate® Graft
MI-OFPR-S	OrganoFlow® S
MI-OFPR-L	OrganoFlow® L
MI-OT-1	OrganoTEER®

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