1. Objective

Seeding of cells against extracellular matrix (ECM) gel in an OrganoPlate® 3-lane for tubule formation.

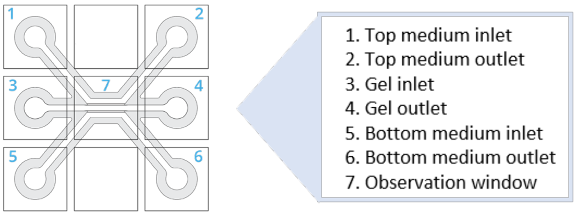


***Figure 1**: Schematic representation of an OrganoPlate® 3-lane tissue chip.*

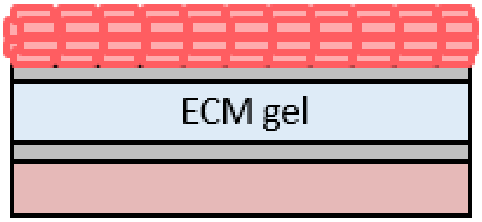


***Figure 2**: Illustration of a tubule of cells (top channel) grown against an ECM gel (middle channel) in an OrganoPlate® 3-lane tissue chip.*

2. Background

Tubular structures, such as endothelial or epithelial barrier tissues, are established in the OrganoPlate® by growing cells against an ECM gel. Morphology and function of the tubule can be assessed by microscopy, a barrier integrity assay, or other functional assays. This protocol describes the culturing of a tubule against ECM in the top lane of an OrganoPlate® 3-lane (see figure 1 and 2). The resulting tubular culture allows for access to apical and basal side of the barrier tissue.

3. Materials

- OrganoPlate® 3-lane (MIMETAS, 4004-400-B)
- OrganoFlow® S or L (MIMETAS, MI-OFPR-S or MI-OFPR-L)
- 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)
- Medium: 10 mL per OrganoPlate®
- Cells: seeding density is dependent on the cell type
- Repeating pipette for gel loading and cell seeding, we recommend:
  - The Eppendorf® Multipette® M4 with the Eppendorf® Biopur® 0.1 mL tip (VWR #613-2067) for dispensation of 2 µL, <u>or</u>
  - The Sartorius eLINE® electronic pipette (Sartorius, #735021 (previously #730021)) for accurate dispensation of volumes ranging from 0.2 to 10 µL. Use with corresponding Sartorius tips or with Eppendorf® ep Dualfilter tips (Eppendorf, 022491211 / 0030077512)
- HBSS (Sigma H6648)
- Multichannel pipette (1200 µL and 300 µl) and multichannel tips
- Crushed ice

4. Tubule seeding in the OrganoPlate®

A collagen-I ECM gel is loaded in the gel inlet of the OrganoPlate® and fills the gel channel. After polymerization of the gel, a cell suspension is seeded in the top medium inlet and fills the top medium channel. After cell attachment, medium perfusion is started to aid the formation of a tubule (figure 3).

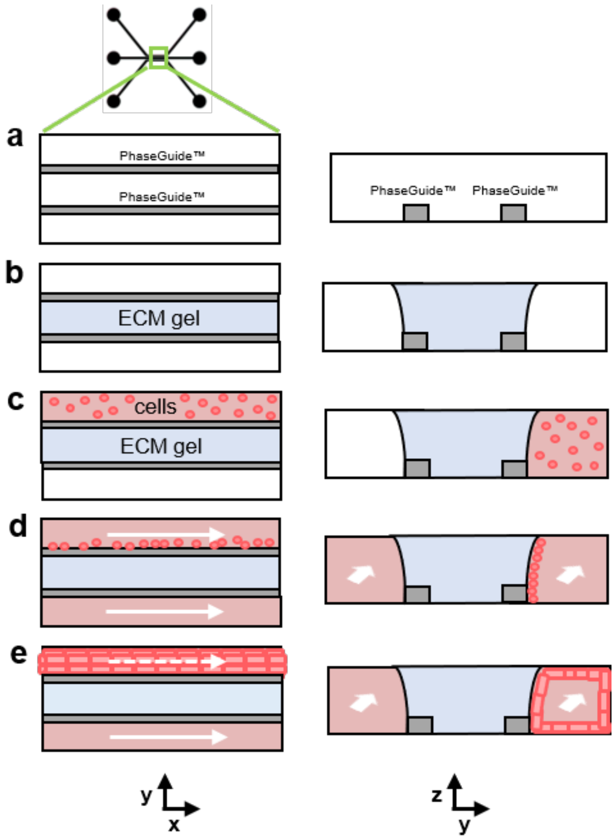


Figure 3*: Schematic representation of tubule culture against ECM gel in the OrganoPlate® 3-lane*

Load ECM gel in the OrganoPlate®

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

1. Take the OrganoPlate® from the packaging
2. Add 50 µL of HBSS to all wells in columns 2, 5, 8, 11, 14, 17, 20, and 23 using a multichannel repeating pipette. These columns contain the chips' observation windows
3. Prepare the required amount of ECM gel (e.g. 2 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, quickly spin the tube down (~5 seconds)
vi. Use gel immediately after preparation (within 10 minutes)

4. Dispense the gel into the gel inlet (columns 1, 4, 7, 10, 13, 16, 19, 22; rows B, E, H, K, N) using the Eppendorf® Multipette® M4 or 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 µL gel per gel inlet
   d. In case of incomplete gel filling, increase the loading volume (i.e. to 2.3 µL)
   e. In case the gel overflows from the gel channel into the adjacent medium channel, reduce the loading volume (i.e. to 1.7 µL)
   f. For examples of correct gel filling in the OrganoPlate® 3-lane, see figure 5.

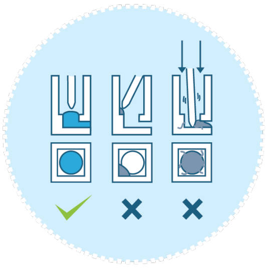


***Figure 4**: Gel loading*

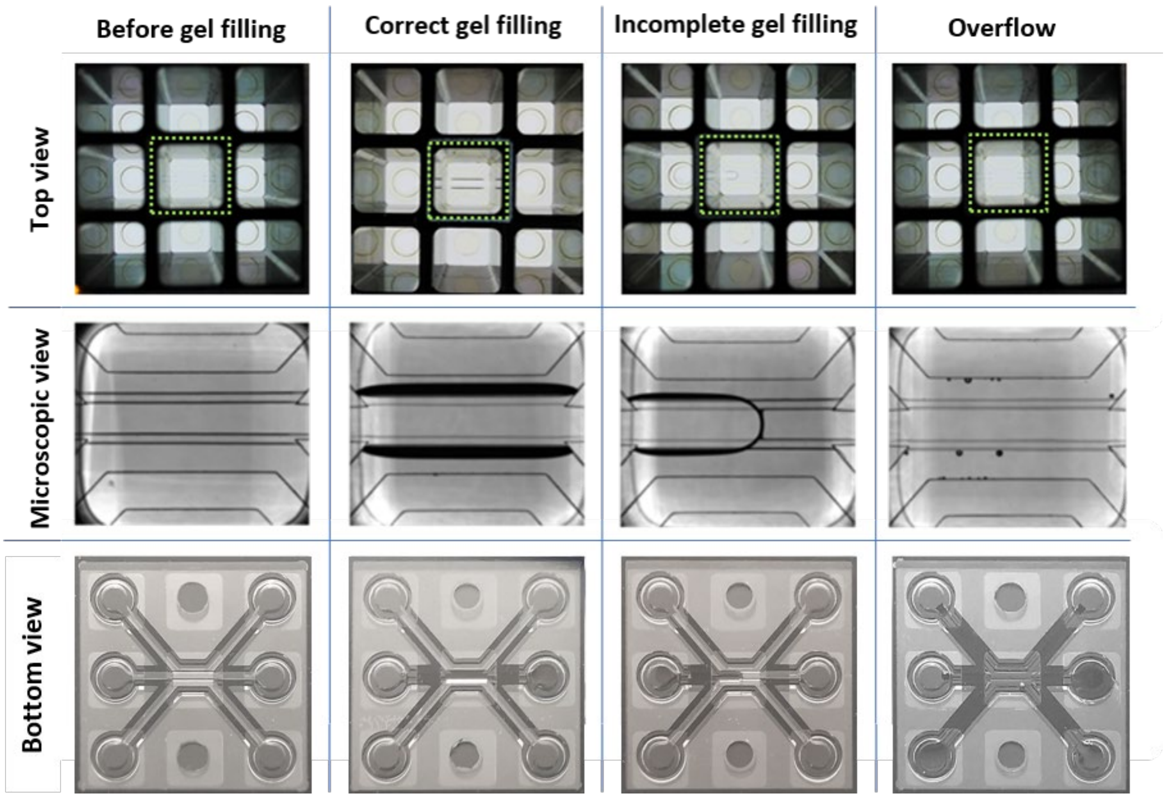


***Figure 5**: Overview of correct gel filling, incomplete gel filling, and overflow in the OrganoPlate® 3-lane.*

Both the Eppendorf® Multipette® M4 and the Sartorius eLINE electronic pipette can successfully be used to load the OrganoPlate®. Tabel 1 shows an overview of each pipette's advantages and disadvantages for gel loading.

Table 1

Pipette for gel loading	Advantage	Disadvantage
Eppendorf® Multipette® M4	Allows user to load many chips in one go without having to reload the pipette tip*	Only allows whole-microliter volumes (1 µl, 2 µL, etc.), making it more difficult to correct incomplete gel filling or overflow
Sartorius eLINE® electronic pipette	Allows user to select the loading volume with 10 nL steps, such as 1.75 µL, 1.80 µL, 1.85 µL, etc.	Total volume of pipette is 10 µL, allowing user to load approximately 5 chips at a time before having to reload the pipette

*We recommend loading a maximum of 20 chips at once before emptying and reloading the pipette tip with cold gel. This will avoid gelation of the gel while it is in the pipette tip.

5. 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
6. Add 30 µL of HBSS to the <u>gel inlet</u> (columns 1, 4, 7, 10, 13, 16, 19, 22; rows B, E, H, K, N) to prevent the gel from drying out
    a. For examples of dried out gel, see section *Trouble Shooting, figure 8.*
7. Place the OrganoPlate® 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.

Seed cells against the ECM gel

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® and pellet them
    a. The optimal cell density for seeding against ECM in the OrganoPlate® is cell type dependent (generally between 5,000 and 20,000 cells/µL)
    b. For example:
        i. Number of chips to seed: 40
        ii. Volume of cell solution to seed per chip: ~2 µL
        iii. Seeding density: 10,000 cells/µL
        iv. You need: 40 x 2 x 10,000 = $8.0*10^5$ cells
        v. Prepare 25% extra: pellet $1.0*10^6$ cells
4. Resuspend pellet in [$1.0*10^6$ / 10,000 =] 100 µL medium to obtain a 10,000 cells/µL cell suspension
5. Remove HBSS from the gel inlets
6. Seed 2 µL of cell suspension in the <u>top medium inlet</u> (columns 1, 4, 7, 10, 13, 16, 19, 22; rows A, D, G, J, M) using the same pipetting procedure as previously used for gel loading (see figure 3)
    a. Regularly resuspend the cell suspension during seeding to ensure homogenous cell density.

b. In case you want to include cell-free controls, seed 2 µL of medium without cells in the top medium inlet of these chips (instead of the cell suspension)

7. Add 50 µL of medium to the top medium inlet (columns 1, 4, 7, 10, 13, 16, 19, 22; rows A, D, G, J, M)
8. Place the OrganoPlate® on its side in the MIMETAS plate stand in the incubator to allow the cells in the top channel to settle onto the ECM gel and attach (see figure 6).
    a. The time cells need to attach is cell type dependent and generally varies between 2-6 hours

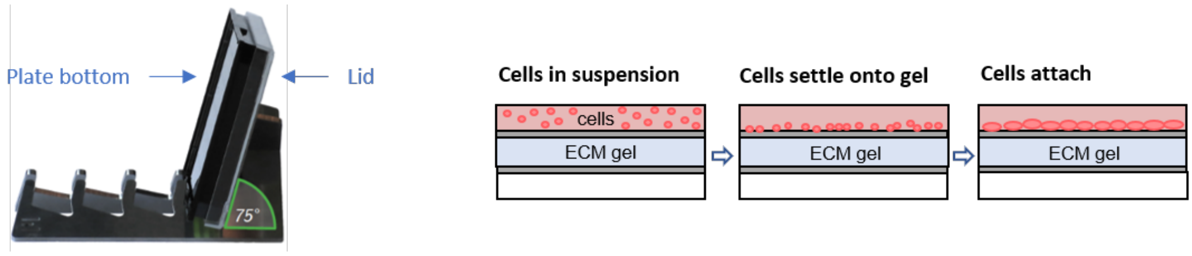


***Figure 6**: Incubate the OrganoPlate® on the side to allow cells to attach to the ECM gel*

9. After cells have attached, add 50 µL of medium to the top medium outlet (columns 3, 6, 9, 12, 15, 18, 21, 24; rows A, D, G, J, M).
10. Add 50 µL of medium to the bottom medium inlet (columns 1, 4, 7, 10, 13, 16, 19, 22; rows C, F, I, L, O)
    a. Ensure that the medium has filled the channel completely
    b. Ensure that no air bubbles are trapped on medium inlet and outlet. If bubbles are trapped, remove the bubbles gently with a pipette tip
    c. **For many endothelial cell types, perfusing only the channel that contains the tubule instead of both medium channels results in improved barrier integrity of the tubule. In this case, perfuse only the top channel by adding 50 µL of medium to the top medium inlet and outlet and do not add medium to the bottom medium inlet and outlet (i.e. proceed from step 9 of the protocol to step 12).**
11. Add 50 µL of medium to the bottom medium outlet (columns 3, 6, 9, 12, 15, 18, 21, 24; rows C, F, I, L, O)

12. Place the plate on the OrganoFlow® in a humidified incubator to start cell culture (see figure 7)
    a. An inclination of 7° and an interval of 8 minutes is optimal for most cultures

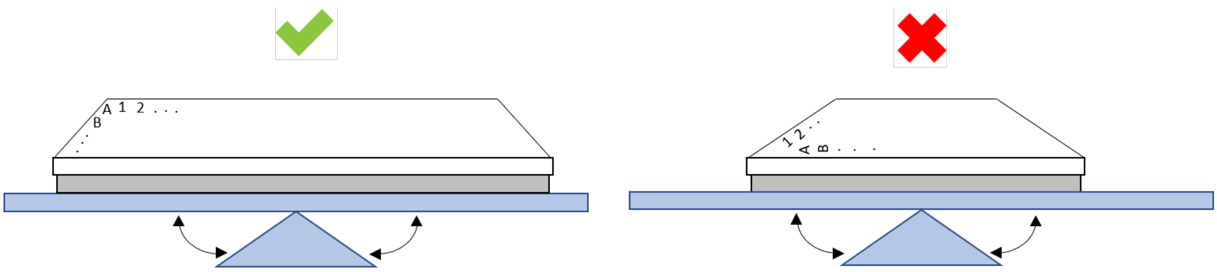


***Figure 7**: Place the OrganoPlate® on the OrganoFlow® rocker in the correct orientation*

13. 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
    An example of a tubule culture against ECM in the OrganoPlate® 3-lane is shown in figure 8.

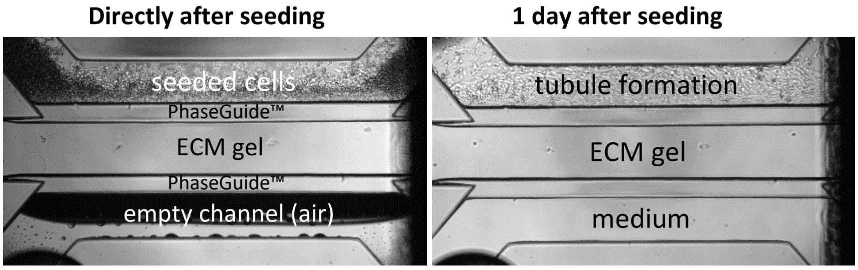


Figure 8*: Kidney epithelial cells form a tubule against an ECM gel in the OrganoPlate® 3-lane*

5. Troubleshooting

ECM drying

In some cases, the ECM gel can dry out during the gel loading and polymerization process (figure 9). This generally happens when the gel 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 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.

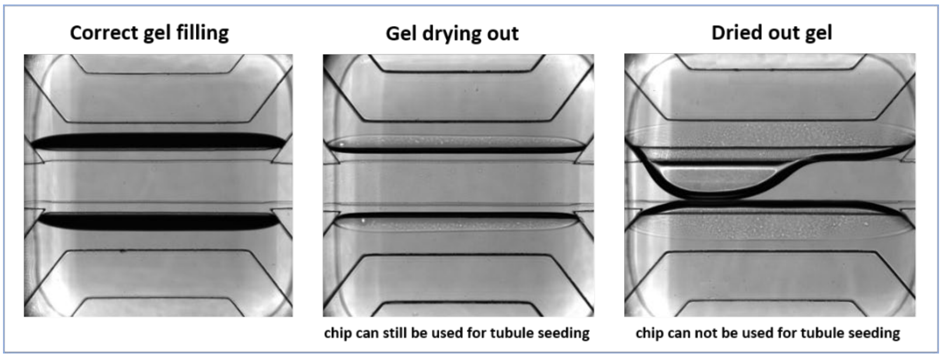
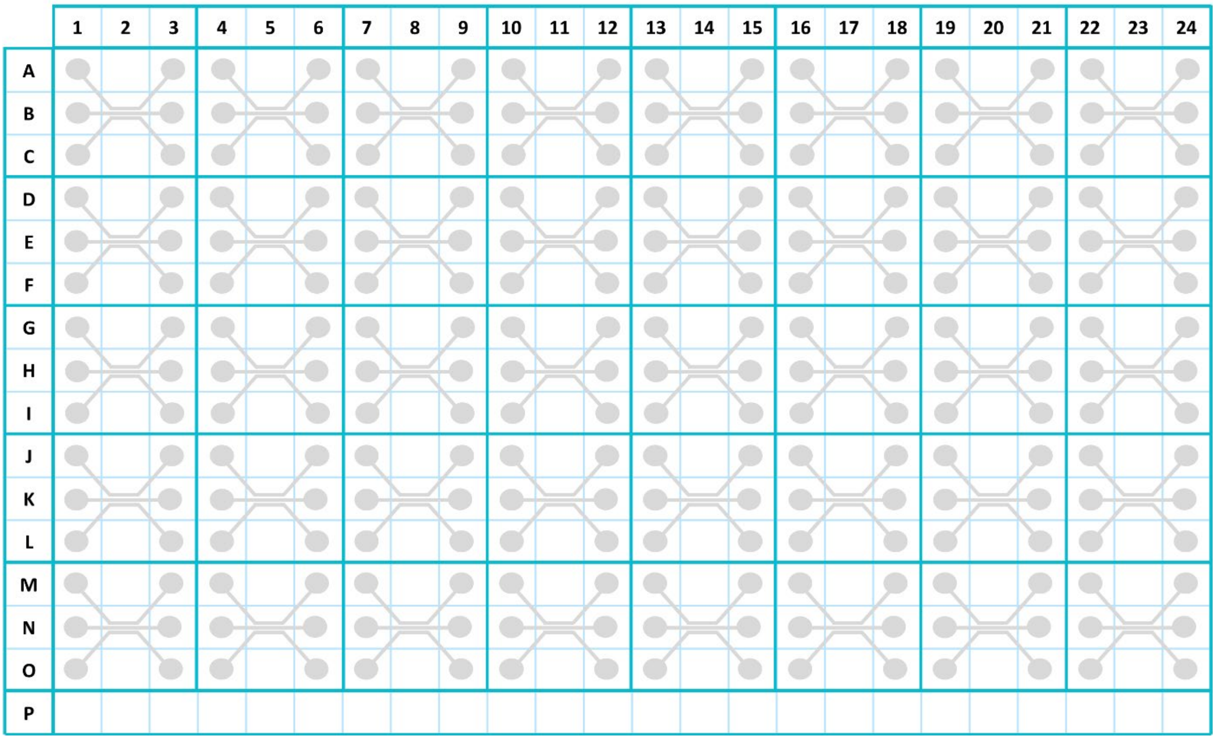


Figure 9*: ECM gel drying out due to prolonged gel loading or polymerization*

Cell invasion

In case of undesired cell invasion into the gel, the use of MMP inhibitors is recommended (e.g. addition of 10 µM of MMP-I inhibitor GM6001 (Abcam, ab120845) to the culture medium).

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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