1. Objective

This protocol describes the procedure for immunostaining of tubular cultures and vascular networks in the OrganoPlate® Graft. This protocol will efficiently stain endothelial tubules and angiogenic sprouts. Staining of a microtissue cultured in the graft chamber above the vascular network may require different conditions and optimization.

2. Background

Immunofluorescent staining is a technique that uses antibodies to target specific cellular biomolecules expressed on cells. Detection of the bound antibodies is done using fluorescence microscopy.

3. Materials

- OrganoPlate® Graft (MIMETAS, 6401-400-B) with or without a vascular network
- Tween-20 (Sigma, cat# P9616)
- FBS (Gibco/ATCC, cat# A13450)
- Triton™ X-100 (Sigma, cat# T8787)
- HBSS (+Ca/Mg) (Sigma, cat# 55037C-1000ML)
- BSA (Sigma, cat# A2153)
- Crushed ice
- Repeating multichannel pipets and tips
- 1x PBS (Gibco, cat# 70013065)
- Rocker platform (optional)
- Fixative (see table 1)

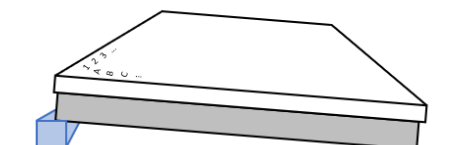
Fixative	Incubation time
3.7% formaldehyde in HBSS	10-15min RT
0.4% formaldehyde in HBSS	10-15min RT
-20°C 100% acetone	5min RT
-20°C 100% methanol	10-15min RT
-20°C 95% methanol, 5% acetic acid	5-10min RT

Table 1. Fixation methods compatible with the OrganoPlate® Graft

- Permeabilization buffer: 0.3% Triton X-100
- Blocking solution: 2% FCS, 2% BSA, 0.1% Tween20 in PBS
- Washing solution: 4% FCS in PBS
- Antibodies and nuclear stain (Hoechst™/DraQ5™)

4. Assay

Ensure that perfusion in the microfluidic channels of the OrganoPlate® Graft is induced during all steps of the immunostaining procedure. This will guarantee correct entry of all staining reagents into the channels. Perfusion can be created by placing the OrganoPlate® Graft on a regular rocker platform and having it switch sides. Use a small angle and a low switching interval (i.e. 5° angle, 2-5 min interval). Alternatively, flow can be induced by placing the OrganoPlate® Graft under an angle by positioning one end on top of an object (see figure on the right) and regularly switching sides.



Fixation

All steps are performed at room temperature

1. Prepare fixative (see table 1).
    a. Standard fixative is 3.7% formaldehyde in PBS (dilute 1:10 from 37% stock)
    b. For a complete OrganoPlate® Graft you will need 25 mL
2. Aspirate medium from the chips and add 50 µL of fixative to the gel chamber, all inlets and outlets (see below)

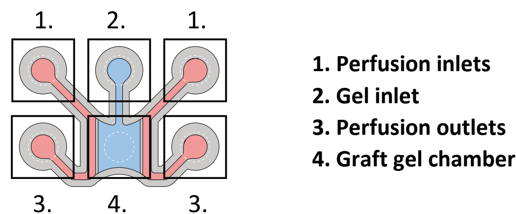


Figure 1: *Schematic representation of the OrganoPlate® Graft.*

3. Incubate the fixative for the appropriate amount of time (see table 1)
4. Aspirate fixative and wash the chips 3x (5 min each) with PBS (50 µL in the gel chamber, all inlets and outlets)
5. Proceed to immunostaining or seal the plate around the edges with Parafilm® and wrap the plate in aluminum foil. The plate can be stored at RT up to 2 weeks.

Immunostaining

All steps are performed at room temperature

1. Prepare permeabilization buffer, blocking solution, and washing solution (see materials list)
2. Wash chips 1x 5 min with washing solution (50 µL in the gel chamber, all inlets and outlets)
3. Permeabilize cells for 10 minutes with permeabilization buffer (50 µL in the gel chamber, all inlets and outlets)
4. Wash chips 1x 5 min with washing solution (50 µL in the gel chamber, all inlets and outlets)
5. Block cells for 30-45 min with blocking solution (50 µL in the gel chamber, all inlets and outlets)
6. Meanwhile, prepare primary antibody in blocking solution in the appropriate dilution
    a. 120µl antibody per chip
7. Incubate primary antibody 1-2 hours at RT or overnight at 4˚C (20 µL in the gel chamber, all inlets and outlets)
8. Wash chips 3x (5 min each) with washing solution (50 µL in the gel chamber, all inlets and outlets)
9. Prepare secondary antibody in blocking solution in the appropriate dilution
10. Incubate secondary antibody 30 min in the dark at RT (20 µL in the gel chamber, all inlets and outlets)
11. Wash chips 3x (5 min each) with washing solution (50 µL in the gel chamber, all inlets and outlets)
12. If desired, stain cells with direct stains (e.g. Hoechst or ActinRed), using manufacturer's instructions
    a. Use stains for fixed cells
    b. Incubate stains at least 15 min for cells grown as tubes against the ECM gel and at least 30 min for cells embedded in ECM gel
13. Aspirate all wells and add 50 µL of PBS to all wells
14. Proceed to microscopy or store the plate
    a. Perform microscopy within one week after staining for optimal results
    b. Imaging can be performed on all standard fluorescent microscopes

c. Store plate by sealing edges with Parafilm® and wrapping it in aluminum foil. Store at RT for up to two weeks

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
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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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