1. Objective

This protocol describes the procedure for immunostaining of tubular cultures, vascular networks, and microtissues cultured in the OrganoPlate® Graft. Optimal imaging of the center of the microtissues cultured in the graft chamber may require clearing procedures that need to be optimized depending on the application.

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 endothelial vessels or a vascular network, with or without a microtissue placed in the Graft gel chamber
- Triton™ X-100 (Sigma, cat# T8787)
- HBSS (+Ca/Mg) (Sigma, cat# 55037C-1000ML)
- BSA (Sigma, cat# A2153)
- Repeating multichannel pipets and tips
- PBS (Gibco, cat# 70013065)
- Antibodies and Hoechst (ThermoFisher, #H3570)
- Rocker platform (OrganoFlow® (set at a 7° angle, 0.5 min interval), or a comparable alternative)
- Fixative (see table 1)

Fixative	Incubation time
3.7% formaldehyde in HBSS	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 / blocking buffer** (same solution): 1% Triton X-100 + 3% BSA in PBS
- **Antibody incubation buffer**: 0.3% Triton X-100 + 3% BSA in PBS
- **Washing solution**: 0.3% Triton X-100 in PBS

4. Assay

Fixation

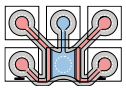
All steps are performed at room temperature

1. Prepare fixative (see table 1).
    a. Standard fixative is 3.7% formaldehyde in HBSS (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 fixative according to *volume scheme 1* on the next page

100µL		100µL
50µL	50µL	50µL

Volume scheme 1



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

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 according to *volume scheme 1*
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.

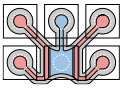
Immunostaining

All steps are performed at room temperature

1. Prepare permeabilization buffer / blocking buffer, and washing solution (see materials list)
2. Aspirate PBS from all wells
3. Add **permeabilization buffer / blocking buffer** according to *volume scheme 2* below and incubate for 2 hours on the rocker platform (see materials list).

50µL		50µL
50µL	50µL	50µL

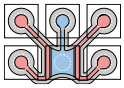
Volume scheme 2



4. Meanwhile, prepare **primary antibody** in antibody incubation buffer in the appropriate dilution
5. Aspirate permeabilization buffer / blocking buffer from all wells and incubate primary antibody according to *volume scheme 3* below overnight on the rocker platform

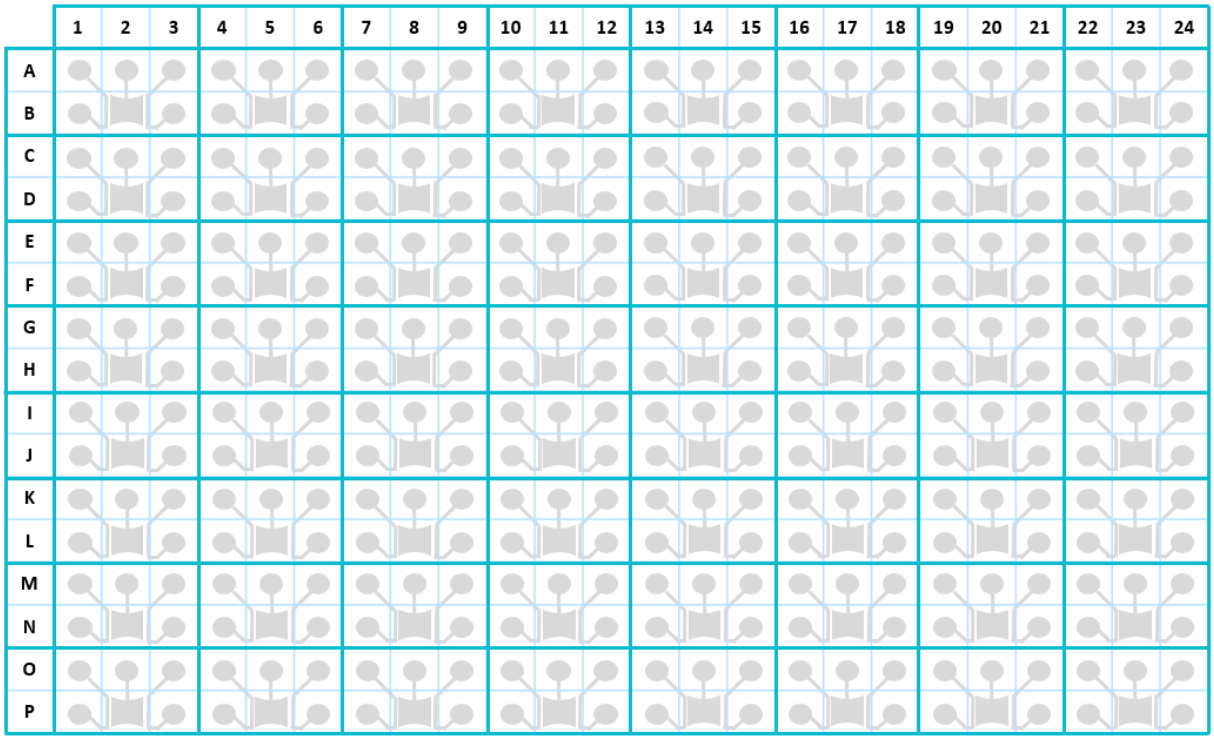
40µL		40µL
40µL	40µL	40µL

Volume scheme 3



6. Wash chips 3x 5 min with **washing solution** according to *volume scheme 1*
7. Meanwhile, prepare **secondary antibody** in antibody incubation buffer in the appropriate dilution
8. Incubate secondary according to *volume scheme 3* for 2h in the dark on the rocker platform
9. Wash chips 3x 5 min with **washing solution** according to *volume scheme 1*
10. Wash chips 1x 5 min with PBS according to *volume scheme 1*
11. Aspirate PBS and add **Hoechst** (1:2000 in BPS) according to *volume scheme 3*
12. Incubate Hoechst for 30-60 min on the rocker platform
13. Wash cells 1x 5 min with PBS according to *volume scheme 1*
14. Aspirate, and add fresh 50 µL PBS to all wells
15. 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 the plate by sealing the edges with Parafilm® and wrapping the plate in aluminum foil. Store at RT for up to two weeks.

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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