1. Objective

This protocol describes the perfusion of fluorescently labeled monocytes through a tubule of endothelial cells in the OrganoPlate® (as published by Poussin et al., DOI: 10.14573/altex.1811301). The number of adhered monocytes is quantified and normalized to the number of endothelial cells in each test condition.

2. Background

The OrganoPlate® allows the culture of in-gel tissues (e.g. neuronal networks or liver cells), the culture of tubular tissues (e.g. endothelial or epithelial barriers), or combinations of both. Endothelial cells grown in the OrganoPlate® form an endothelial tubule by placing the OrganoPlate® on a rocker platform. To study endothelial activation, monocytes are labeled with green-fluorescent Calcein-AM and perfused through the endothelial tubules, of which the nuclei are stained with Hoechst™. Fluorescent images are acquired and allow for quantification of the number of adhered monocytes per endothelial cell in different conditions.

3. Materials

- OrganoPlate® 2-lane (MIMETAS, 9605-400-B) with endothelial tubules
- Endothelial cell specific medium
- Monocytes (i.e. MM6 or THP-1) and monocyte specific medium
- Calcein-AM (1 mg/mL in DMSO, Thermo Fisher Scientific, C3099)
- Hoechst™ 33342 (10 mg/mL in water, Thermo Fisher Scientific, H3570)
- Human recombinant TNF-α (ImmunoTools, 11343015)
- HBSS (Sigma, H6648)

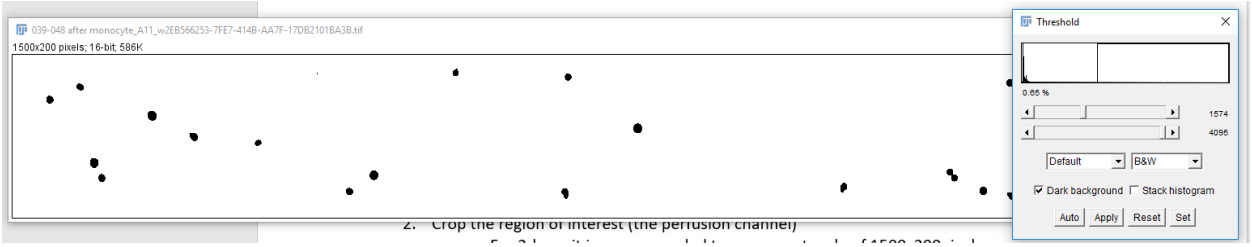
4. Procedure

1. Expose a subset of chips to a positive control condition: 1 ng/mL TNF-α in endothelial cell medium
    a. Short term (4 hours) or long term (16-24 hours)
2. Label monocytes with green-fluorescent Calcein-AM
    a. Prepare a $1x10^5$ cells/mL suspension in monocyte medium (prepare 120 µL per chip)
    b. Label monocytes with 0.5 µg/mL Calcein-AM (dilute 2000x from stock)
    c. Incubate monocyte suspension with Calcein-AM for 15 minutes at 37˚C (tube in incubator)
3. Stain nuclei of endothelial tubule:
    a. Prepare a 5 µg/mL Hoechst™ solution (dilute 2000x from stock) in endothelial cell medium
    b. Aspirate medium from medium in and outlets of the OrganoPlate® chips
    c. Add 25 µL Hoechst™ solution to medium inlet and medium outlet of each chip
    d. Incubate 20 minutes on the rocker at 37˚C, 5% CO_2
4. Pellet the calcein-labeled monocyte suspension (x*g* and minutes to centrifuge is cell type specific)
5. Resuspend the monocytes in medium without Calcein-AM in a final concentration of $1x10^5$ cells/mL
    a. Use medium specific for the endothelial cells to resuspend the monocytes in, as endothelial cells can be very sensitive to changes in medium composition
6. Aspirate the Hoechst™ solution from the chips' medium inlets and outlets
7. Wash chips 5 min with fresh endothelial cell medium (75 µL medium on inlets, 25 µL on medium outlets)

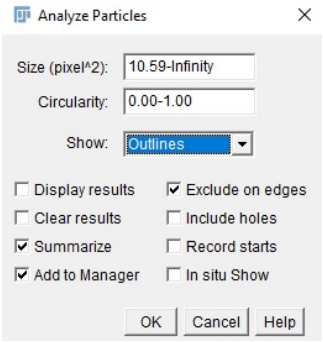
8. Aspirate medium from the chips' medium inlets and medium outlets
9. Add 50 µL monocyte cell suspension to the chips' medium inlets and outlets
10. Incubate the OrganoPlate® on the rocker (7° inclination, 4 min interval) at 37˚C, 5% CO_2 for 15 min
11. Aspirate medium from the chips' inlets and outlets
12. Wash chips 5 min at RT with HBSS (100 µL in medium inlets, 50 µL in medium outlets)
13. Aspirate HBSS from the chips' inlets and outlets
14. Wash the chips 1 min at RT with HBSS (50 µL in medium inlets, 50 µL in medium outlets)
15. Aspirate HBSS from the chips' inlets and outlets
16. Add fresh HBSS to medium inlets (50 µL) and medium outlets (50 µL)
17. Image plate with 4x objective using DAPI and FITC filters
18. After imaging you can fix the plate, see protocol *Immunostaining in the OrganoPlate®* section washing and fixation.

5. Data analysis

1. Import the acquired images (DAPI and FITC) in Fiji (ImageJ) software
2. Crop the region of interest (the medium channel)
3. Duplicate the stack (Control+Shift+D), duplicate only the DAPI channel (usually channel 1)
4. Set a threshold (Control+Shift+T), check "dark background", ensure that only the endothelial cells appear dark and press "apply" (see image below)
    a. In case the window *Convert stack to Binary* appears, select the following settings: Method: Default, Background: Dark, and deselect "Calculate threshold for each image".
    b. Press ok
5. Count the nuclei of the endothelial cells by clicking "Analyze" → "Analyze particles" (for settings see image below)
6. Copy the results section to Excel
7. Duplicate the stack (Control+Shift+D), duplicate only the FITC channel (usually channel 2)
8. Set a threshold (Control+Shift+T), check "dark background", make sure that only the monocytes appear dark and press "apply" (see image below)



    a. In case the window *Convert stack to Binary* appears, select the following settings: Method: Default, Background: Dark, and deselect "Calculate threshold for each image"
    b. Press ok
9. Count monocytes by clicking "Analyze" → "Analyze particles" using the settings depicted in the image on the right
10. Copy the results section to Excel. The analysis only requires the information in the columns with chip names and counts
11. Copy the results from the nuclei count to a separate column
12. Divide the number of counted monocytes by the number of counted endothelial cells and multiply the resulting number by 100 to obtain the percentage of counted monocytes per endothelial cell



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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