1. Objective

This assay describes the assessment of neurite outgrowth in neuronal cultures in the OrganoPlate® 2-lane

2. Background

Neurite outgrowth provides information about neuronal health and network integrity. Healthy neurons show long neurites protruding from their cell bodies. Exposure to toxic compounds can prevent the formation of neurons after cell seeding in the OrganoPlate® or can cause retraction of neurites in already established neuronal networks.

This protocol describes the use of Calcein-AM, a cell-permeant dye that is converted to green-fluorescent Calcein after being taking up by the cells. The networks can be detected using confocal imaging and subsequent analysis using neurite outgrowth software.

3. Materials

- OrganoPlate® 2-lane (9605-400-B) with a neuronal culture (see figure 1)
- Medium (cell type specific)
- Calcein-AM (1 mg/mL in DMSO, Thermo Fisher Scientific, C3099)
- Hoechst™ 33342 (10 mg/mL in water, Thermo Fisher Scientific, H3570)
- Optional: methylmercury(II) chloride (Sigma, 33368)

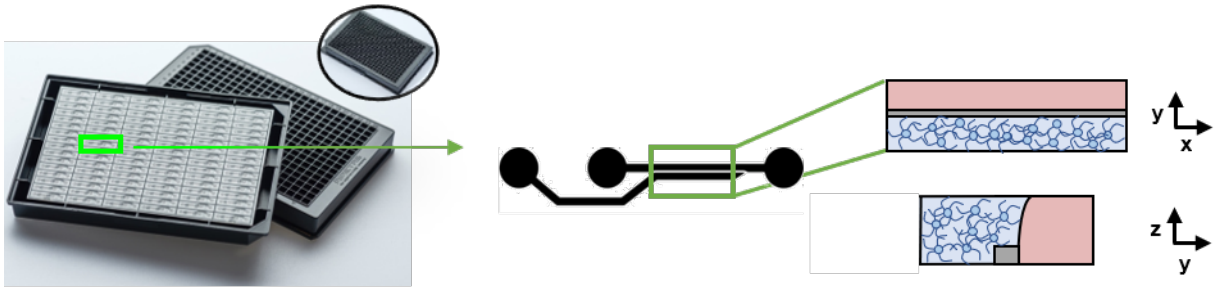


Figure 1: Neuronal cultures in the OrganoPlate® 2-lane. The OrganoPlate® 2-lane holds 96 microfluidic chips that can be used to culture miniaturized tissue- and organ models. Each chip holds two channels: one is used to seed an extracellular matrix (ECM) gel and the other supplies the cells with medium. To obtain neuronal cultures, neural cells (neural stem cells or mature neurons/astrocytes) are seeded in ECM gel in the gel channel, where they form networks. Medium and assay reagents can access the cells through the adjacent medium channel.

4. Assay

Optional: compound treatment

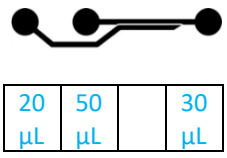
If the goal is to assess compounds' effects on neurite outgrowth and/or neurite stability, compounds can be added to the culture medium and pipetted into the chips' inlets and outlets. Compounds can be added upon cell seeding, to assess their effect on neurite outgrowth, or added later – after networks have already been established – to assess their effect on neurite stability. Concentrations and exposure times are compound specific and require optimization. However, the conditions described below can serve as relevant controls for an experiment assessing compound effects on neurite outgrowth:

- Positive control: 24h exposure to 10 µM methyl mercury (diluted from 100 mM in DMSO stock)
- Vehicle control: 24 exposure to 0.01% DMSO
- Negative control: 24h exposure to medium

Dye loading into the cells

1. Calculate the volume of Calcein-AM/Hoechst solution you will need
    a. You need 100 µL per chip
2. Warm medium in a 37°C water bath
3. Prepare the Calcein-AM/Hoechst solution as followed:
    a. Dilute the Calcein-AM stock (see *Materials*) 1:2000 + Hoechst stock (see *Materials*) 1:2000 in warm medium in an amber Eppendorf tube
    b. The resulting **Calcein-AM/Hoechst solution** contains 0.5 µg/mL Calcein-AM and 5 µg/mL Hoechst

4. Remove medium from the chips that you want to use
5. Add the Calcein-AM/Hoechst solution to the chips by pipetting the solution into the gel inlets (20 µL), medium inlets (50 µL), and medium outlets (30 µL) of each chip, as depicted on the right



6. Incubate the Calcein-AM/Hoechst solution for 30-60 min at 37°C, 5% CO_2
    a. Incubate static
    b. The different volumes in inlets and outlets ensure a slow flow through the chip that aids in proper dye loading in all cells
7. Proceed to imaging

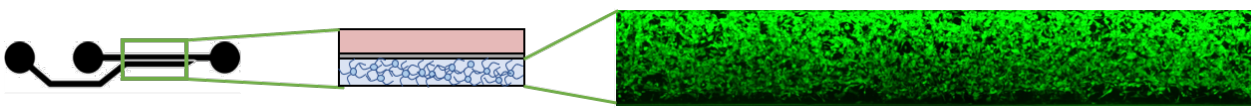


Figure 2: Detection of neuronal networks in the OrganoPlate® using Calcein-AM. Calcein-AM is added to the cultures. Calcein-AM is converted to green-fluorescent Calcein after acetoxyethyl ester hydrolysis by intracellular esterases. Fluorescent microscopy is used to image the cells.

5. Imaging & Analysis

Imaging settings depend on the microscope that is used. To give an example of settings that are compatible with the assay, the procedure that we use at MIMETAS is described below:

- Microscope: ImageXpress Micro XLS-C HCI System (Molecular Devices)
- Confocal mode (42 µm pinhole)
- 10x objective
- 1x binning
- 16-bit range
- FITC filter for Calcein detection
- DAPI filter for Hoechst detection
- 3 µm step size for z-stacks

The OrganoPlate® is also compatible with other (high-content) imaging systems. Setting up an imaging protocol on another microscope should be straightforward, but some optimization may be required.

Maximum projection images of each chip's network can be analyzed using standard neurite outgrowth software and applications. We use the *Neurite Outgrowth Application Module* incorporated in the Molecular Devices MetaXpress software.

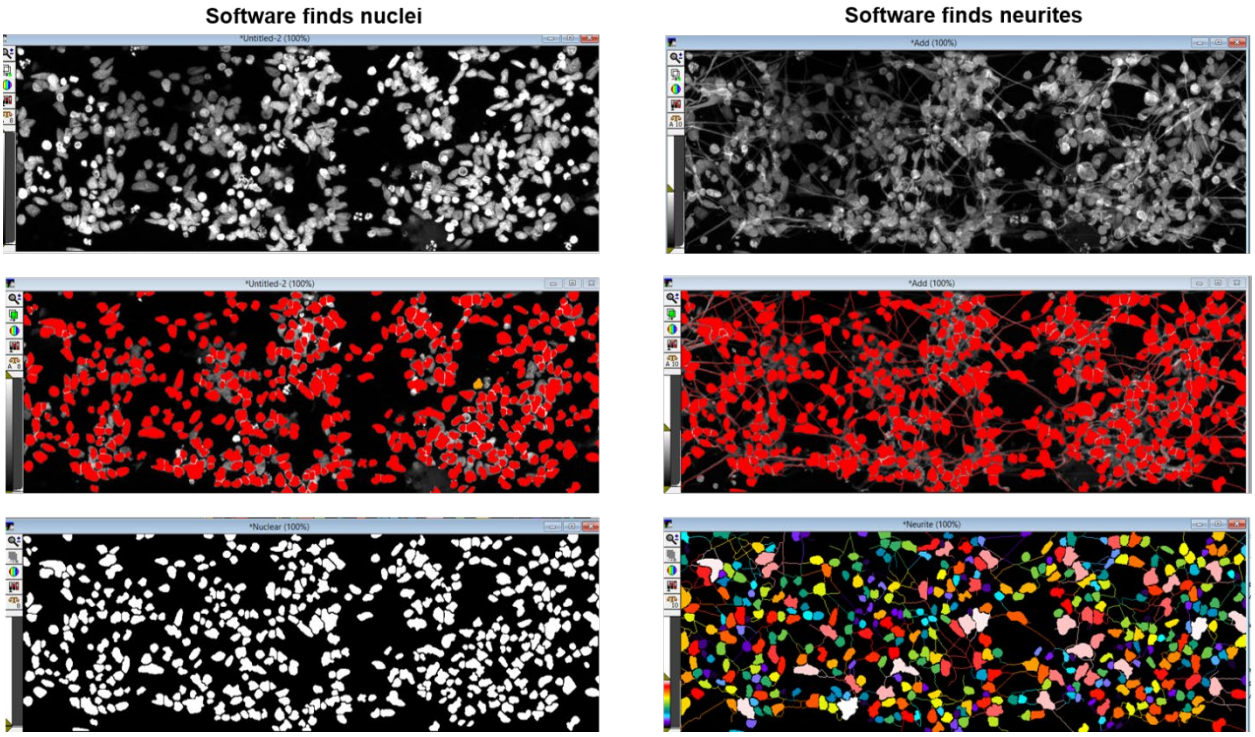


Figure 3: Analysis of neurite outgrowth in neuronal cultures in the OrganoPlate® 2-lane. Top images show pictures acquired of a network's nuclei (left) and cell bodies/neurites (right). The MetaXpress Neurite Outgrowth Application module finds the network's nuclei, cell bodies, and neurites and extracts the network form the maximum projection images.

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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