High-Content Assay for Morphological Characterization of 3D Neuronal Networks in a Microfluidic Platform

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INTRODUCTION

The absence of physiologically relevant *in vitro* models of the nervous system is an important limitation in understanding mechanisms of neurological diseases and drug development. This has generated an increasing interest in using three-dimensional (3D) cultures for assay development applicable for neurodegenerative diseases and neurotoxicology screens.[1,2] The goal of the present study was to develop a model for 3D neurite outgrowth assessment using iPSC-derived neurons in the microfluidic, high-throughput OrganoPlate® platform.

The OrganoPlate® was developed as an organ-on-a-chip platform allowing the formation of three-dimensional (3D), microfluidic-based, long-term cultures of live cells suitable for screening.[3,4] Neuronal cultures were treated for five days with several compounds including methyl mercury and other selected chemicals that are known to inhibit neurite outgrowth. To assess the neuronal viability and complexity of networks, cells were stained using a combination of three dyes: Calcein AM, MitoTracker Orange, and Hoechst nuclear dye. We optimized the methods for assessing morphology and viability of neurons in 3D matrix using automated confocal imaging and analysis. Disintegrations of neuronal connections were visible in a dose-dependent manner after treatment with neurotoxic compounds. A series of confocal images were automatically acquired at different planes separated by 3-10 µm, covering approximately 150-300 µm in depth. Images were analyzed using our 3D analysis module in MetaXpress analysis software.

Phenotypic readouts allowed quantitative characterization of the extent and complexity of the neural networks in 3D. Multiple measurements were used for defining effective concentrations for neurotoxicity. In addition, we evaluated assay reproducibility and tested a set of known neurotoxic compounds. The proposed method is extensively applicable for compound screening for prediction of neurotoxicity in a high-throughput manner.

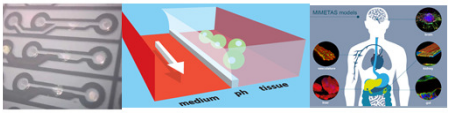
GOAL

The focus of the present study was to develop a high- throughput 3D neurite outgrowth assay using iPSC-derived neurons developing in the microfluidic OrganoPlate® platform (MIMETAS, the Netherlands), with the goal of establishing 3D models for neurodegenerative diseases and neurotoxicology screens.

METHODS

- ImageXpress® Micro Confocal High-Content Imaging System
  - Equipped with Widefield and Confocal (60µm pinhole) Optics
- MetaXpress® High-Content Image Acquisition and Analysis Software

The OrganoPlate® is a high-throughput platform that combines the most recent advancesin3D cell culture and microfluidics[3]. The OrganoPlate contains 96 tissue chips suitable for long-term cultures of live cells, is amenable for screening purposes, and is compatible with standard laboratory equipment or automated systems.



Formation of 3D Neural Networks in OrganoPlate:

- Human iPSC-derived neurons (iCell® Neurons) and the appropriate media were purchased from Cellular Dynamics International, Fujifilm CO (CDI, Madison, WI). Cells were plated at a density of 30,000 neurons/ chip after premixing with Matrigel (Corning) to a final concentration of 7 µg/ml Matrigel in the OrganoPlate®. The cell suspension in Matrigel was kept on ice during plating. The seeding volume of this solution varied between 1µL-1.4µL depending on cell density and Matrigel concentration. After seeding, the plate was placed into a tissue culture incubator (37°C, 5% CO_2) for 30min to facilitate solidification of the Matrigel. Next, 50 µL of growth media was added to both the medium inlets and outlets on the plate, and then placed back into the tissue culture incubator. Neurite outgrowths began to form ~24h after plating and extended for up to 14 days in culture.
- Treatment with compounds was performed 24h post plating; cultures were exposed to treatment for 5 days during which the culture media containing the compounds was changed every second day.

Staining

- Formation of neurite networks was monitored over time using transmitted light imaging. To assess neuronal viability, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1µM), the mitochondria potential dye MitoTracker Orange (1 µM), and the Hoechst nuclear dye (2 µM) (all from Life Technologies). The dye mixture was added to the chips and incubated for 60 min followed by replacement with culture medium.

References

1. Chang TT, Hughes-Fulford M. Tissue Eng Part A. Mar 2009;15(3):559-567.
2. Kunz-Schughart LA at al.,. J Biomol Screen. 2004;9(4):273-285.
3. van Duinen V et al., Curr Opin Biotechnol. 2015;35:118-26.
4. Wevers NR at al.,, Sci Rep. 2016 Dec 9;6:38856.
5. Sirenko et al., Assay and Drug Dev Technologies12(9-10):536-47

MIMETAS
the organ-on-a-chip company

RESULTS:

Phenotypic Assays in 3D Cultures

High-content imaging and analysis were utilized for evaluation of treatment effects on neuronal networks. We optimized confocal imaging and analysis protocols for assessing morphology and viability of neurons in this 3D matrix. Images were acquired using ImageXpress Micro Confocal system (Molecular Devices, Sunnyvale, CA), with 10x, 20x or 40x objectives.

A series of images was acquired at different planes along the focal axis (Z-stack) (Figure 1). A stack of 17-30 planes separated by 3-10 µm was acquired, covering approximately 150-300 µm in depth.

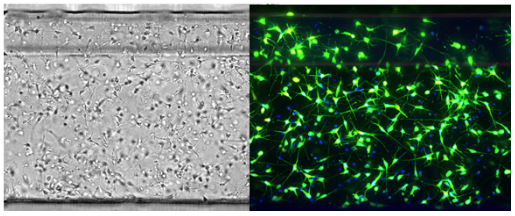


Figure 1: Left. Transmitted light images captured of iCell neurons plated into OrganoPlate capillary wells in Matrigel. Cells were plated at a density of 30,000 cells per well for 72 hours, then imaged using transmitted light with a 20x objective. Right: Live cells were stained with the viability dye Calcein AM, the mitochondrial integrity dye MitoTracker Orange, and the Hoechst nuclear dye. Then Z-stacks of images in 3 colors were taken using the ImageXpress Micro Confocal system in confocal mode, DAPI, FITC, and TRITC channels, 20x magnification, 25 images 6 µm apart. Maximum projection composite images are shown here for the DAPI and FITC channels.

3D Visualization and 3D Image Analysis

MetaXpress software (Molecular Devices) offers a 3D analysis option that allows for combining objects from adjacent Z-planes, as well as 3D visualization of cells and networks. Images were analyzed using a 3D analysis Custom Module using "find fibers" function with which a "fibers" measurement was generated for quantifying neurite outgrowth. Objects are first found in each plane, and then connected in 3D space using the "connect by best match" function. The measurements output from the 3D analysis included the number of neurites (fibers) and processes, cell volumes, total volume of fibers, numbers of processes, nuclei, and branching points.

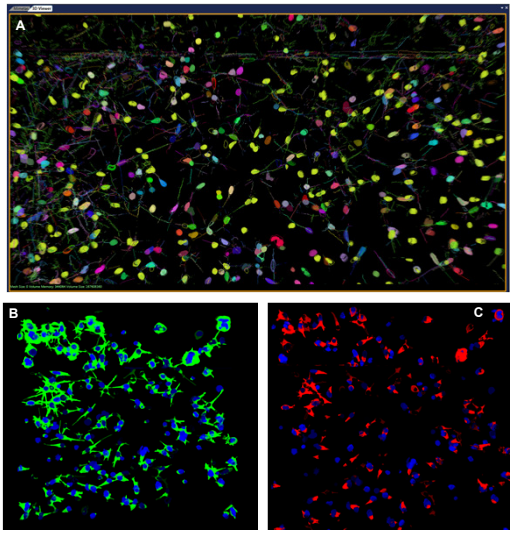


Figure 2: Z-stacks of images were analyzed using MetaXpress Custom Module Editor 3D analysis option. Two different analysis modules were used for evaluation of complexity of neural networks in 3D: one developed to measure fibers (neurites), the other for cell scoring for marker positivity. A. Numbers and volumes of the neurites (fibers), as well as segments and branching points were detected and counted in 3D space using the "find fibers" and "connect by best match" options in the custom module editor (CME). Visualization of fibers (green) and nuclei (blue) are shown. B. The numbers of the total cells, Calcein AM positive "live" cells, as well as volumes of Calcein AM positive cytoplasm were measured in 3D space using "Cell Scoring" and "Connect by best match" functions in CME. Nuclei shown in blue and Calcein AM positive cytoplasm in green. C. The number of MitoTracker Orange positive cells and the volumes of MitoTracker positive cytoplasm were measured using "Cell Scoring" and "connect by best match" options in CME; nuclei visualized in blue, mitochondria stain is shown in red.

A supplementary custom module was used for characterization of cell viability (cell bodies positive for Calcein AM) and mitochondrial integrity (cell bodies positive for MitoTracker Orange). Cell scoring analysis in 3D was applied to count and characterize live cells (Calcein AM positive) and cells with intact mitochondria (MitoTracker positive cells). Results calculated via this analysis method included: cell number (total nuclei), live cell count (Calcein AM positive), number of MitoTracker positive cells, as well as cell volumes or fluorescence intensities.

Compound-Specific Effects on Neurite Outgrowth

Phenotypic readouts included quantitative characterization of the extent and complexity of neural networks in 3D by multiplexed measurements. In this neuronal model system, we have evaluated assay reproducibility, characterized multiple measurements and tested several known neurotoxic compounds. Via these analysis methods, we have accurately measured concentration-dependent inhibitory effects of these compounds on the complexity of neurite networks. Effects on neurite outgrowth, complexity of networks and viable cell number were assessed following 72hr of exposure. Dose-dependent responses to selected toxic compounds shown below. Phenotypic read-outs included characterization of neurite outgrowth, branching, number of processes, and cell viability.

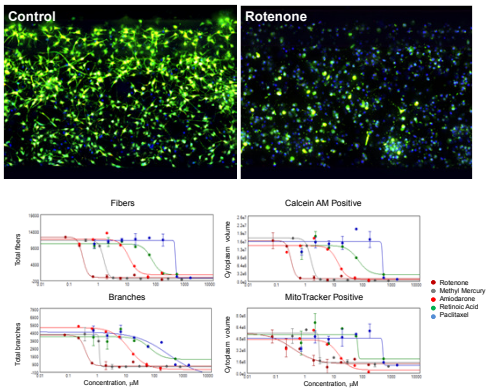


Figure 3: iCells Neurons were cultured as described above and treated with indicated neurotoxic compounds for 5 days. Images collected and analyzed in 3D as described above. Numbers and volumes of the neurites (fibers) and branching points were detected and counted in 3D space. In addition, the numbers of the total cells, Calcein AM positive "live" cells, and MitoTracker Orange positive cells were counted and characterized in 3D. Quantitative effects of compounds on the numbers and total volume of neurites (fibers), numbers of branching points, as well as the numbers of total, live cells, and MitoTracker Orange positive cells are presented.

Comparison between 2D and 3D Models

Disruption of neuronal networks, cell viability, and mitochondria potential markers were compared between conventional (2D) cell cultures and 3D cultures in OrganoPlates. EC_{50} values for toxicity effects for retinoic acid and paclitaxel were relatively higher for 3D cultures, and lower for rotenone, while for others compounds the values were comparable.

3D culture 3D analysis

EC_{50}, µM	Total Fibers	Total Branches	Positive Cytoplasm Volume Sum	Positive Mitochondria Volume Sum
Rotenone	0.29 +/- 0.047	0.336 +/- 0.081	0.30 +/- 0.06	0.38 +/- 0.44
Methyl mercury	1.59 +/- 0.074	1.19 +/- 1.14	3.05 +/- 1.47	3.12 +/- 1.81
Amiodarone	11.8 +/- 3.42	13.3 +/- 1.55	13.7 +/- 4.79	15.3 +/- 8.86
Retinoic acid	74.8 +/- 12.7	70.1 +/- 66.5	70.5 +/- 51.8	68.8 +/- 48.5
Paclitaxel	494.5*	317 +/- 638	497 +/- 9.48	515 +/- 38.9

2D culture 2D analysis

EC_{50}, µM	Total Processes	Total Branches	Positive Cytoplasm Area Sum	Positive Mitochondria Area Sum
Rotenone	4.81 +/- 1.56	0.49 +/- 0.49	4.33 +/- 3.87	10.01 +/- 12.4
Methyl mercury	1.10 +/- 0.21	0.41	0.74 +/- 0.15	1.16 +/- 0.40
Amiodarone	18.3	17.9 +/- 6.21	12.5 +/- 3.84	10.4 +/- 4.33
Retinoic acid	30.9 +/- 9.57	26.7 +/- 16.9	18.1 +/- 9.71	25.5 +/- 12.4
Paclitaxel	23.1 +/- 9.52	19.6 +/- 6.61	73.3 +/- 23.6	78.5 +/- 41.4

Table 1: For 3D cultures iCells Neurons were cultured in OrganoPlates and treated with neurotoxic compounds as described above. For 2D cultures experiments were done in parallel using conventional culture methods and analysis described previously[4]. EC_{50} valuess for decreases in different measurements for both models were determined using 4-parametric data fit.

CONCLUSIONS

- We presented here the methods that will enable neural development and neurotoxicity assays that use 3D models
- Confocal imaging and 3D analysis allows quantitative characterization of complex phenotypic effects
- 3D neuronal models can be successfully used for toxicity evaluation, disease modelling, and compound screening

MOLECULAR DEVICES