APPLICATION NOTE

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

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Benefits

- **Establish a high-throughput 3D neurite outgrowth assay using iPSC-derived neurons**
- **Generate more *in vivo*-like results using the microfludic OrganoPlate® platform**
- **Optimize high-content imaging for evaluation of treatment effects on neuronal networks**

Introduction

Establishment of physiologically-relevant *in vitro* models is crucial to further understanding of the mechanisms of neurological diseases as well as targeted drug development. While iPSC-derived neurons show great promise for compound screening and disease modeling, use of three-dimensional (3D) cultures is emerging as a valid approach for neuronal cell based assay development. 3D cultures are recognized as more closely recapitulating aspects of the human tissues including the architecture, cell organization, as well as cell-cell and cell-matrix interactions[1,2].

The focus of the present study was to develop a high-throughput 3D neurite outgrowth assay using iPSC-derived neurons developed in the microfluidic OrganoPlate® platform, with the goal of establishing 3D models for neurodegenerative diseases and neurotoxicology screens[3]. The OrganoPlate® is a high-throughput platform that combines the most recent advances in 3D cell culture, Phaseguides™ and microfluidics[4,5,6]. The OrganoPlate contains 96 tissue chips suitable for long-term culture of live cells, is amenable for screening purposes, and is compatible with standard laboratory equipment or automated systems, like the ImageXpress® Micro Confocal High-Content Imaging System.

Materials

- OrganoPlate® platform (MIMETAS)
- Human iPSC-derived iCell® Neurons (Cellular Dynamics International)
- Neuronal media (Cellular Dynamics International)
- Matrigel (Corning)
- Calcein AM (Life Technologies)
- MitoTracker Orange (Life Technologies)
- Hoechst (Life Technologies)
- Saponin (Sigma)
- PBS (Sigma)
- Αντι-β-tubulin III (TUJ-1) antibodies (BD Biosciences)
- ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices)
- MetaXpress High-Content Image Acquisition and Analysis Software, version 6.2 (Molecular Devices)

Formation of neurite networks in a 3D matrix

Cell culture, compound treatment, and cell staining steps were performed using the microfluidics assay format. Confocal imaging and analysis protocols were optimized for assessing morphological phenotypes and viability of neurons cultured in a 3D matrix. Human iPSC-derived neurons were plated at a density of 30,000 cells/chip after premixing with Matrigel to a final concentration of 7 mg/ml Matrigel in the OrganoPlate®. The Matrigel cell suspension was kept on ice during plating. The seeding volume of this solution varied between 1-1.4 µL per chip depending on cell density and Matrigel concentration. After seeding, the plate was placed into a tissue culture incubator (37°C, 5% CO_2) for 30 min to facilitate polymerization of the Matrigel. Next, 50

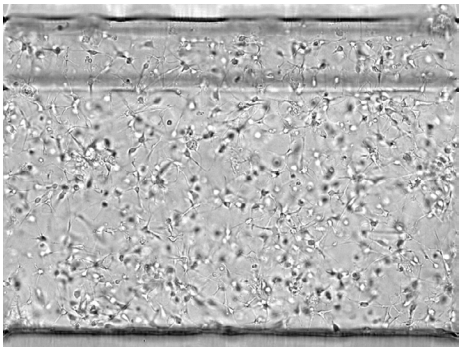

Figure 1. Neurons in OrganoPlate. Transmitted light images captured of iCell neurons plated into OrganoPlate capillary wells in Matrigel. Cells were plated at a density of 30,000 cells per chip for 72 hours, then imaged using transmitted light with a 20x, Plan Fluor objective.

µL of growth media was added to both the medium inlets and outlets of the chips , and then placed back into the tissue culture incubator. Neurite outgrowths formed ~24h after plating and extended for up to 14 days in culture.

To evaluate the feasibility of the OrganoPlate platform for testing neurotoxicity and neuronal development, these neuronal cultures were treated with a selection of compounds known to inhibit neurite outgrowth[7]. 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. Compound dilutions were performed by applying the appropriate final dilution in the media before addition to the medium inlets and outlets of the OrganoPlate chips.

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 (1 µM), the mitochondria potential dye MitoTracker Orange (1 µM), and the Hoechst nuclear dye (2 µM). The dye mixture was added to the chips and incubated for 60 min followed by replacement with culture medium. Alternatively, cells were fixed in 4% formaldehyde, permeabilized with 0.01% of saponin solution in PBS and stained using fluorophore-conjugated antibodies against β-tubulin III (TUJ-1) neuronal marker (1:100 dilution). Nuclei were stained with Hoechst. Staining of fixed cells with primary antibodies was performed overnight at 4°C.

Phenotypic analysis of 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 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,

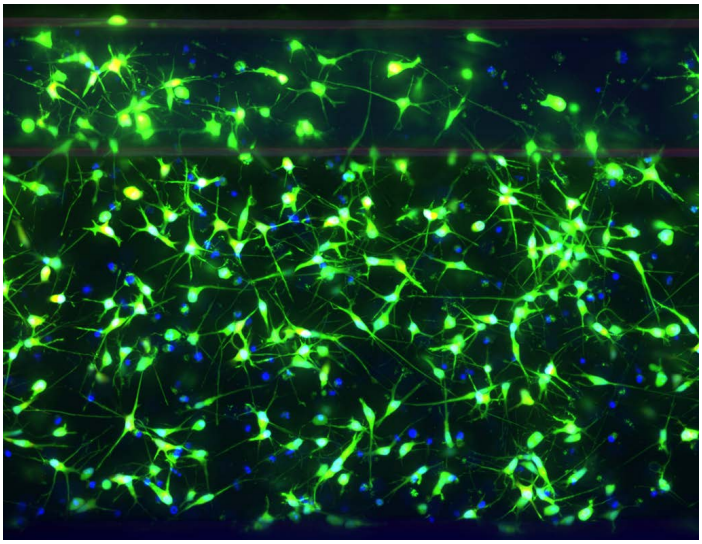

Figure 2. Live cell staining of neurons. iCell neurons plated into OrganoPlate capillary wells, in Matrigel, at 30,000 cells per chip. Cells were maintained for 5 days, and then live cells were stained with the viability dye Calcein AM, the mitochondrial integrity dye MitoTracker Orange, and the Hoechst nuclear dye. Then Z-stacks of images in 3 colors were taken using the ImageXpress Micro Confocal system in confocal mode using the 60 µm pinhole spinning disc configuration, 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.

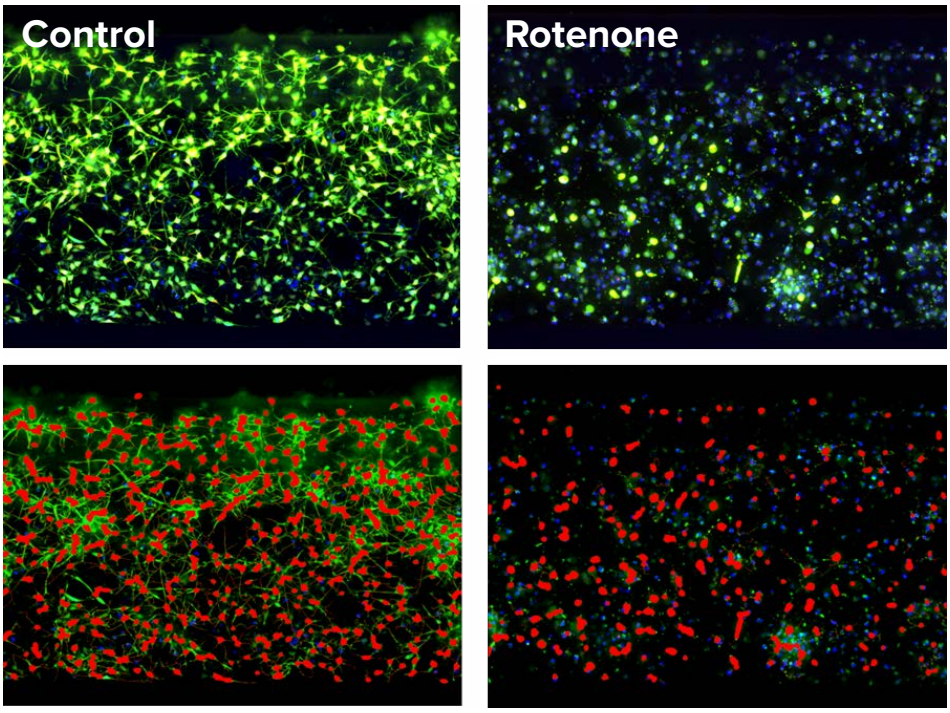

Figure 3. 2D analysis of neural networks. iCell neurons were plated at a density of 30,000 cells per chip for 24 hours, then treated with compounds for 5 days, and then live cells were stained with Calcein AM, MitoTracker Orange, and Hoechst. Z-stacks of images in 3 colors were taken using the ImageXpress Micro Confocal in confocal mode using the 60 µm pinhole spinning disc configuration at 20x magnification. Maximum projection images were analyzed using the MetaXpress Neurite Outgrowth application module. Masks of the cell bodies and neurites are shown in red. Images of control (DMSO) and rotenone (10 mM) treated cells are shown. Disintegration of neuronal connections is detectable as a result of compound treatment.

covering approximately 150-300 µm in depth. All individual images were saved and used for 3D analysis, as well as 2D projection (Maximum Projection or Best Focus) images.

Image analysis of Maximum Projection images in 2D

Images were analyzed using MetaXpress® High-Content Image Acquisition and Analysis Software. Automated quantitative analysis of these images was performed using two methods: analysis of projection images (2D) or 3D image analysis on Z-stacks. For the analysis of projection images, Z-stacks of images were converted into 2D maximum projection images at the time of acquisition, then, analyzed using the MetaXpress Neurite Outgrowth application module. Phenotypic readouts included quantitative characterization of the extent and complexity of neural networks via several endpoint measurements including: total neurite outgrowth, number of processes and branches, as well as cell number and viability. Best focus projection images were used for analysis of transmitted light images. Analysis of projection images is rapid, while providing an accurate estimation of the extent of neural networks. However, 2D analysis has its limitations, as this method does not calculate volume measurements and typically counts only a fraction of the objects. Figures 2 and 3 show composite fluorescent images of the control and rotenone treated neuronal cultures with the 'analysis masks' used to measure neurite outgrowth. Measurements derived include total neurite outgrowth, number of branches, processes, and the number of total cells (cell bodies). Figure 4 illustrates a decrease of neurite outgrowth length, number of branches and cell viability in response to exposure with neurotoxic compounds including: triphenylphosphate, tetraethyl thiuram, hexachlorophene, rotenone and methyl mercury (10 µM each).

3D visualization and 3D image analysis

MetaXpress software offers a 3D analysis option that allows for combining objects from adjacent Z-planes, as well as 3D visualization of cells and networks.

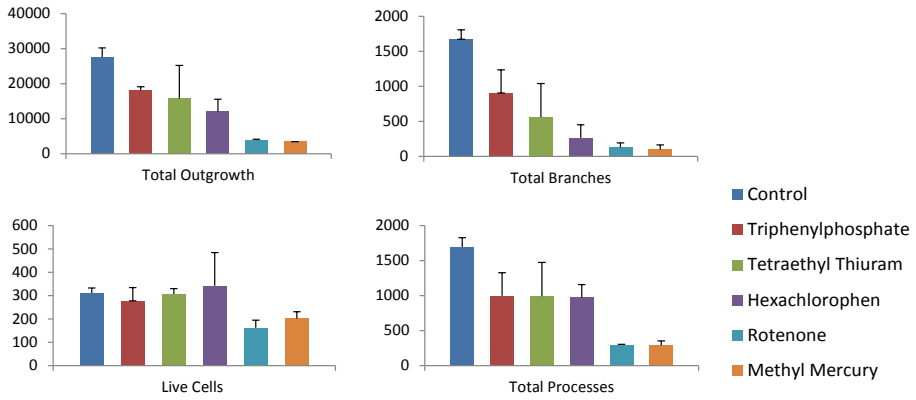

Figure 4. 2D measurements characterizing compound effects on neurite networks. iCell Neurons were cultured as described in Figure 2 and treated with the indicated neurotoxic compounds for 5 days. Z-stacks of images in 3 colors were taken using the ImageXpress Micro Confocal system in confocal mode using the 60 spinning disc configuration 20x magnification. Maximum projection images (2D compressions from 3D image stuck) were analyzed using the Neurite Outgrowth application module. Quantitative effects of compounds (10 µM) are shown on the total outgrowth, numbers of live cells, as well as the numbers of branches and processes.

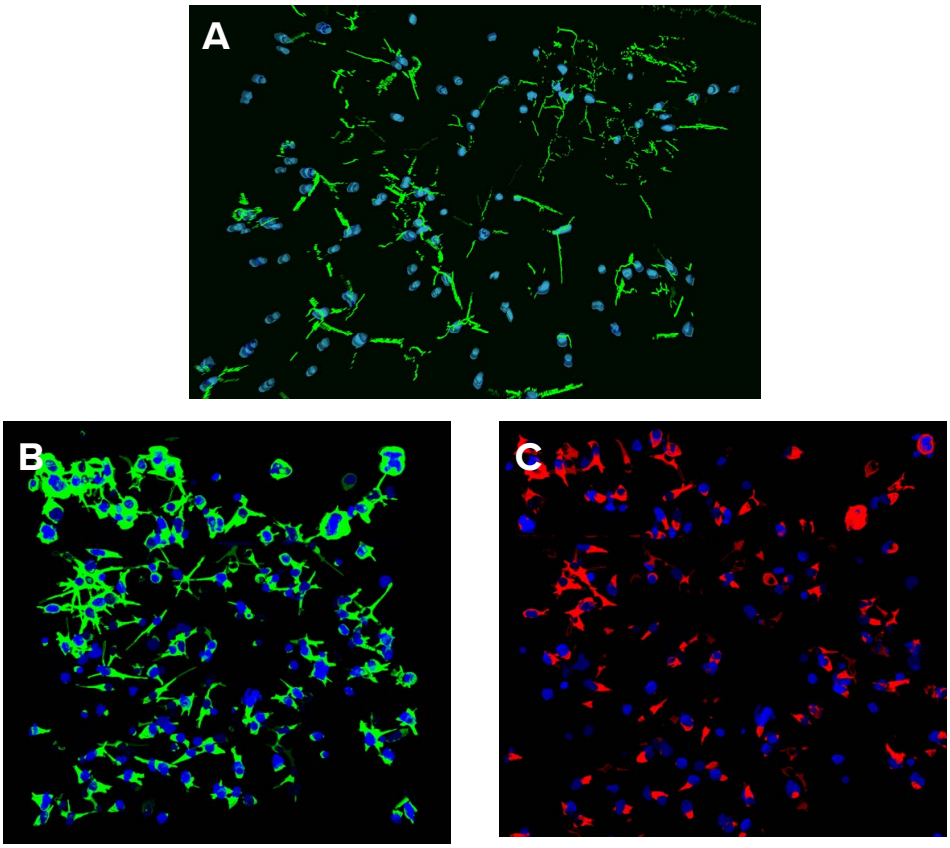

Figure 5. 3D analysis of neurite networks. Cells were treated and stained as described in Figure 2. Z-stacks of images were analyzed using MetaXpress Custom Module Editor 3D analysis option. Two different analysis modules were used for evaluation of complexity of neural networks in 3D: measuring fibers (neurites) and positive cell scoring (marker expression). **(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 andvolumes 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.

Images were analyzed using a 3D analysis Custom Module using “find fibers” function with which a “fibers” measurement was generated for quantifying neurite outgrowth; cell nuclei were also counted. Objects are first found in each plane, and then connected in 3D space using the “connect by best match” function. 3D analysis is typically more processing-intensive, however delivers improved resolution and quantitation of objects in the 3D space (including multiple overlapping objects). The measurements output from the 3D analysis included the number of neurites (fibers), cell volumes, total volume of fibers, numbers of processes, and branching points. Total numbers of cells (nuclei) were defined by finding “spherical objects” positive for Hoechst stain. Figure 5A shows the analysis masks for the “fibers” and “nuclei”.

An alternate 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. Figures 5B and 5C show the analysis masks for the “Calcein AM positive cytoplasm”, “MitoTracker positive cytoplasm” and “nuclei”. Figure 6 presents several measurements characterizing the compound effects on neurite networks: decreases in the numbers of processes (‘fibers”) and branching points, as well as the numbers of live cells or cells with intact mitochondria.

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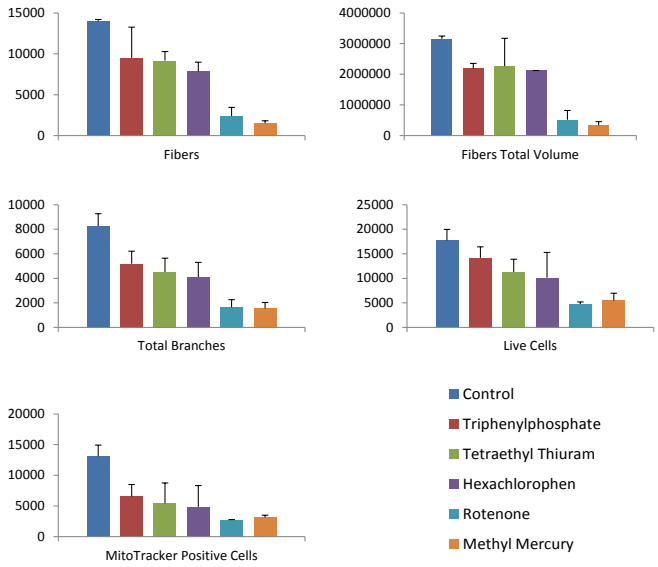

Figure 6. 3D measurements characterizing compound effects on neurite networks. iCells Neurons were cultured as described in Figure 2 and treated with indicated neurotoxic compounds for 5 days. The effects of 10 µM concentrations are shown for various measurements from 3D analysis. Numbers and volumes 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 the numbers of total, live cells, and MitoTracker Orange positive cells are presented.

Neurotoxicity assay in 3D using OrganoPlates

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. Therefore, this proposed method can be used for high-throughput, high-content compound screening for prediction of neurotoxicity.

Conclusion

We have developed a quantitative, confocal high-content imaging method that enables high-throughput phenotypic assessment of treatment effects upon the viability and morphology of 3D neuronal cultures using MIMETAS OrganoPlates®. Confocal imaging and multi-parametric 3D analysis allows for counting and characterization of neurites and also provides statistical characterization of neurite development and branching in 3D. Analysis methods enable characterization of neuronal networks and provide quantitative measurements that can be used for defining EC_{50} values and comparing toxic effects of selected compounds.

References

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