APPLICATION NOTE

High-content assay for morphological characterization of neuronal development in a 3D matrix using human iPSC-derived neuronal cultures

Benefits

- **Develop 3D neurotoxicity assays for compound screening**
- **Leverage hydrogels and high-content imaging for high- throughput 3D neurite outgrowth assays**
- **Generate quantitative measurements that can be used to define IC_{50} values and compare toxicities of various compounds**

Introduction

Development of more complex, biologically relevant, and predictive cell-based assays for compound screening is a primary challenge in drug discovery. The development and integration of three-dimensional (3D) assay models are becoming more popular to drive translational biology. Specifically, 3D cultures offer the advantage of closely recapitulating aspects of human tissues including the architecture, cell organization, cell-cell and cell-matrix interactions, and more physiologically-relevant diffusion of characteristics.

Hydrogels are widely used to simulate extracellular matrices to grow neural cells in a 3D environment. The hydrogel is poly (ethylene glycol) (PEG)-based, fully synthetic, and transparent. The hydrogel is made permissive to cell migration via the incorporation of MMP-cleavable sites and contains RGD motives to support cell adhesion[1]. The fully synthetic hydrogels were previously developed pre-casted in a 96-well plate which features an in-depth surface density gradient promoting the infiltration in 3D of cells deposited on the hydrogel surface (3DProSeed™ hydrogels, Ectica Technologies). This hydrogel platform is simple to use and compatible with automation[2,3] (Figure 1).

In conjunction, human induced pluripotent stem cells (iPSC)- derived neurons are also increasingly used for the development of physiological cell models; their human origin, prolonged viability in culture and availability in unlimited volumes makes them more advantageous compared to primary cell and animal models for neuroscience applications.

Formation of 3D neurite networks in multi-well micro titer plates

We optimized cell culture and staining in a 96-well format and developed confocal imaging and analysis protocols for assessing morphological phenotypes and viability of neurons grown in a 3D matrix.

Cells used for the assay were CNS.4U™ (Axiogenesis AG), a human iPSC-derived cell mix composed of neurons (glutamatergic , dopaminergic and GABAergic) and astrocytes. CNS.4U™ cells were cultured for up to 14 days in 3DProSeed™ hydrogel (Ectica Technologies). Cells were plated at 40,000 neurons/well in 200 µL media. For selected studies, the effect of cell seeding density was also evaluated (5,000-80,000 cells/well). The medium composition included a 50:50 mix of neurobasal media + DMEM/F12 + supplements (Axiogenesis).

Cells were seeded on the surface of hydrogels and then allowed to penetrate inside the hydrogel (Figure 1). Neurite outgrowths began to form ~24h after plating and continued over 14 days in culture. The formation of neurite networks were monitored over time using transmitted light imaging. For the endpoint measurements, cells were fixed using 4% formaldehyde, then permeabilized with 0.1% of Triton X-100 and stained using fluorophore-conjugated antibodies against TuJ-1 neuronal marker, plus Hoechst nuclear stain.

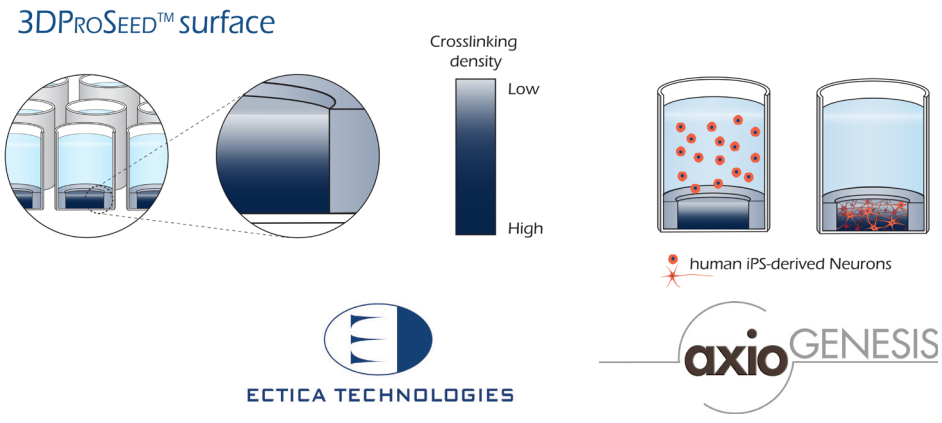


Figure 1. Schematic presentation of 3DProSeed™ hydrogel and seeding of neuronal cells into the matrix.

Generate 3D images from a stack of images

High-content imaging and analysis were used to evaluate the treatment effects on the formation of neuronal networks. A series of images was acquired at different planes along the focal axis (Z-stack) (Figure 2). Images were acquired using the ImageXpress® Micro Confocal High-Content Imaging System, with 10X or 4X objectives. A z-stack of 11-33 planes separated by 5-10 µm was acquired, covering 150-300 µm in depth. In addition to automatically saving the 2D projection (Maximum Projection or Best Focus) images, all individual images were saved and used for 3D analysis.

Generate measurements from 2D or 3D image sets

Images were analyzed using the 3D analysis module available in the integrated MetaXpress software. Quantitative analysis was done using 2 methods: analysis of projection image (2D) or 3D analysis. 2D maximum projection images (Hoechst staining and TuJ-1 fluorescence) were analyzed using a neurite outgrowth algorithm. Phenotypic readouts included quantitative characterization of the extent and complexity of neural networks by multiplexed read-outs including measuring 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 enables rapid and accurate quantitation of these phenotypes. Figure 3 shows image examples of neuronal networks using transmitted light, fluorescence (TuJ-1 and nuclear stain), and the analysis masks used to measure neurite outgrowth.

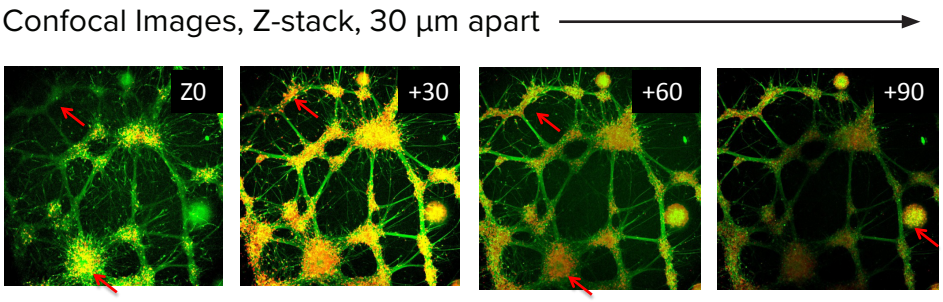


Figure 2. CNS.4U™ cells were seeded on 3DProSeed™ at 40,000cells/gel and imaged at 14 days in confocal mode. Nuclei were stained with Hoechst (red) and tubulin –TuJ-1 (green). Four panels represent different focal planes. Network of neurites extends several hundreds of micrometers into the hydrogel.

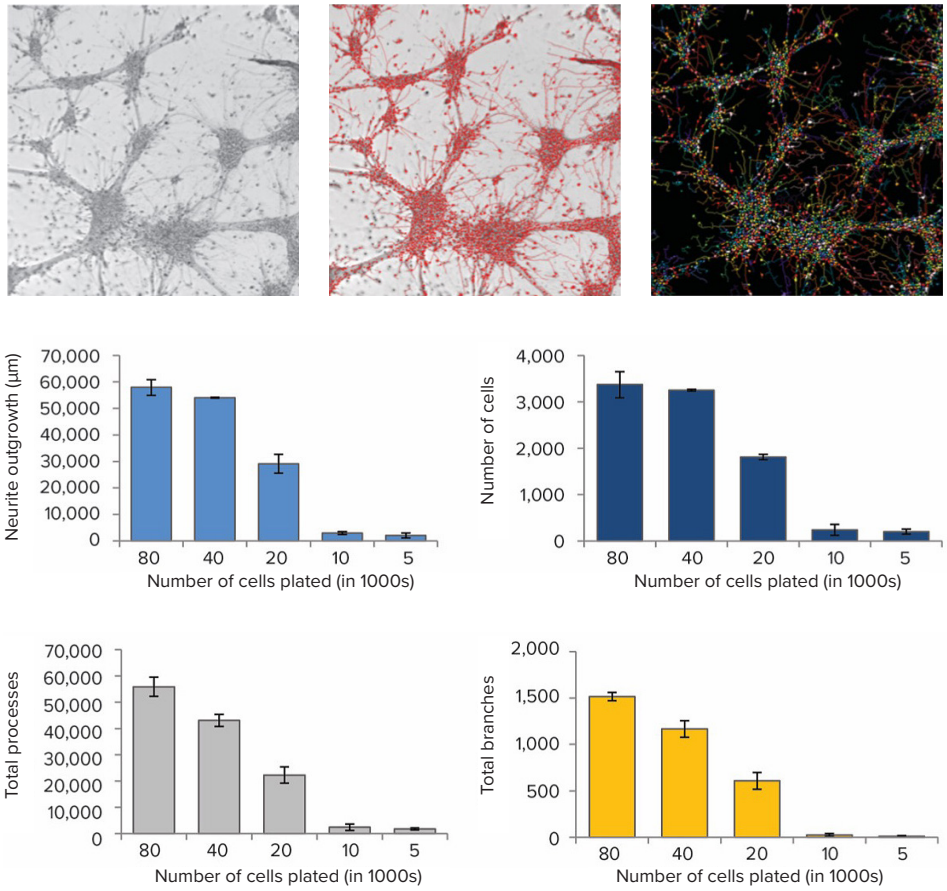


Figure 3. Analysis of 2D projection image. (Top) Transmitted light projection images and overlaying analysis masks for neuronal detection. CNS.4U™ cells were seeded on 3DProSeed™ at different seeding densities (5,000-80,000 cells/gel). Cells were kept in culture for 14 days followed by staining and imaging on the ImageXpress Micro Confocal system. Imaging in 3D (Z-stacking) was performed and 2D projection images were used for analysis: maximum projection for fluorescent images and best focus for the transmitted light images. Analysis automatically measured cell number, neurite outgrowth (length of neurites), as well as numbers of processes and branches. Graphs demonstrate dependencies of various measurements from the number of plated neurons. Averages from triplicate wells presented.

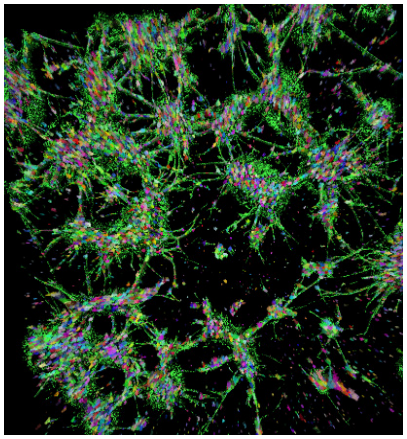
The MetaXpress 3D analysis module combines objects from different planes to create a 3D visualization of cells and networks. 3D visualization represents individual cell nuclei (pseudocolored) and TuJ-1-positive neurites and cell bodies (green) (Figure 4). The MetaXpress Custom Module Editor was used to define neurites for outgrowth and cell nuclei. Objects are first found in each plane and then connected in 3D space using the "connect by best match" function. 3D analysis provides more accurate quantitation of cells throughout the volume of the matrix including overlapping objects.

Compounds can be screened using 3D hydrogels

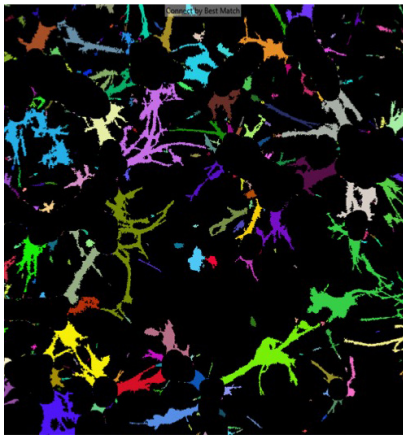
Phenotypic readouts included quantitative characterization of the extent and complexity of neural networks by multiplexed read-outs. We evaluated assay reproducibility, characterized multiple measurements, and tested a series of compounds that are known neurotoxicants. Two methods for analysis were compared: analysis of projection images using standard neurite outgrowth algorithm and 3D analysis using custom module defining neurites, branches, and nuclei.

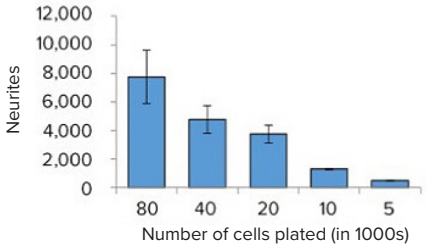
We evaluated feasibility of neurotoxicity studies in hydrogel by treating cells grown in this format with compounds at 72 hours post-plating, measuring effects for 7-10 days post-treatment. (Figure 5). The culture media with compounds were changed every 2 days. Post-treatment, these cultures were fixed, stained, and imaged as described above. We then measured concentration–response effects of selected compounds on neural networks (neurite outgrowth and other read-outs). These experiments provide proof of concept for using the assay system for neurotoxicity assessment.

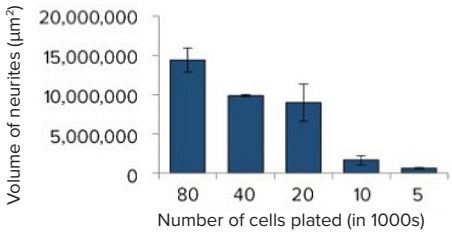
3D visualization of cell nuclei and TuJ-1-positive neurites and cell bodies

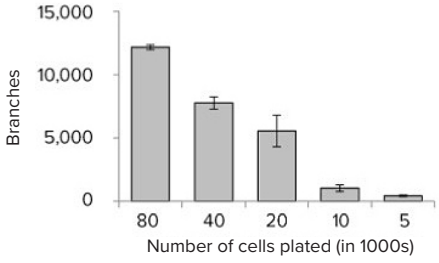


Analysis masks for outgrowths defined in the individual plane









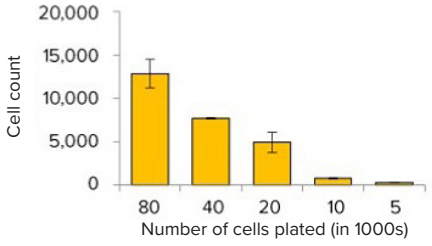


Figure 4. 3D evaluation of cells and networks in hydrogel by MetaXpress software. (Top Left) 3D visualization shows cell nuclei (pseudocolored) and TuJ-1-positive neurites and cell bodies (green). **(Top Right)** Analysis masks for outgrowths defined in the individual plane. 3D analysis was performed defining the number of neurites per well, total volume of neurites, number of branching points, and number of cells (nuclei). Graphs demonstrate dependencies of measurements from the number of plated neurons (triplicates).

Summary

We developed a quantitative high-throughput assay which enables assessment of the viability and morphological changes in 3D neuronal cultures. Using 3D ProSeed™ hydrogels, CNS.4U™ human iPSC-derived neural cells, and confocal high content imaging, this proposed method can be used for high-throughput compound toxicity screening and safety evaluation.

High resolution imaging and multi-parametric analysis allows neurite and single cell counting and statistically characterizes neurite development and branching in 3D. Using 2D and 3D analysis provides quantitative measurements that can be used for calculating IC_{50} values and comparing toxicities of various compounds..

References

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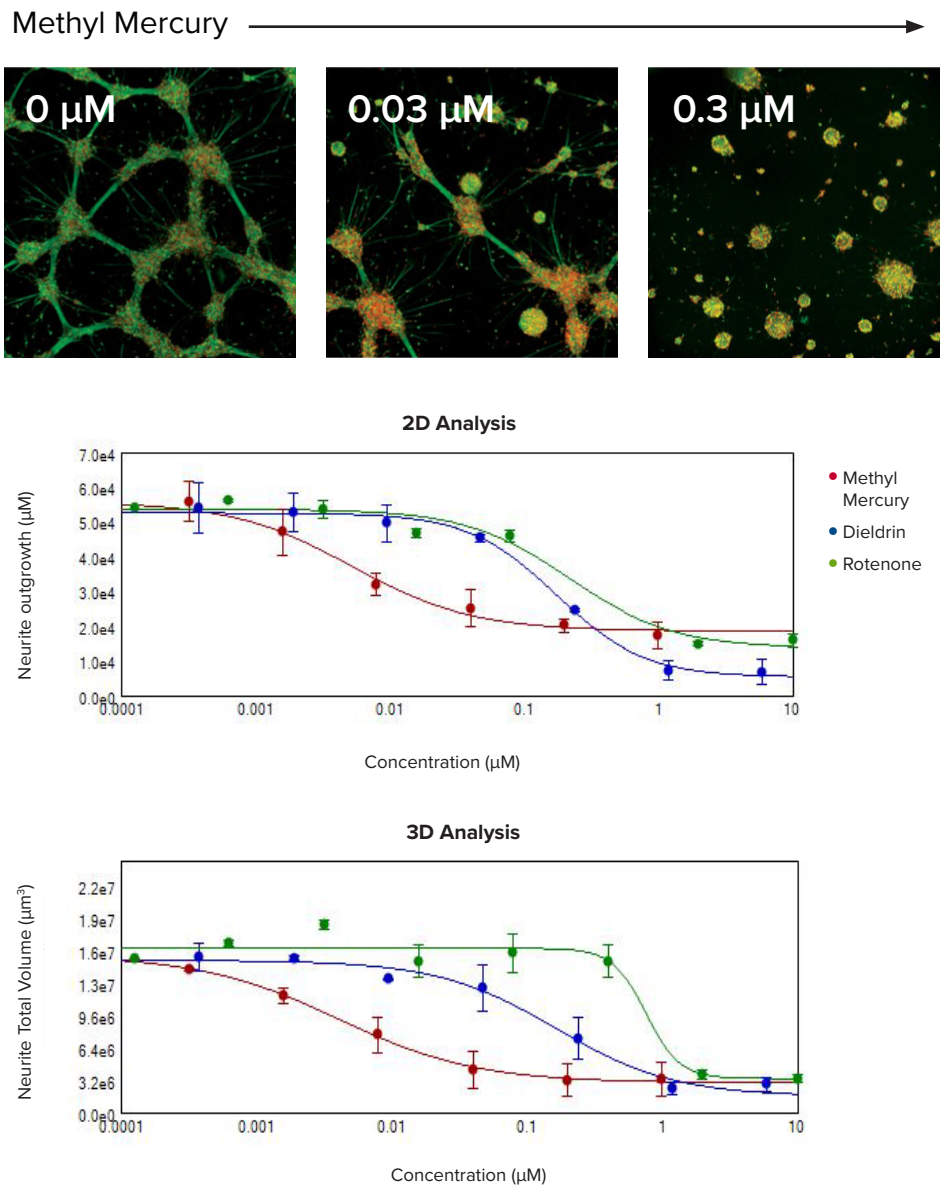

Figure 5. Measuring dose responses of chemical compounds. At 72h post-seeding three compounds with known neurotoxic effect were added to the neuronal cultures at different concentrations ranging from 0 to 10 µM. **(Top)** Inhibitory effects of compound on neurite development is readily visualized. **(Upper graph)** 2D compressed images (maximum projections) were used for analysis. Concentration-response curves are shown for neurite outgrowth: methyl mercury (IC_{50} 5 nM), dieldrin (IC_{50} 170 nM), and rotenone (IC_{50} 220 nM). The IC_{50} values were comparable with 3D analysis and previously published data[4]. **(Lower graph)** 3D analysis results. The total volume of neurites was measured. Concentration-response curves shown for total branches: methyl mercury (red, IC_{50} 2 nM), dieldrin (blue, IC_{50} 170 nM), and rotenone (green, IC_{50} 750 nM).

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