**INTRODUCTION**

Development of more complex, biologically relevant, and predictive cell-based assays for compound screening is one of the main challenges in drug discovery. There is an increasing interest in using three-dimensional (3D) cultures for assay development and translational biology. 3D cultures are believed to have the advantage of closely recapitulating aspects of the human tissues including the architecture, cell organization, cell-cell and cell-matrix interactions, and more physiologically relevant diffusion characteristics.

Hydrogels are widely used as an artificial extracellular matrix to grow neural cells in a 3D environment. The fully synthetic hydrogels were developed pre-casted in a 96-well plate featuring an in-depth surface density gradient promoting the infiltration in 3D of cells deposited on the hydrogel surface (3DProSeed™ hydrogels). This platform offers high simplicity of use and high compatibility to automation.

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

**METHODS**

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

**ASSAY DEVELOPMENT**

- Cells used for the assay were CNS-4U™ (Ncardia), an iPSC-derived cell mix comprised of neurons (glutamatergic, dopaminergic, and GABAergic), as well as astrocytes.
- 3DProSeed™ 96-well plate provided by Ectica Technologies. The plate contains pre-casted fully synthetic PEG-based hydrogels. The hydrogel feature an in-depth surface density gradient promoting the infiltration in 3D of neurites and establishment of a 3D network. This hydrogel platform offers high simplicity of use and high compatibility to automation (Simona et al. and Zhang et al.).

** Formation of 3D Neural Networks in Hydrogels:**

- CNS-4U cells were cultured for up to 14 days in 3DProSeed hydrogel. Cells were plated at 40,000 neurons/well, in 200 µL media. Seeding density can be varied. Medium composition included 50:50 mix of neurobasal media + DMEM/F12 + supplements (Ncardia)
- Cells were settled first on the surface of hydrogels, and then penetrated inside the hydrogel. Neurite outgrowth started to form ~24h after plating and extended over 14 days in culture. Formation of neurite networks was monitored over time using transmitted light and confocal imaging.

**Staining**

For the end-point measurements cells were fixed using 4% formaldehyde, then permeabilized with 0.01% of Triton X-100 and stained using fluorophore-conjugated antibodies against TuJ-1 neuronal marker, plus Hoechst nuclear stain.

**RESULTS: Phenotypic Analysis of 3D Cultures**

**Imaging**

High-content imaging and analysis were used for evaluation of neuronal networks. We optimized cell imaging and staining, and also developed confocal imaging and analysis protocols for assessing morphology and viability of neurons in 3D matrix. A series of images were acquired at different planes along the focal axis (Z-stack) using ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices, Sunnyvale, CA), with 10X or 4X objectives. A stack of 11-33 planes separated by 5-10 µm was acquired, covering approximately 100-300 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**

Automatic quantitative analysis was done using 2 methods: analysis of projection image (2D) or 3D analysis. 2D maximum projection images were analyzed using a neurite outgrowth algorithm. Phenotypic read-outs 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. Analysis of projection image is faster and allows a good quantitation.

**Neurotoxicity Assay in 3D Hydrogels**

Phenotypic readouts included qualitative 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 fibers, branches, and nuclei.

**Summary**

Using 3DProSeed™ hydrogels for 3D culture of iPSC-derived CNS-4U™ neural cells and confocal high-content imaging, we have developed a quantitative high-throughput assay which allows assessment of the viability and morphological changes in 3D neuronal cultures.

Higher resolution and multi-parametric analysis allows neurite and single cell counting and statistically characterizes neurite development and branching in 3D. 2D and 3D analysis enable characterization of neuronal networks and provide quantitative measurements that can be used to define IC50 values and compare toxicities of various compounds.