

High-Content Assay for Morphological Characterization of Neuronal Development in 3D Matrix Using Human iPSC-Derived Neuronal Cultures

Oksana Sirenko¹, Grisha Chandy¹, Greta Thompson-Steckel², Thomas Palm³, Felix Haniel³, Benjamin Simona⁴ and Vincent Milleret⁴
¹Molecular Devices, LLC, Sunnyvale, CA, USA ²Laboratory of Biosensors and Bioelectronics, ETH Zurich, Switzerland
³Ncardia, Cologne, Germany ⁴Ectica Technologies AG, Zurich, Switzerland

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.

GOAL

The focus of the present study was to develop a high-throughput 3D neurite outgrowth assay using iPSC-derived neurons developing in the hydrogel matrix, with the long term goal of establishing automation-compatible 3D models for neurodegenerative and neurotoxicology screens.

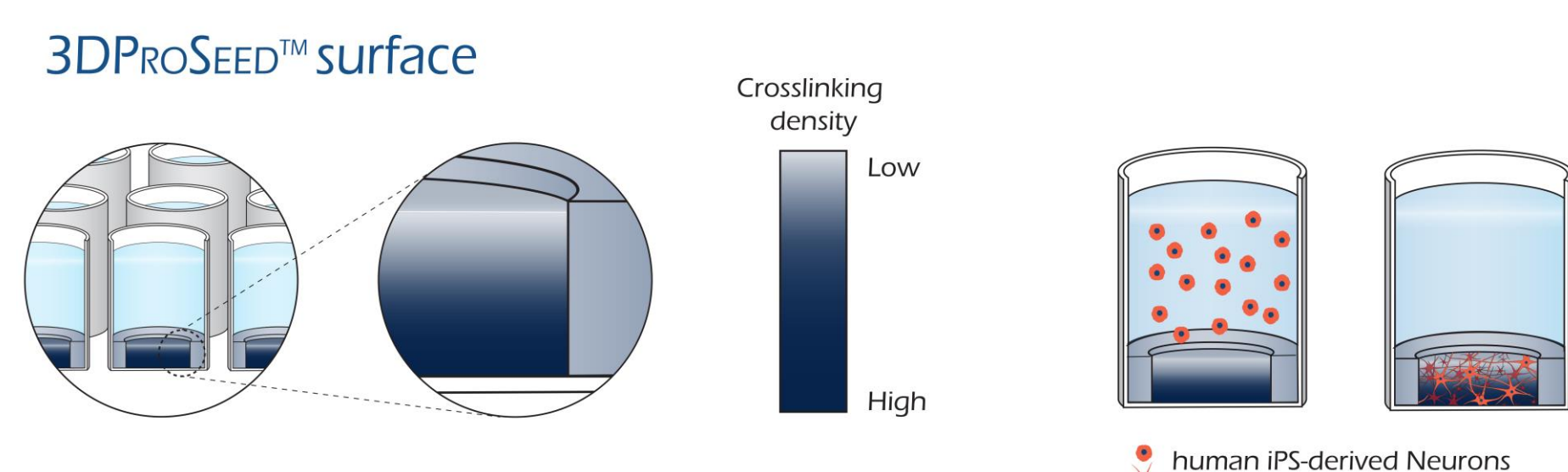
METHODS

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



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.

References

- Simona B.R. et al., Biomater. Sci., 3:586-591, 2015.
- Zhang N. & Milleret V., SLAS Discovery, accepted, 2017.
- Sirenko et al., Assay and Drug Dev Technologies 12(9-10):536-47

RESULTS: Phenotypic Analysis of 3D Cultures

Imaging

High-content imaging and analysis were used for evaluation of neuronal networks. We optimized cell culture 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.

Confocal Images, Z-stack, 30 µm apart

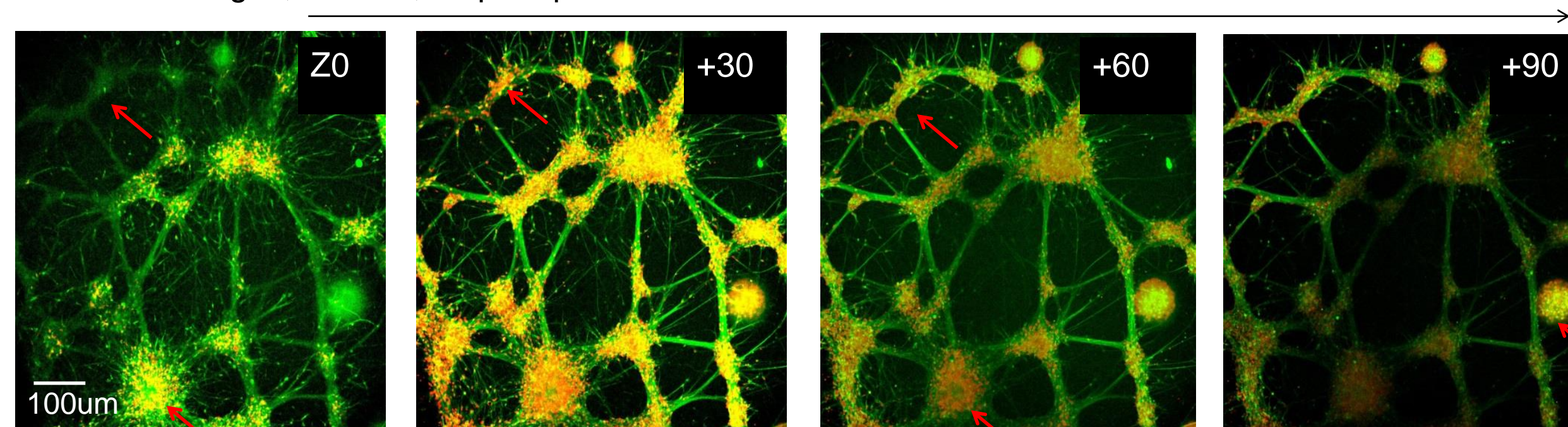


Figure 1. CNS.4U cells were seeded on 3DProSeed and imaged at 14 days in confocal mode. Nuclei are stained with Hoechst (red) and tubulin -TuJ-1 (green). Four panels represent different focal planes in the gel. Neurite networks extend several hundred micrometers into the hydrogel.

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.

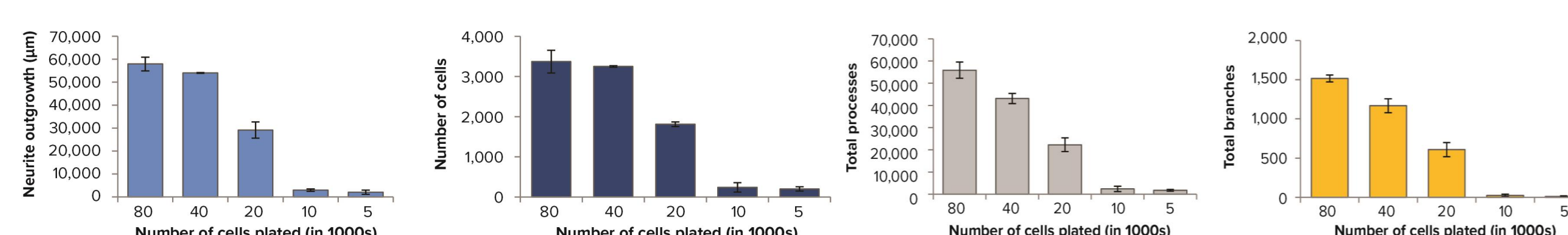
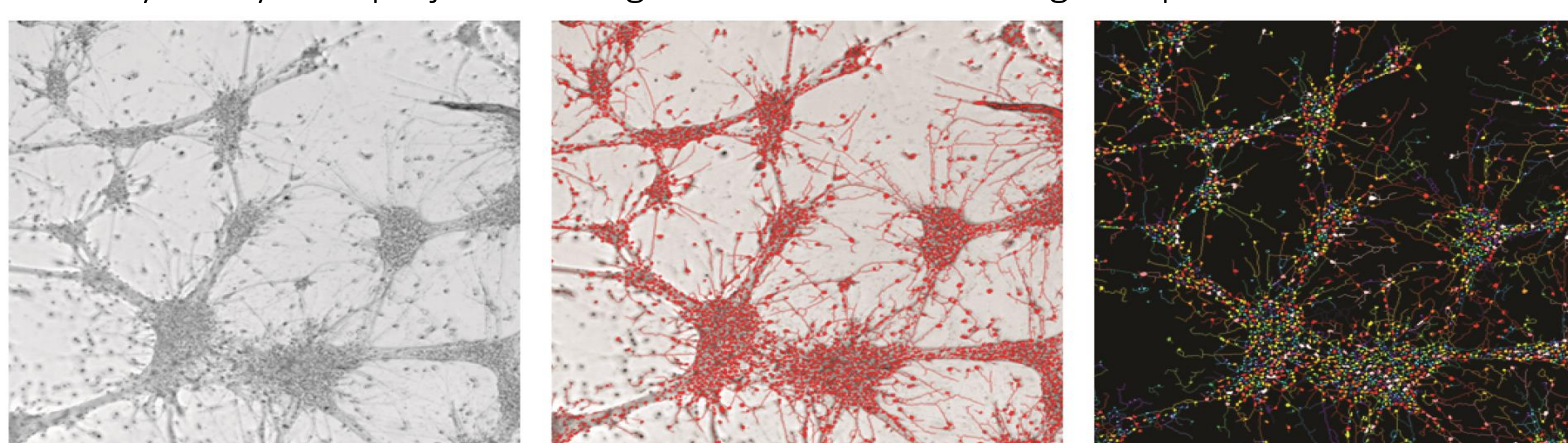


Figure 2. CNS.4U cells were seeded on 3DProSeed at different seeding densities (5,000-80,000 cells/gel). Cells were kept in culture for 14 days and imaging was performed using ImageXpress Micro Confocal system. Imaging in 3D (Z-stacking) was performed and maximum projections were used for analysis. Analysis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

3D Visualization and 3D Image Analysis

Analysis software allows combining objects from different planes and 3D visualization of cells and networks. Custom Module was used to define “fibers” for outgrowth and cell nuclei. Objects were first found in each plane and then connected in 3D space using “connect by best match” function. 3D analysis typically takes longer, but allows better resolution and quantitation of objects in the volume (including overlapping objects).

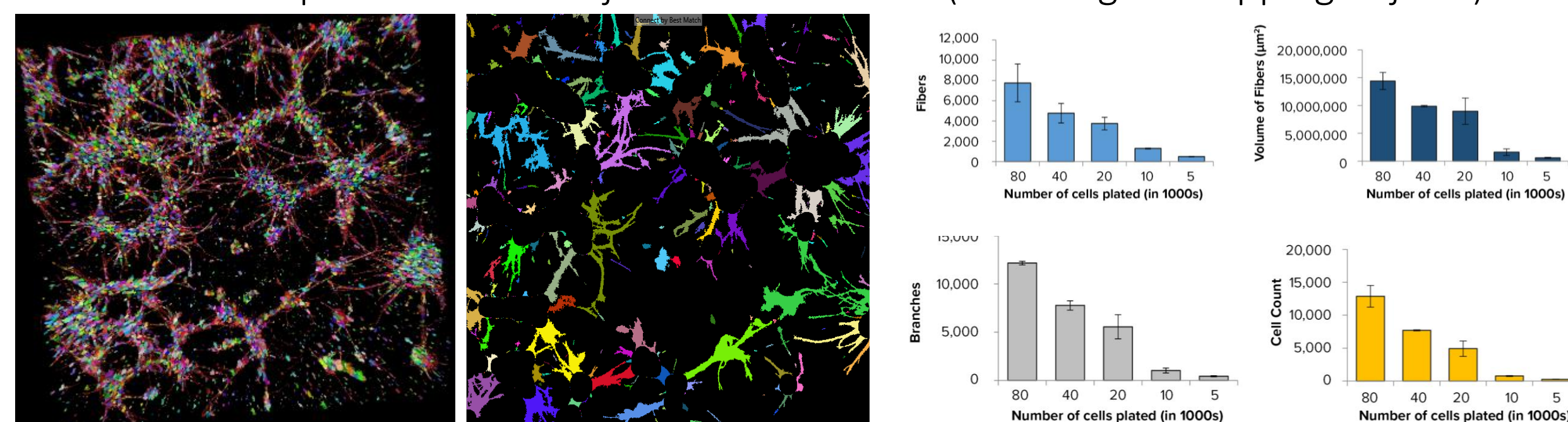


Figure 3. Left: 3D visualization of cells and networks in hydrogel by MetaXpress software. 3D visualization shows cell nuclei (pseudocolored) and TuJ-1 positive neurites and cell bodies (red). Middle: Analysis masks for “fibers” defined in the individual plane. 3D analysis was performed to define the number of fibers (neurites) per well, total volume of fibers (neurites), number of branching points, and number of cells (nuclei). Graphs demonstrate dependencies of measurements from the number of plated neurons (triplicates).

Neurotoxicity Assay in 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 fibers, branches, and nuclei.

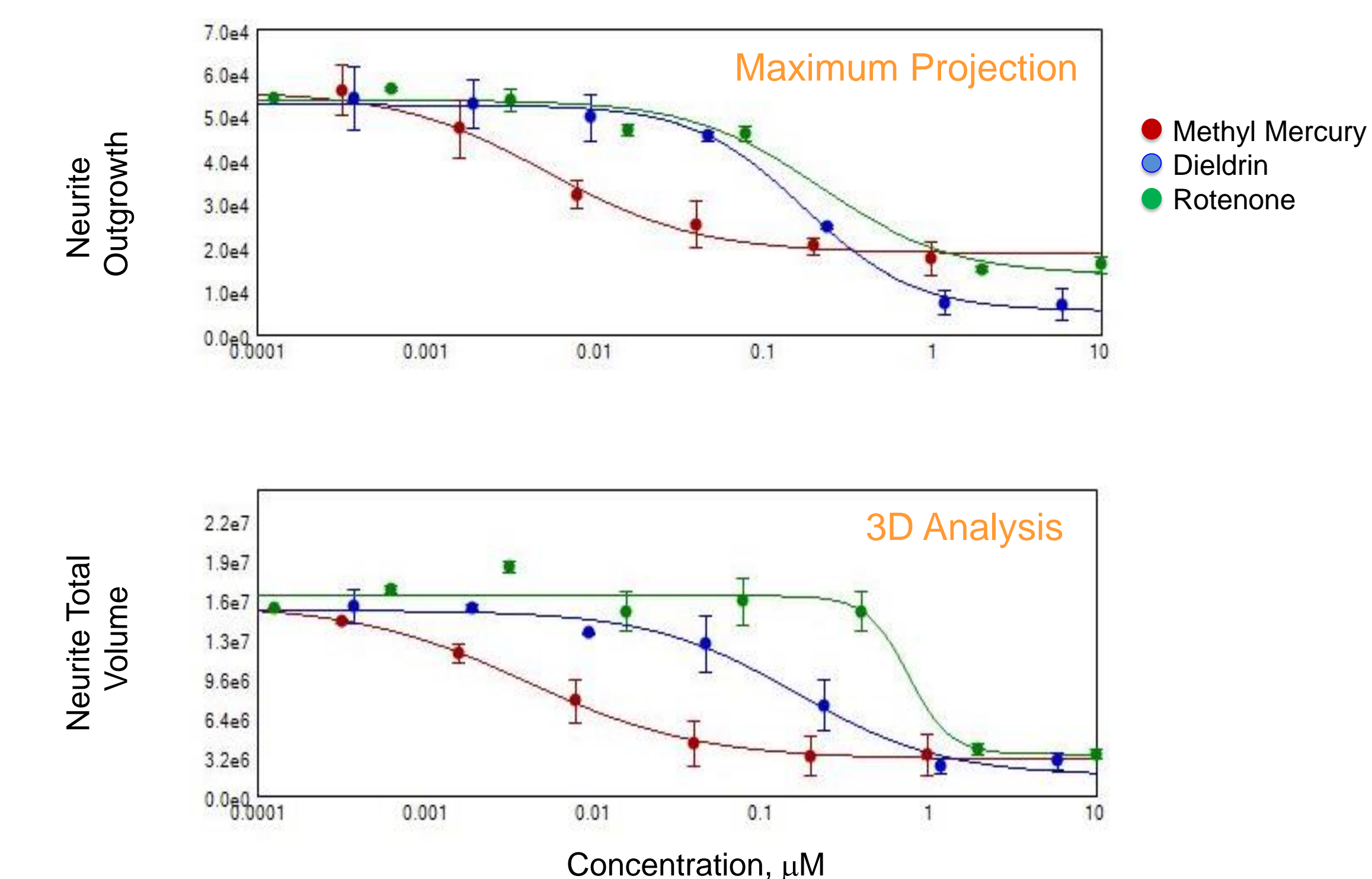
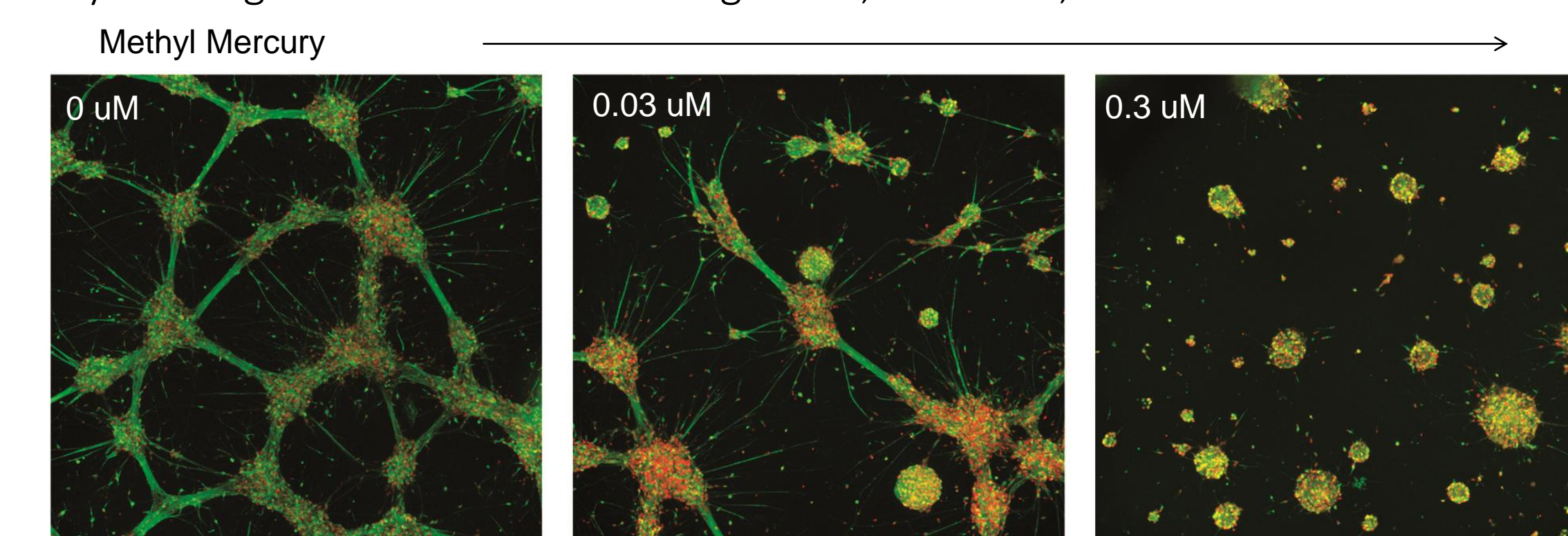


Figure 4. At 72h post-seeding three compounds with known neurotoxic effect were added to the neuronal cultures at different concentrations ranging from 0 to 10 µM.

Top panel: Inhibitory effect of compound on neuronal development. Cells were stained and imaged as described above.

Upper graph: 2D compressed images (maximum projections) were used for analysis. The neurite outgrowth, number of cells, processes and branches were measured. Concentration-response curves are shown for neurite outgrowth: methyl mercury (red, IC₅₀ 5nM), dieldrin (blue, IC₅₀ 170nM), and rotenone (green, IC₅₀ 220nM). The IC₅₀ values were comparable with 3D analysis (Lower Graph) and results were obtained using standard 2D neuronal cell cultures (Sirenko et al.).

Lower graph: Results of 3D analysis. The number of neurites (fibers), volume of fibers (neurites), branching points, and total cell numbers were measured. Concentration-response curves shown for total volume of neurites (µm³): methyl mercury (red, IC₅₀ 2nM), dieldrin (blue, IC₅₀ 170nM), and rotenone (green, IC₅₀ 750nM).

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 enables 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.

