Image-Based Analysis of a Human Neurosphere Stem Cell Model for the Evaluation of Potential Neurotoxicants

Brad Larson[1], Hilary Sherman[2], Hannah Gitschier[2], Alexandra Wolff[3], Wini Luty[3]

[1]BioTek Instruments, Inc.|Winooski, VT|USA • [2]Corning Incorporated, Life Sciences|Kennebunk, ME|USA • [3]Enzo Life Sciences|Farmingdale, NY|USA

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Abstract

Developmental neurotoxicity (DNT) of environmental chemicals has long been identified as a threat to the health of the human population, as the developing nervous system is particularly susceptible to toxicant exposure. The resulting neurological deficits can have long-term effects on families and society both financially and emotionally. Current DNT testing guidelines involve the use of animal models; primarily rodents. The testing strategy incorporates large numbers of animals, which can be extremely time- and cost-intensive; particularly due to the backlog of chemicals needing to be tested (Lein *et al.*, 2005). This demand, in addition to current and future proposed regulations on the use of animals for testing makes it imperative that new models be found to reduce animal experimentation while providing a suitable method to test new chemicals.

Three-dimensional cell models, which incorporate human neural stem cells (hNSCs) aggregated into neurospheres, have been proposed as a viable alternative for DNT testing. The *in vitro* system has the ability to recapitulate the processes of brain development, including proliferation, migration, differentiation and apoptosis (Salma *et al.*, 2015). Inclusion of human cells, as opposed to murine, also meets recommendations to circumvent the drawback of species differences between *in vivo* testing and actual exposure effects.

Here we demonstrate the use of a 3D neurosphere model, composed of hNSCs, to conduct toxicity testing of potential neurotoxicants. A spheroid microplate was used to create and maintain cells in the 3D model. 3D neurosphere proliferation, multipotency, along with the continued capacity to differentiate into neurons, astrocytes, and oligodendrocytes was initially validated. Neurotoxicity testing was then performed using neurospheres maintained in the 3D spheroid plate. Detection of induced levels of oxidative stress, apoptotic, and necrotic activity within treated neurospheres, compared to negative control spheres, was evaluated. Monitoring of cell proliferation, differentiation, multipotency and experimental testing was performed using a novel cell imaging multi-mode reader.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays. Integrated Gen5™ Data Analysis Software controls Cytation 5. The instrument was used to image spheroids using brightfield and fluorescence microscopy, as well as individual differentiated cells plated in two dimensional format.

Materials

Cells: StemPro® Neural Stem Cells (Catalog No. A15654) were purchased from ThermoFisher (Waltham, MA).

Plates: 384 Well Black, Clear, Round Bottom Ultra-Low Attachment Spheroid Microplates (Catalog No. 3830), 384 Well High Content Imaging Film Bottom Microplates (Catalog No. 4681), and 6 Well Clear, TC-Treated Microplates (Catalog No. 3516) were donated by Corning Life Sciences (Corning, NY).

Neurotoxicants and Plate Coatings: Mercury (II) Chloride (Catalog No. 215465), Methylmercury (II) Chloride (Catalog No. 442534), Hydrogen Peroxide Solution (Catalog No. 216763), Poly-L-Ornithine (Catalog No. P3655), and Laminin (Catalog No. L2020) were purchased from Sigma-Aldrich (Saint Louis, MO).

Antibodies: Nestin (human) monoclonal antibody (2C1 3B9) (Catalog No. ENZ-ABS109-0100), Oct4 monoclonal antibody (9B7) (Catalog No. ENZ-ABS271-0100), βIII-Tubulin monoclonal antibody (TU-20) (Catalog No. ALX-804-405-C100), GFAP monoclonal antibody (GF2) (Catalog No. ENZ-30934), Goat anti-mouse IgG1 (ATTO 590 conjugate) (Catalog No. ALX-211-204TM-C100), and Goat anti-mouse IgG (ATTO 647N conjugate) (Catalog No. ALX-211-205TS-C100) were donated by Enzo Life Sciences (Farmingdale, NY). Anti-oligodendrocyte specific protein antibody (ab53041) was purchased from abcam (Cambridge, MA).

Assay Components: ROS-ID™ Total ROS detection kit (Catalog No. ENZ-51011), GFP CERTIFIED® Apoptosis/Necrosis detection kit Apoptosis detection reagent (Catalog No. ENZ-51002), and NUCLEAR-ID® Blue/Red cell viability reagent (GFP Certified) (Catalog No. ENZ-53005-C100) were donated by Enzo Life Sciences (Farmingdale, NY).

Image-Based Tracking of Neurosphere Growth

Neural stem cells were propagated on 6 well plates previously coated with poly-L-ornithine and laminin. Cells were then removed and added to wells of a 384 well spheroid microplate at concentrations of 32,000-100 cells/well, with 12 replicates per concentration. Single neurospheres formed after a 48 hour period. The plate was inserted into the Cytation 5, previously set to 37 °C/5% CO_2, and all 96 wells containing spheroids were imaged (Figure 1A). The process was repeated at regular intervals over a 9 day period to determine the ability of neural stem cells to propagate in a 3D configuration.

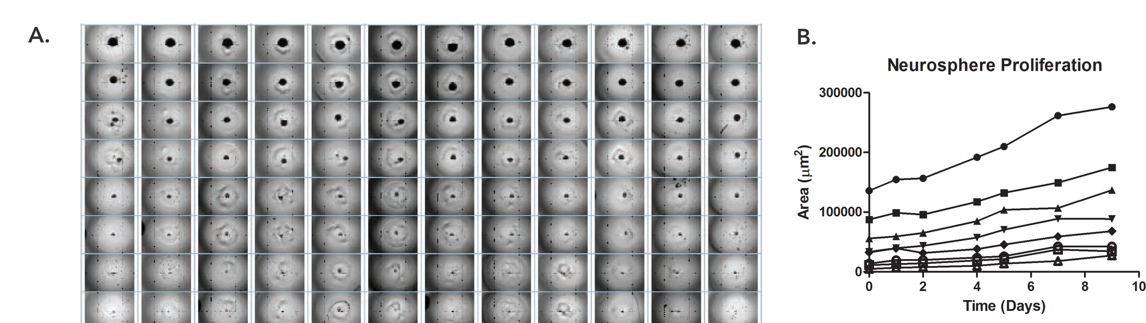

Figure 1. Imaging and area analysis of 3D neurosphere growth. **(A)** Thumbnail brightfield images of aggregated neurospheres captured using a 4x objective. Twelve replicates per row of neurospheres formed from neural stem cells dispensed at 32,000, 16,000, 8000, 4000, 2000, 1000, 500, and 100 cells/well. **(B)** Plot of average spheroid area per dispensed cell concentration during incubation period as determined by Gen5 Data Analysis Software.

Brightfield imaging, using a 4x objective, was performed on the spheroid set with the Cytation 5 on day 0 (after spheroid formation was complete) and again after 1, 2, 4, 5, 7, and 9 additional days of incubation. The cellular analysis capabilities of the Gen5 Data Analysis Software were used to automatically place object masks around each entire spheroid. Average area inside the masks for the 12 replicate neurospheres, as calculated by the software, was plotted over time (Figure 1B). All neurospheres showed active growth in the spheroid plate, as witnessed by an increase in average spheroid area from day 0 to day 9. Fold increases in area compared to original values ranged from a 2X increase for neurospheres initially containing 32,000 cells to a 5X increase for neurospheres initially containing 100 cells.

Neurosphere Proliferation and Multipotency Validation

The ability of neural stem cells, cultured into neurospheres, to maintain high proliferative and multipotency capability was further tested through immunocytochemistry. Nestin is an intermediate filament protein necessary for neural stem cell self-renewal. Oct4 is a transcription factor linked to multipotency in stem cells. Primary antibodies specific for each were added to separate wells containing cultured neurospheres to assay for the two biomarkers. Fluorescently labeled secondary antibodies were then added to the wells, in addition to negative control wells containing no primary antibody.

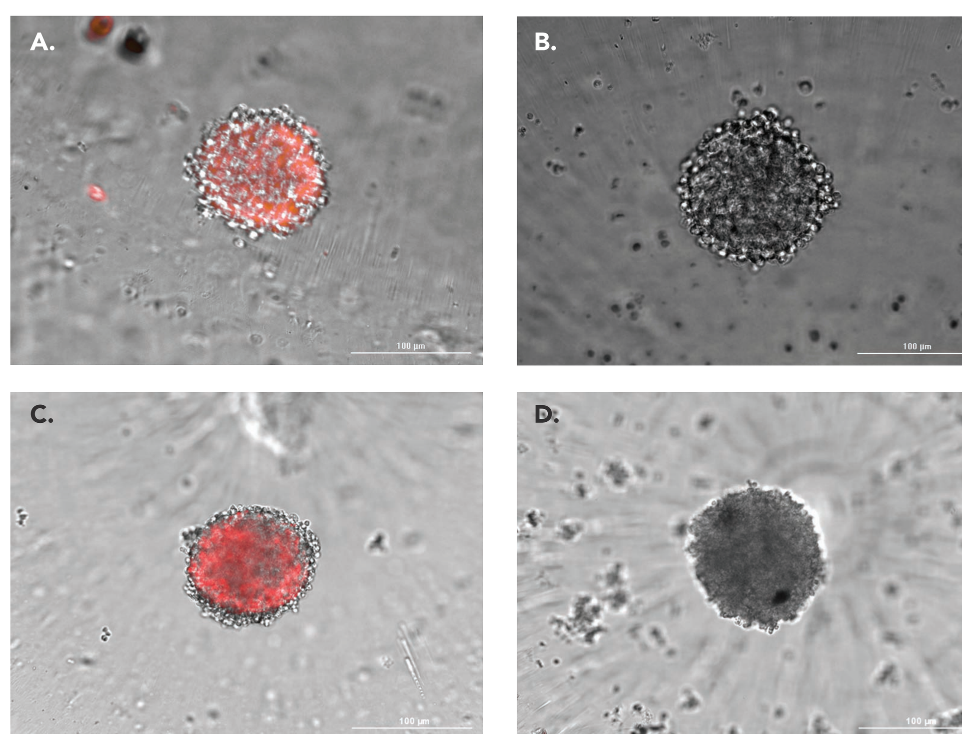

Figure 2. Detection of proliferation and multipotency markers. Overlaid brightfield and fluorescence 20x images of positive and negative control wells. **(A)** Proliferation positive control: Nestin (human) monoclonal antibody (2C1 3B9) plus Goat anti-mouse IgG1 (ATTO 590 conjugate) antibody. **(B)** Proliferation negative control: Goat anti-mouse IgG1 (ATTO 590 conjugate) antibody alone. **(C)** Multipotency positive control: Oct4 monoclonal antibody (9B7) plus Goat anti-mouse IgG (ATTO 647N conjugate) antibody. **(D)** Multipotency negative control: Goat anti-mouse IgG (ATTO 647N conjugate) antibody alone. RFP Channel: ATTO 590 goat anti-mouse IgG1; Texas Red Channel: ATTO 647 goat anti-mouse IgG.

Expression of nestin and Oct4 proliferation and multipotency proteins within 3D cultured neural stem cells is confirmed by the fluorescent signal emanating from primary and secondary antibody binding in Figure 2A and C. These findings are further validated by the fact that no fluorescence is seen from negative control wells containing no primary antibody, demonstrating that secondary antibody binding takes place only in the presence of bound primary antibody.

Neural Stem Cell Differentiation

The ability of 3D cultured neural stem cells to differentiate into neurons and glial cells was also evaluated using two separate methods. First, Accutase® cell detachment solution (Corning Catalog No. 25-058-CI) was added to specific wells to break apart neurospheres. Individual neural stem cells were then added to separate wells of a poly-L-ornithine/laminin coated 384 well high content imaging plate in the presence of specific media optimized by Corning Life Sciences to differentiate cells into neurons, astrocytes, and oligodendrocytes. Second, whole neurospheres were also transferred to wells of the 384 well plate containing differentiation medias. Upon completion of prescribed differentiation protocols, immunocytochemistry was again performed including primary antibodies for markers expressed in each lineage (βIII-Tubulin:Neuron; GFAP: astrocyte; Oligodendrocyte specific protein: oligodendrocyte) and previously described secondary antibodies.

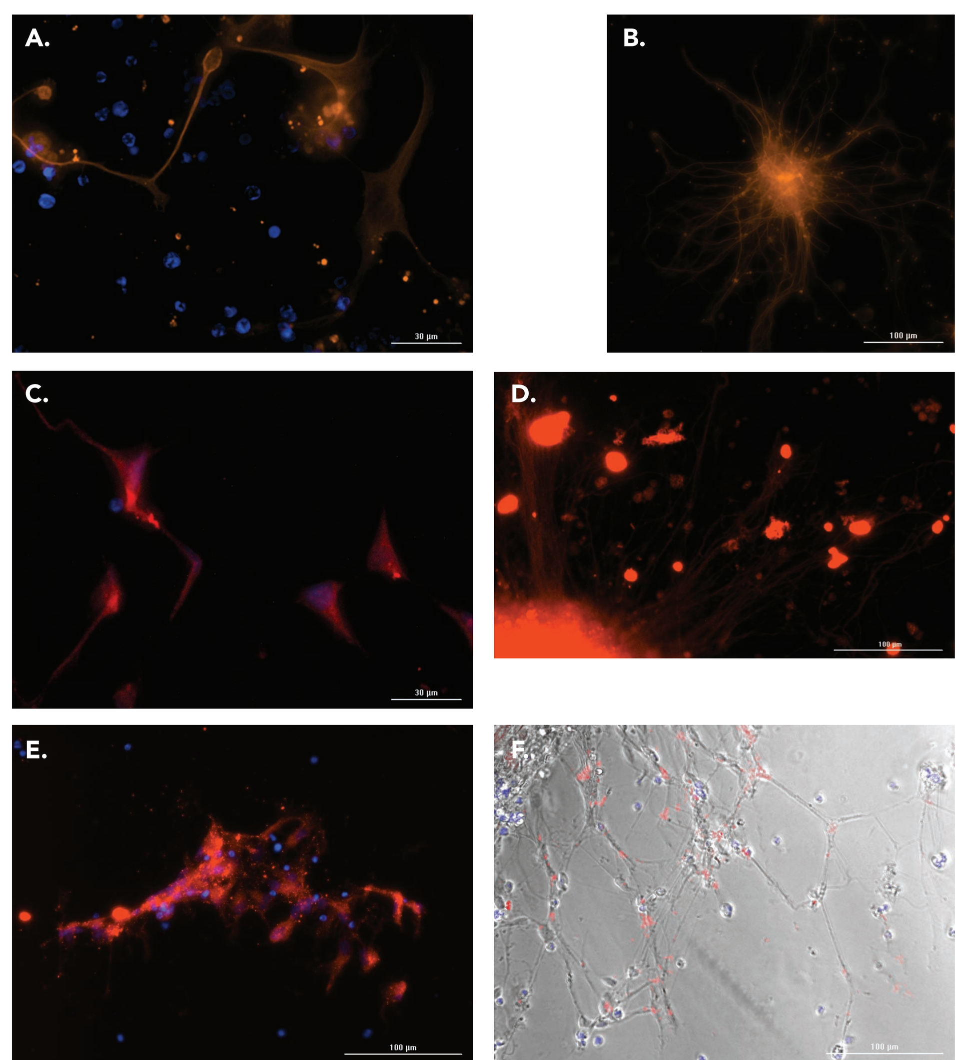

Figure 3. 2D and 3D neural stem cell differentiation. (A) 2D differentiated neurons and **(B)** 3D neurogenesis; 40x objective; RFP Channel: βIII-Tubulin 1° Ab/ATTO 590 2° Ab; DAPI Channel: Hoechst 33342. **(C)** 2D differentiated astrocytes and **(D)** 3D astrogenesis; 40x or 20x objective; Texas Red Channel: GFAP 1° Ab/ATTO 647 2° Ab; DAPI Channel: Hoechst 33342. **(E)** 2D differentiated oligodendrocytes and **(F)** 3D oligodendrogenesis; 20x objective; Texas Red Channel: oligodendrocyte specific protein 1° Ab/ ATTO 647 2° Ab; DAPI Channel: Hoechst 33342; Phase contrast channel overlay also shown in **(F)**.

Differentiation into neurons (Figure 3A), astrocytes (Figure 3C), and oligodendrocytes (Figure 3E) was seen from 3D cultured neural stem cells differentiated in 2D format. Neuro- (Figure 3B), astro- (Figure 3D), and oligodendrogenesis (Figure 3F) was also seen from differentiated neurospheres when exposed to the same incubation conditions

The combination of the neurosphere validation experiments performed confirms that neural stem cells exhibit no ill effects from 3D culture, maintain proliferative, multipotency, and differential capabilities, and represent a suitable model for neurotoxicity testing.

Neurotoxin Analysis

Evaluation of the effect of compounds to cause a toxic effect on 3D neurospheres was then performed. Neural stem cells, at a concentration of 2000 cells/well, were added to a 384 well spheroid plate and allowed to aggregate. Neurospheres were then exposed to varying concentrations of three well known neurotoxins methylmercury chloride, mercury chloride, and hydrogen peroxide for 1, 2, 4, or 7 days. Spheres were dosed with fresh media and compound daily. Following incubation, media containing compound was removed, wells were washed with fresh media, and media containing total ROS, apoptosis, and live/dead cell probes was added for four hours. Wells were washed 2X with fresh media to remove unbound probe and imaged by the Cytation 5 using a 4x objective and DAPI, GFP, RFP, and Texas Red fluorescent imaging channels.

Cellular analysis was then carried out using Gen5 to accurately detect the signal from each probe emanating from the neurospheres. As the fluorescence from the live cell probe remains relatively consistent despite potential changes in all other probes, this signal as measured with the DAPI channel, was used by the software to automatically draw object masks around each neurosphere (Figure 4A). Minimum and maximum object size values were also increased appropriately to allow the spheres to be seen as single objects, as opposed to individual cells. The total fluorescent signal within the object mask, captured with the GFP, RFP, and Texas Red imaging channels (Figure 4B-G), was then calculated to determine the effect each compound treatment had to change oxidative stress, apoptosis, and necrosis levels, respectively, within 3D cultured neural stem cells.

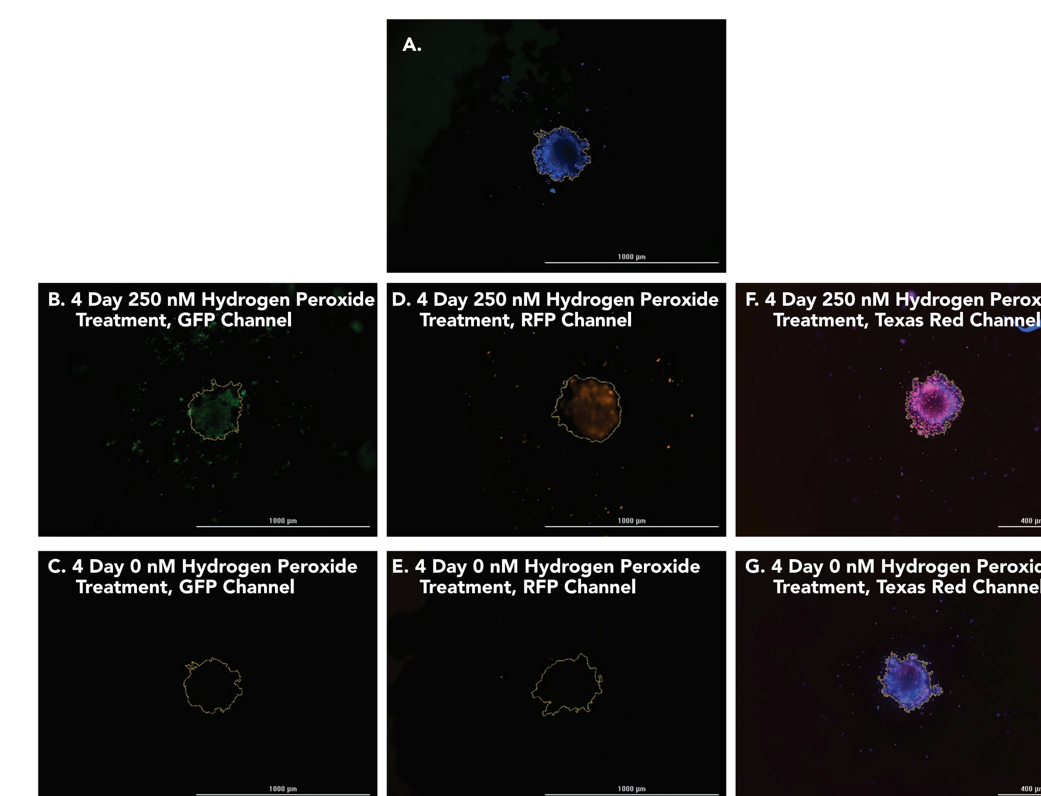

Figure 4. Image-based cellular analysis of fluorescent probes. 4x images captured following neurotoxin treatment and fluorescent probe incubation. **(A)** Gen5 placed object mask around Nuclear-ID live cell probe signal emanating from treated neurosphere captured using DAPI channel. Fluorescent signal from positive or negative control wells, treated for four days with 250 or 0 nM hydrogen peroxide, respectively, shown for **(B-C)** total ROS probe (GFP channel); **(D-E)** apoptosis detection reagent (RFP channel); and **(F-G)** dead cell probe (Texas Red channel).

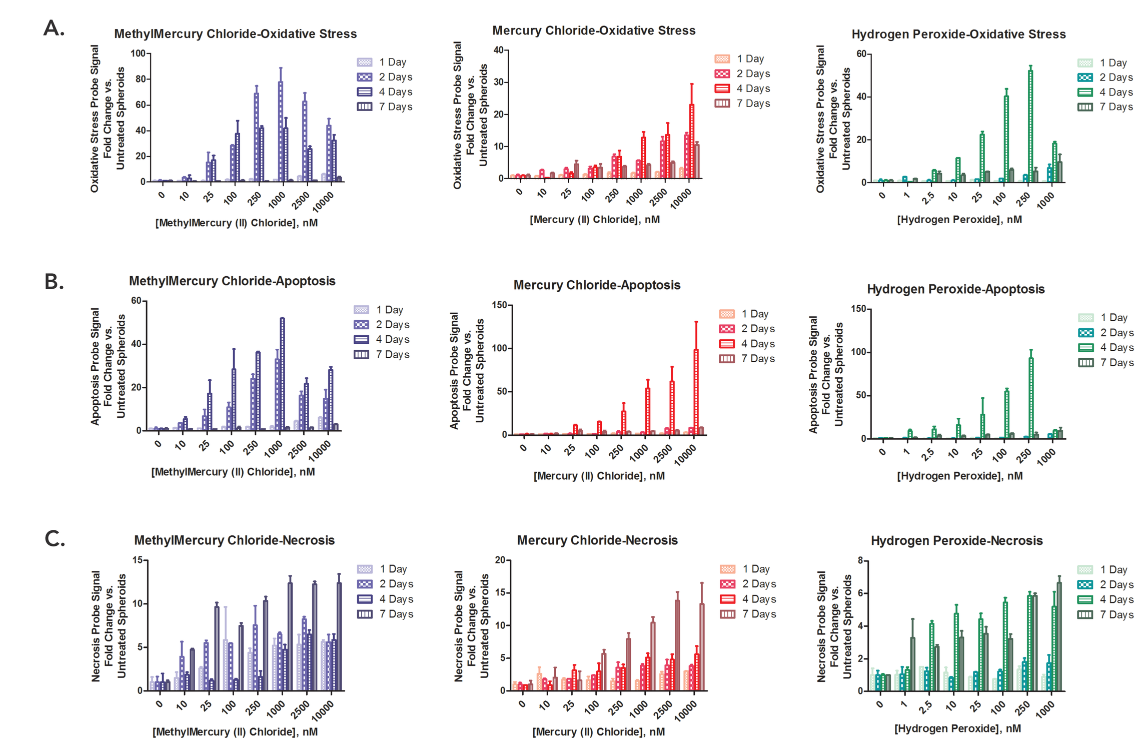

Figure 5. Test compound neurotoxic effect calculation. Fold change in total fluorescent signal calculated by dividing signal from test wells by the average from untreated wells for each specific compound and length of compound incubation. Results shown for **(A)** total ROS; **(B)** apoptosis; and **(C)** fluorescent probes.

The images in Figure 4 illustrate how incubation with the known neurotoxicants cause a dramatic increase in signal generated by fluorescent probes, when compared to signal from untreated wells. By incorporating cellular analysis, generated object masks focus solely on fluorescence emanating from each neurosphere, eliminating background signal and creating a more sensitive measurement. Fold change can then be calculated as previously described. By using this method, the final graphs in Figure 5 confirm the unmistakable, yet unique, toxic effect that each compound elicits on the 3D neurospheres.

Conclusions

1. Neural stem cells cultured into 3D neurospheres, with the use of 384 well spheroid microplates from Corning, represent a viable, robust cell model that can be easily and consistently created.
2. The configuration of the microplate allows for simple media replacement, compound dosing, and assay performance, in addition to affording the ability to perform cellular imaging in the same plate.
3. Primary antibodies from Enzo Life Sciences, specific for unique targets, partnered with fluorescently labeled secondary antibodies, provide a sensitive method to assess the presence of essential proteins in cultured neurospheres.
4. Fluorescent probes from Enzo Life Sciences offer a rapid and easily discernible method to detect changes in important toxic biomarkers.
5. The Cytation 5 Cell Imaging Multi-Mode Reader affords a sensitive, yet flexible system to perform brightfield, phase contrast, and fluorescent imaging of 2D neural stem cells and 3D neurospheres using a wide range of magnification. Gen5 Software then supplies the capability to assess changes in multiple whole spheroid analysis parameters to provide accurate results in real time.
6. The combination of 3D cell model and microplate, assessment methods, and instrumentation together create a powerful solution to perform accurate, high throughput assessments of the potential neurotoxic effects of test molecules.