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

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Introduction

Developmental neurotoxicity (DNT) of environmental chemicals is a threat to human health, as the developing nervous system is particularly susceptible to toxicant exposure. Resulting neurological deficits may have long-term financial and emotional impacts on families and society. Current DNT testing guidelines involve use of animal models; primarily rodents, and the large quantities needed can be extremely time- and cost-intensive; particularly due to the backlog of chemicals requiring testing[1,2,3]. This demand, in addition to current and future proposed regulations on the use of animal test models makes it imperative to find new models that reduce animal experimentation while providing a suitable method to test new chemicals.

Three-dimensional cell models, incorporating human neural stem cells (hNSCs) aggregated into neurospheres, are proposed as a viable alternative for use in DNT testing. The *in vitro* system can recapitulate the processes of brain development, including proliferation, migration, differentiation and apoptosis[2]. By including human instead of murine cells, the system also meets recommendations to circumvent the drawback of species differences between *in vivo* testing and actual exposure effects.

Here we demonstrate a 3D neurosphere model, composed of hNSCs, to conduct toxicity testing of several potential neurotoxicants. A specialized spheroid microplate was used to create and maintain cells in the 3D model. 3D neurosphere proliferation, multipotency, and continued differentiation into neurons, astrocytes, and oligodendrocytes was initially validated. Neurotoxicity testing was then performed using neurospheres maintained in the 3D spheroid plate. Induced levels of oxidative stress, apoptotic and necrotic activity within treated neurospheres were evaluated and compared to negative control spheres.

Materials and Methods

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 hydrobromide (Catalog No. P3655), and laminin (Catalog No. L2020) were purchased from Sigma-Aldrich (Saint Louis, MO).

Media and Antibodies

glutaGRO™ DMEM (Catalog No.10-101-cv) and 1% fetal bovine serum (Catalog No. 35-010-cv) were obtained by Corning Life Sciences. Neurobasal media (Catalog No. 21103-049), 1% N-2 supplement (Catalog No. 17502-048), 2% B-27 supplement (Catalog No. 17504-044) and 1x glutaMAX™ (Catalog No. 35050-061) were obtained from Life Technologies (Carlsbad, CA). T3 supplement (Catalog No. T5516) and dcAMP (Catalog No. D0627) were purchased from Sigma-Aldrich. Nestin (human) monoclonal antibody (2C1 3B9) (Catalog No. ENZ-ABS109-0100), Oct4 monoclonal antibody (9B7) (Catalog No. ENZ-ABS271-0100), βIII-Tubulin (human) 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 (Catalog No. 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 (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). Accutase® Cell Detachment Solution was obtained from Corning Life Sciences.

Cytation™ 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, and is controlled by integrated Gen5™ Data Analysis Software. The instrument was used to image spheroids using brightfield and fluorescence microscopy, as well as individual differentiated cells plated in two dimensional format.

Methods

Specialized Media Optimization

Astrocyte media was created by supplementing glutaGRO™ DMEM with 1% N-2 supplement and 1% fetal bovine serum. Oligodendrocyte media was created by adding 2% B-27 supplement, 1x glutaMAX and 30 ng/mL T3 supplement to neurobasal media. Neuron media was created by combining neurobasal media, 2% B-27 supplement and 1x glutaMAX; additionally, 1mM dcAMP was added after two days.

Neurosphere Formation

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 twelve replicates per concentration. Single neurospheres formed after a 48-hour period. Wells containing spheroids were imaged in Cytation 5, previously set to 37 °C/5% CO_2 using a 4x objective and brightfield imaging.

Neurosphere Proliferation and Multipotency Validation

Primary antibodies specific for Nestin and Oct4 were added to separate wells containing cultured neurospheres. Fluorescently labeled secondary antibodies were then added to the wells, in addition to negative control wells containing no primary antibody. Wells were then imaged in the Cytation 5, previously set to 37 °C/5% CO_2 using a 20x objective and RFP and Texas Red fluorescent imaging channels.

Neural Stem Cell Differentiation

Accutase, 50 µL 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 the aforementioned optimized media to differentiate cells into neurons, astrocytes, and oligodendrocytes. At the same time, whole neurospheres were also transferred to wells of the 384-well plate containing differentiation medias. Astrocytes were incubated for 4-5 days, oligodendrocytes were incubated for 5-6 days, and neurons were incubated for 7 days. Upon completion of prescribed differentiation protocols, immunofluorescence 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. Wells were then imaged in the Cytation 5, previously set to 37 °C/5% CO_2 using a 20x or 40x objective and RFP, DAPI, Texas Red fluorescent imaging channels, as well as a phase contrast channel overlay.

Neurotoxin Analysis

Neural stem cells, at a concentration of 2000 cells/well, were added to a 384-well spheroid plate and allowed to aggregate. The resulting neurospheres were then exposed to varying concentrations of 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, the 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 two times with fresh media to remove unbound probe, and imaged in the Cytation 5, previously set to 37 °C/5% CO_2, using a 4x objective and DAPI, GFP, RFP, and Texas Red fluorescent imaging channels.

Results and Discussion

Image-Based Tracking of Neurosphere Growth

Replicate cell concentrations cultured into spheroids were imaged at regular intervals (Figure 1) to determine the ability of neural stem cells to propagate in a 3D configuration, starting at day 0, when spheroid formation was complete, and repeating again on Days 1, 2, 4, 5, and 7. During brightfield imaging in Cytation 5, integrated Gen5 Data Analysis Software was used to automatically place object masks around each entire spheroid.

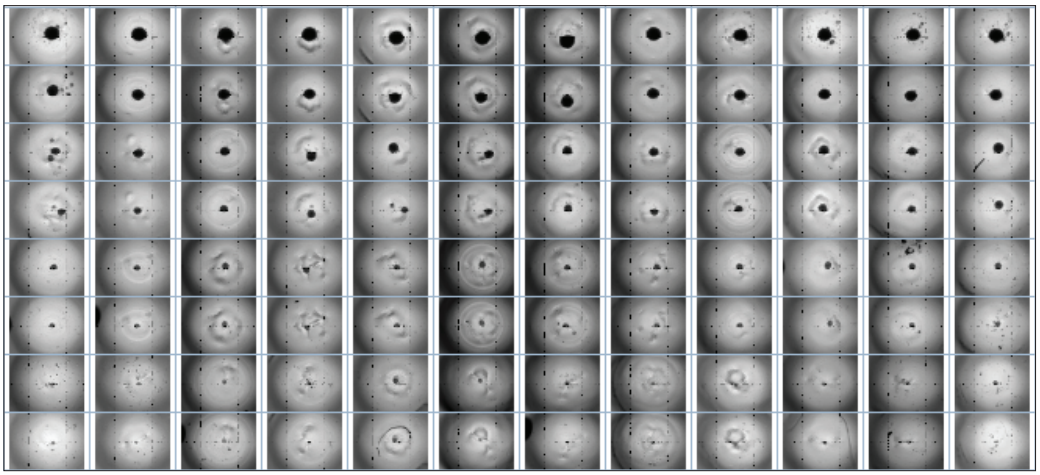

Figure 1. **Imaging and area analysis of 3D neurosphere growth.** Thumbnail 4x aggregated neurosphere brightfield images. Twelve replicates per row of neurospheres formed from neural stem cells dispensed at 32,000, 16,000, 8000, 4000, 2000, 1000, 500, and 100 cells/well (top to bottom).

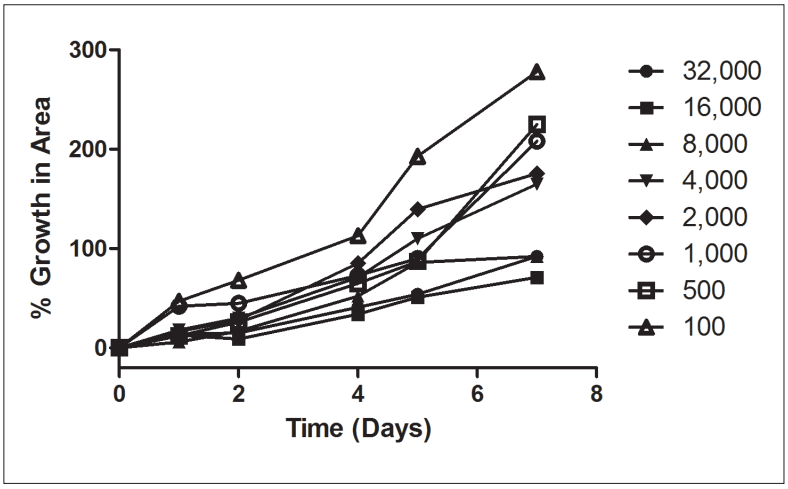

Figure 2. **Plot of** average percent growth in spheroid area compared to initial area measurement **per dispensed cell concentration during incubation period.**

Average percent growth in area inside the object masks for the 12 replicate neurospheres, as calculated by the software, was plotted over time (Figure 2). All neurospheres showed active growth in the spheroid plate, as witnessed by an increase in average percent spheroid growth from Day 0 to Day 7. Percent growth in area, compared to original values, ranged from approximately 100% for neurospheres initially containing 32,000 cells to over 275% for neurospheres initially containing 100 cells illustrating that spheroids starting from a lower number of aggregated cells demonstrate the highest degree of proliferation over time.

Neurosphere Proliferation and Multipotency Validation

The ability of neurospheres derived from neural stem cells to maintain high proliferative and multipotency capability was further tested through immunofluorescence using Nestin and Oct4 fluorescent probes. Nestin is an intermediate filament protein that is necessary for neural stem cell self-renewal, while Oct4 is a transcription factor linked to multipotency in stem cells.

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 Figures 3A and C. These findings are further validated as no fluorescence is seen from negative control wells containing no primary antibody, thereby demonstrating that secondary antibody binding takes place only in the presence of bound primary antibody.

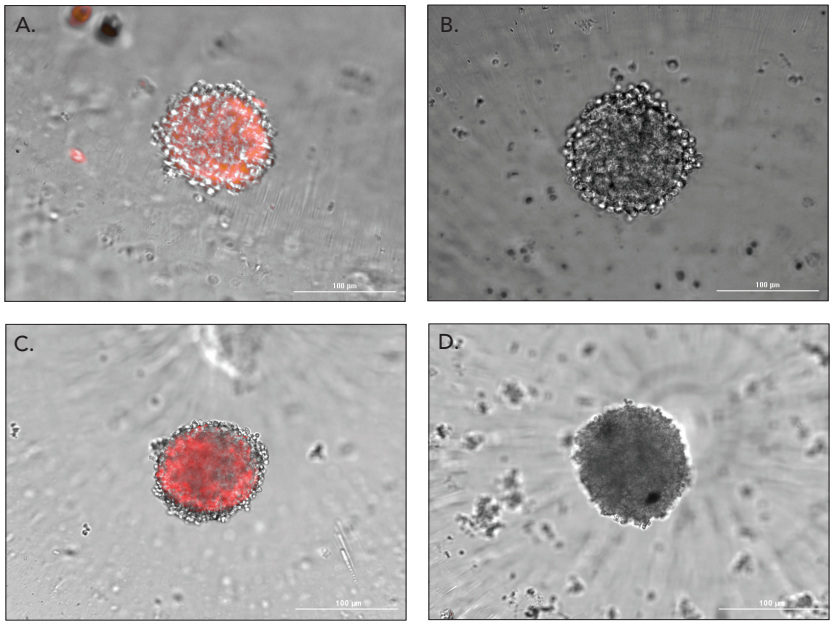

Figure 3. **Detection of proliferation and multipotency markers.** Overlaid 20x brightfield and fluorescence images of positive and negative control wells. (**A**) Proliferation positive control: Nestin (human) monoclonal antibody (2C1 3B9) plus Goat anti-mouse IgG1 (ATTO 590 conjugate) antibody. (**B**) 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.

Neural Stem Cell Differentiation

The ability of 3D cultured neural stem cells to differentiate into neurons and glial cells was evaluated using two separate methods. First, Accutase was used to break the neurospheres into individual neural stem cells, which were then added to separate wells of the imaging plate containing specific media. This allowed the cells to differentiate into neurons, astrocytes, and oligodendrocytes. Separately, whole neurospheres were transferred to the imaging plate wells containing individual differentiation medias. Upon completion of prescribed differentiation protocols, immunofluorescence 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.

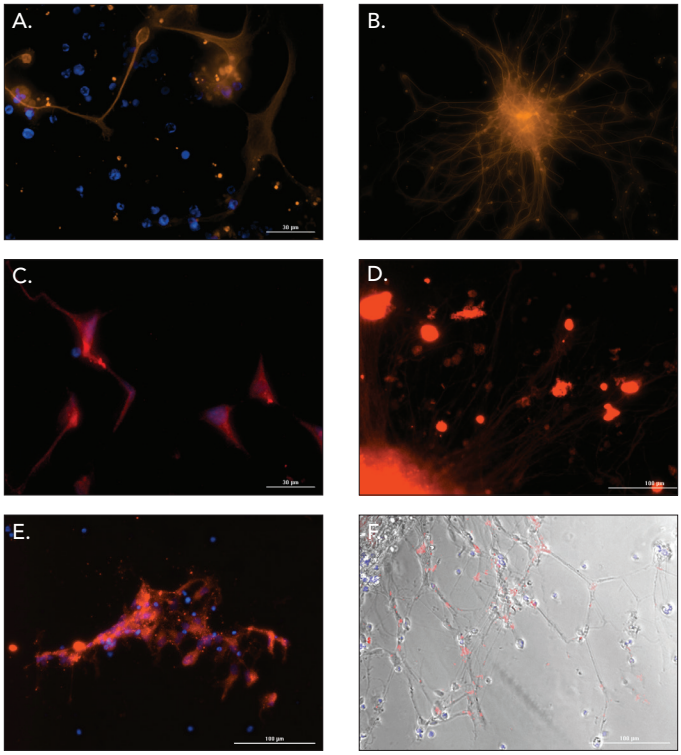

Figure 4. **2D and 3D neural stem cell differentiation.** (**A**) 2D differentiated neurons and (**B**) 3D neurogenesis; 40x objective; RFP Channel: βIII-Tubulin 1° Ab/ATTO 590 2° Ab; DAPI Channel: Hoechst 33342. (**C**) 2D differentiated astrocytes, 40x objective and (**D**) 3D astrogenesis, 20x objective; 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, astrocytes, and oligodendrocytes (Figures 4A, 4C and 4E, respectively) was seen from 3D cultured neural stem cells differentiated in 2D format. Neuro-, astro-, and oligodendrogenesis (Figures 4B, 4D and 4F, respectively) was also seen from differentiated neurospheres when exposed to the same incubation conditions.

The combined neurosphere validation experimental results confirms that neural stem cells exhibit no ill effects from 3D culture, maintain proliferative, multipotency, and differential capabilities, and therefore represent a suitable model for neurotoxicity testing.

Neurotoxin Analysis

Finally, we evaluated the toxic effect of compounds on 3D neurospheres using the well-known neurotoxins: methylmercury chloride (MeHgCl), mercury chloride (HgCl) and hydrogen peroxide (H_2O_2). Cellular analysis was carried out using Cytation 5's integrated Gen5 Data Analysis Software to accurately detect the signal from each probe emanating from the neurospheres (Figure 5). Fluorescence from the live cell probe remains consistent despite potential changes in all other probes. Therefore, this threshold signal, as measured with the DAPI channel, was used to automatically draw object masks around each neurosphere (Figure 5A). Cellular analysis criteria were set, and minimum and maximum object size values were increased appropriately so the spheres registered as single objects as opposed to individual cells. Total fluorescent signal inside the object mask, captured with the GFP, RFP, and Texas Red imaging channels (Figures 5B-G), was then calculated to determine the effect of each compound treatment on oxidative stress, apoptosis, and necrosis levels, respectively, within 3D cultured neural stem cells. Fold change was calculated using the following formula:

$$RFU_{Treated\ (Time\ X)}/RFU_{Untreated\ (Time\ X)}$$

Where $RFU_{Treated\ (Time\ X)}$ is the fluorescence value emanating from neurotoxicant treated spheroids and $RFU_{Untreated\ Time\ X)}$ is the fluorescence from negative control wells containing untreated spheroids after 1, 2, 4, or 7 days of incubation.

Figure 5 images illustrate how incubation with the known neurotoxicants causes 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 highly sensitive measurement. Fold change can then be calculated as previously described.

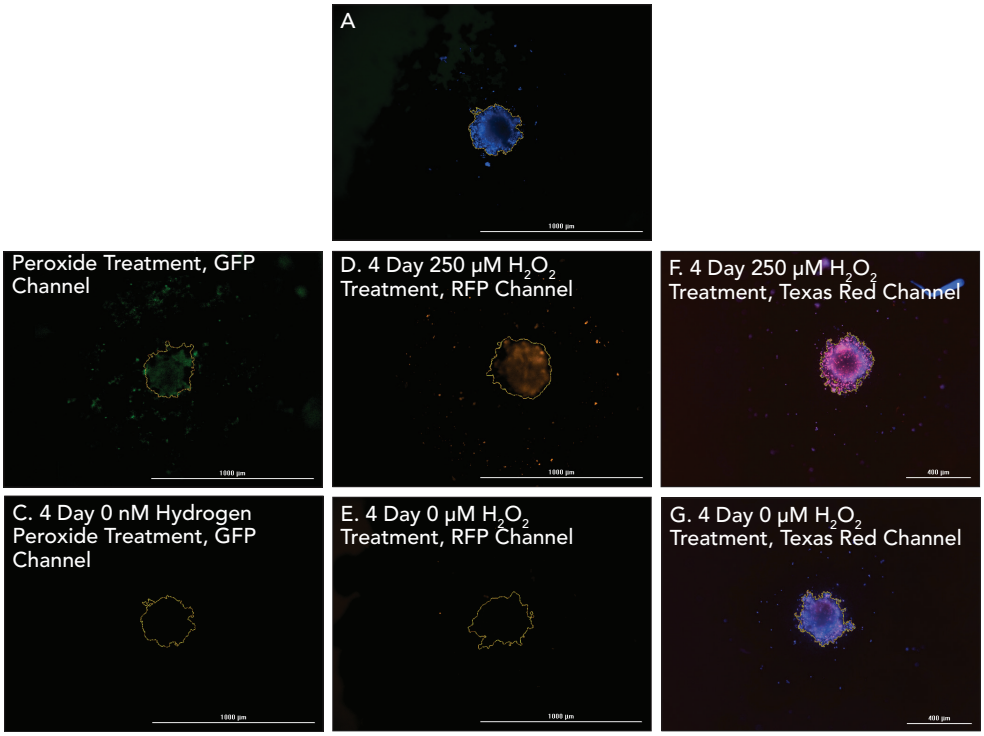

Figure 5. **Image-based cellular analysis using 4x images following neurotoxin treatment and fluorescent probe incubation.** (**A**) Object mask automatically placed around Nuclear-ID live cell probe signal emanating from treated neurosphere captured using DAPI channel. Fluorescent signal from positive or negative control wells following four-day treatment with 250 or 0 µM H_2O_2, 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).

The test well signal from each specific compound at various compound incubation times was divided by the average from the untreated wells to calculate fold change (Figure 6).

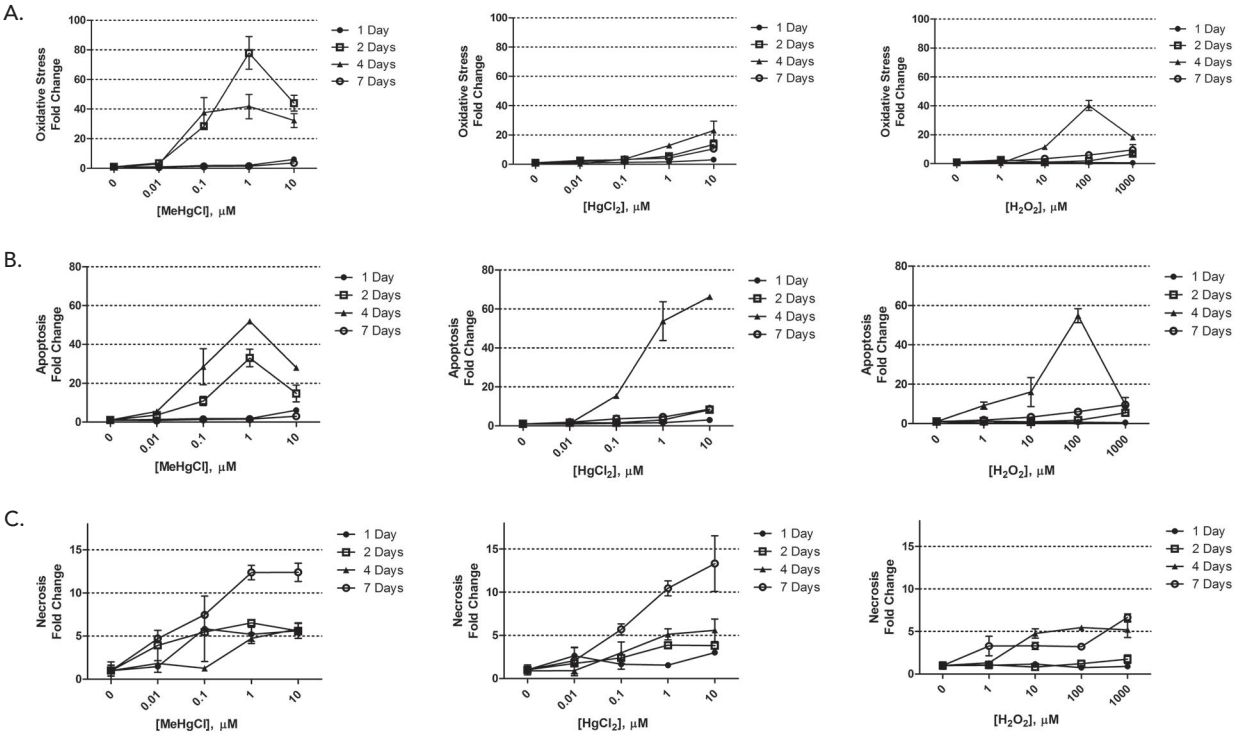

Figure 6. **Test compound neurotoxic effect calculations.** Results shown for (**A**) total ROS; (**B**) apoptosis; and (**C**) necrosis.

Per results in Figure 6A, while an eventual increase in oxidative stress on the neurospheres is elicited by each compound by day 7 of incubation, methylmercury chloride demonstrates the largest and earliest effect, peaking on day 2. Treatment with mercury chloride, in comparison, leads only to a peak fold change in signal from the Total ROS detection kit that is 4x lower than methylmercury chloride and 2x lower than hydrogen peroxide.

Upon examination of the data generated by the apoptosis reagent (Figure 6B), it can then be seen that oxidative stress caused by each test molecule leads to an eventual increase in apoptotic activity within the cells of the same spheres. Maximum apoptosis induction peaks after four days of incubation, with similar results witnessed after incubations with each neurotoxicant. Signal from the assay reagent then decreases by day 7, most likely due to the fact that cells have then become mostly necrotic. This is confirmed by the results from the necrosis reagent (Figure 6C). Fold changes from the signal generated by this fluorescent probe increase as the amount of time each neurotoxicant is incubated with the neurospheres, peaking on day 7.

Conclusions

We have shown that neural stem cells cultured into 3D neurospheres, using a Corning 384-well spheroid microplate, represent a viable, robust cell model that is easily created and reproducible. The efficient microplate configuration allows for simple media replacement, compound dosing and assay performance while also enabling cellular imaging without transfer to a separate plate. Primary antibodies from Enzo Life Sciences, specific for unique targets, when partnered with fluorescently labeled secondary antibodies, provide a sensitive method to assess the presence of essential proteins in cultured neurospheres. Additionally, fluorescent probes, also from Enzo Life Sciences, offer a rapid and easily discernible method to detect changes in important toxic biomarkers. The Cytation™ 5 Cell Imaging Multi-Mode Reader is a sensitive, yet flexible system when performing brightfield, phase contrast, and fluorescent imaging of 2D neural stem cells and 3D neurospheres using a wide magnification range. Integrated Gen5™ Data Analysis Software accurately detects and analyzes changes in multiple whole spheroids in real time. Finally, the combination of 3D cell model and microplate, assessment methods, and instrumentation together create a powerful solution to perform accurate, high throughput assessments of potential neurotoxic effects on test molecules.

References

1. U.S. Environmental Protection Agency. *Guidelines for Neurotoxicity Risk Assessment*. Washington, D.C. The National Academy Press, 1998.

2. Organisation for Economic Co-operation and Development. OECD Guidelines for Testing of Chemicals, Section 4: Health Effects. Paris, France. OECD iLibrary. 2007.

3. Lein, P.; Silbergeld, E.; Locke, P.; Goldberg, A.M. In vitro and other alternative approaches to developmental neurotoxicity testing (DNT). *Environ Toxicol Pharmacol*. **2005**, *19*(3), 735-744.

4. Salama, M.; Lotfy, A.; Fathy, K.; Makar, M.; El-emam, M.; El-gamal, A.; El-gamal, M.; Badawy, A.; Mohamed, W.M.Y.; Sobh, M. Developmental neurotoxic effects of Malathion on 3D neurosphere system. *Applied & Translational Genomics*. **2015**, *7*, 13-18.