Multiparametric High Content Analysis for assessment of neurotoxicity in differentiated neuronal cell lines and human embryonic stem cell-derived neurons

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A B S T R A C T

The potential for adverse neurotoxic reactions in response to therapeutics and environmental hazards continues to prompt development of novel cell-based assays to determine neurotoxic risk. A challenge remains to characterize and understand differences between assays and between neuronal cellular models in their responses to neurotoxicants if scientists are to determine the optimal model, or combination of models, for neurotoxicity screening. Most studies to date have focused on developmental neurotoxicity applications. This study reports the development of a robust multiparameter High Content Analysis (HCA) assay for neurotoxicity screening in three differentiated neuronal cell models – SH-SY5Y, PC12 and human embryonic stem cell-derived hN2TM cells. Using a multiplexed detection reagent panel (Hoechst nuclear stain; antibodies against βIII-Tubulin and phosphorylated neurofilament subunit H, and Mitotracker® Red CMXRos), a multiparametric HCA assay was developed and used to characterize a test set of 36 chemicals. HCA data generated were compared to data generated using MTT and LDH assays under the same assay conditions. Data showed that multiparametric High Content Analysis of differentiated neuronal cells is feasible, and represents a highly effective method for obtaining large quantities of robust data on the neurotoxic effects of compounds compared with cytotoxicity assays like MTT and LDH. Significant differences were observed between the responses to compounds across the three cellular models tested, illustrating the heterogeneity in responses to neurotoxicants across different cell types. This study provides data strongly supporting the use of cellular imaging as a tool for neurotoxicity assessment in differentiated neuronal cells, and provides novel insights into the neurotoxic effects of a test set of compounds upon differentiated neuronal cell lines and human embryonic stem cell-derived neurons.

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1. Introduction

The need to test large numbers of chemicals for their risk of damage to the nervous system is well recognized. In addition to drug safety concerns affecting pharmaceutical development, there are thousands of unclassified chemicals that pose potential environmental neurotoxicity concerns. Consequently, assessment of the potential neurotoxicity of chemicals is increasingly required by regulatory testing schemes (Bal-Price et al., 2012). Traditionally the majority of neurotoxicity testing has been performed using in vivo models, which are expensive, low-throughput, and often lacking in sensitivity (Bal-Price et al., 2012). In recent years there have been calls to develop alternative in vitro neurotoxicity testing stratagems to obtain faster, more accurate and cheaper predictions of neurotoxicity, particularly for the effects of environmental chemicals upon the developing nervous system (Bal-Price et al., 2010; Crofton et al., 2011). With regards to drug safety, several adverse neurotoxic reactions to therapeutics have been reported (Arastu-Kapur et al., 2011; Beijers et al., 2012; Cavaletti et al., 2011), suggesting a need to also evolve preclinical safety pharmacology testing strategies for assessment of new chemical entities and prospective novel therapeutics.
Cellular models available to the field of neurotoxicology testing have developed over time to include immortal neuronal cell lines (Mundy et al., 2010), transformed neuronal precursor cells (Krug et al., 2013a), primary central nervous system (CNS) cultures (Harrill et al., 2013), co-culture systems (Anderl et al., 2009), and stem cell-derived neuroprogenitor cells (Breier et al., 2010; Harrill et al., 2010; Sison-Young et al., 2012). The challenge remains to characterize and understand differences between each cellular model in their responses to neurotoxicants if scientists are to determine the optimal model, or combination of models, for predictive chemical screening. Primary CNS cultures may appear to be ideal candidates, however they generally require animal sacrifice, may be difficult or expensive to obtain in sufficient quantities, and variability in quality and composition may render them unsuitable for screening purposes (Bets, 2010). Immortal cell lines commonly used to study neurotoxicity include the human neuroblastoma SH-SY5Y and the rat pheochromocytoma PC-12. These tumor-derived cell lines are easy to maintain and expand, with well-defined differentiation conditions to induce a relatively mature neuronal phenotype, making them a useful model for high throughput chemical screening. However, such transformed cell lines may not be adequately representative of native neural cells (Bets, 2010), and in the case of PC-12 cells, their rodent origin may limit their utility for human neurotoxicity screening. Human stem cell-derived neurons are emerging as an appealing alternative to primary cultures and transformed cells (Bets, 2010; Breier et al., 2010; Sison-Young et al., 2012). One example is the hN2TM cell line, which is derived from cells of WA09 human embryonic stem cell origin and which has shown some promise as a potential model for developmental neurotoxicity studies (Harrill et al., 2010, 2011a). Most of these recent advances in model systems and assays for neurotoxicity screening have focused on developmental neurotoxicity using undifferentiated neuronal cells, thus little information is available on the performance of neuronal cell assays in which the cells have been differentiated to more closely resemble mature neurons prior to toxic challenge.

To perform neurotoxicity assessment, in vitro cell-based cytotoxicity assays have been frequently utilized, in particular assays such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and LDH (lactate dehydrogenase) release have found widespread application in studies quantifying neuronal cell death (Bal-Price et al., 2008; Lobner, 2000; Wogulis et al., 2005). The MTT assay measures the metabolic activity of cells and is simple in application and analysis, leading to its widespread use to assess neuronal cell viability. The LDH assay measures the release of LDH from cells in which the plasma membrane is damaged and is widely used to assess drug-induced cytotoxicity. Similar to MTT this assay is simple and amenable to higher throughput applications. However both the MTT and LDH assays are broad cytotoxicity assays which can provide little information about the mechanisms of action of toxicity; additionally little is known about their ability to detect neuron-specific toxic events, particularly in differentiated neuronal cells.

It is generally accepted that in vitro neurotoxicity screening methods benefit from the inclusion of neuron-specific endpoints (Bal-Price et al., 2008, 2010, 2012; Crofton et al., 2011). High Content Analysis (HCA) technology has emerged as a technology well-suited for high-throughput assessment of neuron-specific endpoints (Anderl et al., 2009; Culbret et al., 2012; Dragonow, 2008). Using HCA, neuronal cells can be exposed to potential toxicants, fluorescently stained for neuronal-specific markers, and images acquired and quantified using image analysis algorithms. It has been demonstrated that automated algorithms for neurite analysis compare closely to results obtained by manual tracing, with the same measurement precision for treatment effects but with throughput that is orders of magnitude higher than with manual methods (Ramm et al., 2003). Pioneering work has extensively validated this technique for analysis of developmental neurotoxicity (Breier et al., 2008; Culbret et al., 2012; Harrill and Mundy, 2011; Harrill et al., 2010, 2011a,b, 2013; Mundy et al., 2010; Radio and Mundy, 2008; Radio et al., 2008, 2010), and its utility has also expanded into studies of neurotoxicity induced by pharmaceutical therapeutics in mature, differentiated neuronal cells (Arasu-Kapur et al., 2011). To date, HCA has primarily been used to study the effects of neurotoxicants on neuron count and neurite outgrowth, as these are well-established endpoints for neurotoxicity studies; however HCA also enables the detection of multiple morphological, intensity and subcellular event localization measurements. This creates the possibility for highly multiplexed imaging-based toxicity assays (Anderl et al., 2009; Giuliano et al., 2010); a capability of HCA which has not yet been widely exploited for neurotoxicity assessment.

This study had three main objectives. Firstly, to determine if development of a robust multiparameter HCA assay for neurotoxicity screening in multiple differentiated neuronal cell models is feasible. Secondly, to compare HCA-based neurotoxicity assessment with data from MTT and LDH cytotoxicity assays using a panel of test compounds. Thirdly, to compare and contrast the responses of widely used neuronal cell lines with human embryonic stem cell-derived neurons in their responses to a panel of test compounds. To address these objectives, a multiplexed HCA-based neurotoxicity screening assay was developed using a combination of four detection reagents per well – antibodies against βIII-Tubulin and the phosphorylated form of the neurofilament subunit NF-H (pNF-H), the DNA-binding Hoechst 33342 nuclear dye, and Mitotracker™ Red CMXRos. This assay was run using a test set of 36 chemicals in differentiated SH-SY5Y, PC-12, and hN2TM cells, and results compared with those obtained under the same conditions with the MTT and LDH assays. The overall aim was to learn lessons which may be applied to future testing paradigms in mature neurons, particularly for neurotoxicity assessment of pharmaceuticals and potential environmental hazards.

2. Materials and methods

2.1. Materials

The human neuroblastoma SH-SY5Y and rat pheochromocytoma PC-12 cells were obtained from ATCC (Manassas, VA); hN2TM, a human embryonic stem cell (hES) derived neuronal cell line, was purchased from ArunA Biomedical, Inc. (Athens, GA). Ham’s F-12 medium, heat-inactivated horse serum and fetal bovine serum (FBS) were obtained from Life Technologies (Carlsbad, CA). F12-K medium was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Eagle’s Minimum Essential Medium (EMEM) was purchased from ATCC (Manassas, VA). AB2 basal neural medium and ANS neural supplement for hN2TM culture were purchased from ArunA Biomedical, Inc. (Athens, GA). Human recombinant Leukemia Inhibitory Factor (LIF), recombinant human β-nerve growth factor (NGF), recombinant human brain derived neurotrophic factor (BDNF), L-glutamine and dimethyl sulfoxide (DMSO) were from EMD Millipore (Billerica, MA). Penicillin-streptomycin (PS), retinoic acid (RA) and the growth substrates poly-l-lysine (PLL), laminin and calf skin collagen were purchased from Sigma–Aldrich (St. Louis, MO). Table 1 provides full details of all test compounds used in this study. Briefly, all test compounds were obtained from either EMD Millipore (Billerica, MA) or Sigma–Aldrich (St. Louis, MO) with the exception of Bortezomib, which was purchased from Selleck Chemicals (Houston, TX), MTT Cell Growth Assay Kit was from EMD Millipore (Billerica, MA). LDH
Table 1
Compounds used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Expected outcome</th>
<th>CAS number</th>
<th>Supplier</th>
<th>Product code</th>
<th>Vehicle</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>Non-toxic</td>
<td>26787-78-0</td>
<td>Sigma–Aldrich</td>
<td>A8523</td>
<td>DMSO</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>Non-toxic</td>
<td>169590-42-5</td>
<td>Sigma–Aldrich</td>
<td>P20008</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>Non-toxic</td>
<td>147-24-0</td>
<td>Sigma–Aldrich</td>
<td>D3630</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>i-Sorbitol</td>
<td>Non-toxic</td>
<td>50-70-4</td>
<td>EMD Millipore</td>
<td>56755</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>l-Ascorbic acid</td>
<td>Non-toxic</td>
<td>50-81-7</td>
<td>EMD Millipore</td>
<td>1831</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Metformin hydrochloride</td>
<td>Non-toxic</td>
<td>1115-70-4</td>
<td>EMD Millipore</td>
<td>317240</td>
<td>DMSO</td>
<td>&gt;97%</td>
</tr>
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<td>Nadolol</td>
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<td>N1892</td>
<td>DMSO</td>
<td>N/A</td>
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<td>Saccharin sodium salt</td>
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<td>82385-42-0</td>
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</tr>
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<td>Indomethacin</td>
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<td>405268</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Quinidine</td>
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<td>56-54-2</td>
<td>Sigma–Aldrich</td>
<td>Q3625</td>
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<td></td>
</tr>
<tr>
<td>Terodiline</td>
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<td>7082-21-5</td>
<td>Sigma–Aldrich</td>
<td>T4577</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Trogitazone</td>
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<td>97322-87-7</td>
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<td>648469</td>
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<td>&gt;98%</td>
</tr>
<tr>
<td>6-Hydroxydopamine</td>
<td>Neurotoxic</td>
<td>28094-15-7</td>
<td>Sigma–Aldrich</td>
<td>H4381</td>
<td>DMSO</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Neurotoxic</td>
<td>79-06-1</td>
<td>Sigma–Aldrich</td>
<td>A9099</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>Neurotoxic</td>
<td>17932-49-7</td>
<td>Selleck Chemicals</td>
<td>S1013</td>
<td>DMSO</td>
<td>&gt;99%</td>
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<td>Chloroquine diphosphate</td>
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<td>50-63-5</td>
<td>Sigma–Aldrich</td>
<td>C6628</td>
<td>H2O</td>
<td>&gt;98%</td>
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<td>Cisplatin</td>
<td>Neurotoxic</td>
<td>15663-27-1</td>
<td>EMD Millipore</td>
<td>232120</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
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<td>Colchicine</td>
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<td>EMD Millipore</td>
<td>234115</td>
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<td>Dehydroxymethylamine</td>
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<td>50-02-2</td>
<td>EMD Millipore</td>
<td>265005</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Dipyriramidone</td>
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<td>EMD Millipore</td>
<td>322328</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
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<td>Imipramine hydrochloride</td>
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<td>401840</td>
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<td>&gt;95%</td>
</tr>
<tr>
<td>K252a</td>
<td>Neurotoxic</td>
<td>99553-80-9</td>
<td>EMD Millipore</td>
<td>420298</td>
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<td>&gt;95%</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>Neurotoxic</td>
<td>6080-56-4</td>
<td>Sigma–Aldrich</td>
<td>11504</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Neurotoxic</td>
<td>137-58-6</td>
<td>Sigma–Aldrich</td>
<td>L7757</td>
<td>DMSO</td>
<td>N/A</td>
</tr>
<tr>
<td>Methylmercury chloride</td>
<td>Neurotoxic</td>
<td>115-09-3</td>
<td>Sigma–Aldrich</td>
<td>33368</td>
<td>DMSO</td>
<td>N/A</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Neurotoxic</td>
<td>31430-18-9</td>
<td>EMD Millipore</td>
<td>487928</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Oxafluprin</td>
<td>Neurotoxic</td>
<td>61825-94-3</td>
<td>Sigma–Aldrich</td>
<td>O9512</td>
<td>DMSO</td>
<td>N/A</td>
</tr>
<tr>
<td>Pacitaxel</td>
<td>Neurotoxic</td>
<td>33089-62-4</td>
<td>EMD Millipore</td>
<td>580555</td>
<td>DMSO</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>Neurotoxic</td>
<td>13731-39-6</td>
<td>EMD Millipore</td>
<td>567540</td>
<td>H2O</td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td>Neurotoxic</td>
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<td>EMD Millipore</td>
<td>574625</td>
<td>DMSO</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Neurotoxic</td>
<td>10540-29-1</td>
<td>Sigma–Aldrich</td>
<td>T5648</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>t-Retinoic acid</td>
<td>Neurotoxic</td>
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<td>EMD Millipore</td>
<td>554720</td>
<td>DMSO</td>
<td>&gt;95%</td>
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<td>U0126</td>
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<td>662005</td>
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<td>Vinblastine sulfate</td>
<td>Neurotoxic</td>
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<td>EMD Millipore</td>
<td>677175</td>
<td>DMSO</td>
<td>N/A</td>
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<td>Vincristine sulfate</td>
<td>Neurotoxic</td>
<td>2068-78-2</td>
<td>EMD Millipore</td>
<td>677181</td>
<td>DMSO</td>
<td>&gt;99%</td>
</tr>
</tbody>
</table>

Cytotoxicity Detection Kit was purchased from Roche Applied Science (Indianapolis, IN). Table 2 provides full details of detection reagents used for HCA in this study. Hoechst HCS nuclear stain was from Sigma–Aldrich (St. Louis, MO). Mitotracker<sup>®</sup> Red CMXRos was from Life Technologies (Carlsbad, CA). Rabbit polyclonal antibody to βIII-Tubulin, mouse monoclonal PhosphoDetect™ Anti-Neurofilament H Mouse (SMI-31) antibody, FITC-conjugated donkey anti-rabbit IgG and Cy5-conjugated donkey anti-mouse IgG secondary antibodies were from EMD Millipore (Billerica, MA), as were all cell fixation, permeabilization and wash reagents used in this study.

2.2. Cell culture

All cells were maintained at 37 °C in a 95% humidified incubator with 5% CO₂. Costar<sup>®</sup> 96 well black, clear bottom plates (Corning, Inc., Corning, NY) were used as the assay plates for all cell types, coated with a cell-specific surface substrate for each cell type used.

For SH-SYSY and PC-12 cells, wells were coated with a 10 μg/ml collagen solution diluted in Dulbecco’s phosphate-buffered saline (PBS) for 2 h at 37 °C. Collagen was subsequently removed by aspiration and cells seeded directly onto the coated plates. For hN2<sup>TM</sup> cells, wells were coated with 50 μg/mL PLL solution in PBS for 2 h at 37 °C, rinsed once with PBS and then coated with a 20 μg/mL laminin solution in PBS for 2 h at 37 °C. Plates were subsequently rinsed once with PBS prior to plating of hN2<sup>TM</sup> cells.

SH-SYSY cells were propagated in T-75 tissue culture flasks with growth media consisting of F-12/EMEM (1:1 ratio) supplemented with 10% FBS and 1% PS. At the time of experimental setup, SH-SYSY cells were seeded onto collagen-coated 96 well assay plates at a density of 5000 cells/well (1.60 × 10<sup>5</sup> cells/cm<sup>2</sup>) in growth media. Sufficient wells were seeded to enable three biological replicates for each condition subsequently tested. To begin differentiation, 24 h post-seeding the media was replaced with growth media containing 10 μM RA and the media was replaced every 2–3 days for 5 days. Subsequently, media was replaced with differentiation media.

Table 2
Detection Reagents used in this study.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Final use concentration</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotracker&lt;sup&gt;®&lt;/sup&gt; Red CMXRos</td>
<td>Life Technologies # M-7512</td>
<td>0.25 μM</td>
<td>15 min</td>
</tr>
<tr>
<td>Rabbit anti-βIII Tubulin</td>
<td>EMD Millipore # A8523</td>
<td>1:1000 dilution (−1 μg/mL)</td>
<td>1 h</td>
</tr>
<tr>
<td>Mouse anti-phospho Neurofilament H</td>
<td>EMD Millipore # A1572</td>
<td>1:200 dilution (−5 μg/mL)</td>
<td>1 h</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>Sigma–Aldrich # A8523</td>
<td>10 μg/mL</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC-donkey anti-rabbit IgG</td>
<td>EMD Millipore # A1822</td>
<td>5 μg/mL</td>
<td>1 h</td>
</tr>
<tr>
<td>Cy5-donkey anti-mouse IgG</td>
<td>EMD Millipore # A1925</td>
<td>5 μg/mL</td>
<td>1 h</td>
</tr>
</tbody>
</table>
replaced with differentiation media consisting of Ham’s F-12/EMEM (50:50) supplemented with 1% PS and 50 ng/mL BDNF and the media replaced every 2–3 days for 5 days.

PC-12 cells were propagated in T-75 tissue culture flasks with growth media consisting of F-12 K supplemented with 15% horse serum, 2.5% FBS and 1% PS. Cells were seeded onto collagen-coated 96 well assay plates at a density of 2500 cells/well (0.8 × 10^5 cells/cm^2) in growth media consisting of F-12 K supplemented with 15% horse serum, 2.5% FBS and 1% PS. Sufficient wells were seeded to enable three biological replicates for each condition tested. To begin differentiation, 24 h post-seeding the media was replaced with differentiation media consisting of F-12 K supplemented with 1% horse serum, 1% PS and 100 ng/mL NGF and the media was replaced every 2–3 days for 6 days. hN2TM are human neuronal cells derived from WA09 human embryonic stem cells by ArunA Biomedical using a proprietary process under defined feeder and serum free conditions. The cells are supplied by the manufacturer in a cryopreserved ready-to-use format. After differentiation, the cells exhibit neuronal morphology and are reported by the manufacturer to be >90% βIII-Tubulin positive, >60% MAP2 positive, and <5% Oct-4 positive. hN2TM were cultured according to manufacturer instructions. After thaw, cells were seeded directly onto PLL/laminin-coated 96 well assay plates at a density of 5000 cells/well (1.60 × 10^5 cells/cm^2) in growth media consisting of AB2 supplemented with 2% ANS neural supplement, 0.1% LIF, 1% L-glutamine and 1% PS. Sufficient wells were seeded to enable three biological replicates for each condition tested. Growth media were replaced 24 h post-seeding and cells were allowed to attach and differentiate for 3 days.

For all cell types, on the final day of differentiation, prior to chemical treatment, the differentiation media was removed and replaced with fresh differentiation media to achieve the precise volume required to allow for accurate 10 × test compound dilution.

2.3. Chemical treatment

A panel of 36 test chemicals was established, consisting of 23 chemicals reported to be neurotoxic, 5 chemicals reported to be toxic in other organ systems but considered to be non-neurotoxic, and 8 compounds generally reported to be non-toxic (Table 1). Compounds were prepared so as to achieve an 8-point logarithmic dose curve with final concentrations ranging from 0.0001 to 1000 μM. All stock solutions and dilutions were prepared in DMSO with the exception of chloroquine diphosphate and sodium orthovanadate, which were prepared in water. Stock solutions were prepared at 125 × the maximal assay dose and transferred to a 96 well plate where a series of 1:10 serial dilutions were performed. These 125 × serially diluted compounds were then further diluted in cell type-specific culture media to obtain 10 × compound stock plates. At the time of compound addition to the cells, final 1:10 dilutions were performed directly from the 10 × compound plates to the cell culture assay plates. Using this scheme, maximum final DMSO concentration in the assay plates was 0.8%. Compounds were assayed in triplicate and vehicle control wells were included on each assay plate. Cells were incubated with the test compounds for 72 h and then either fixed (3.7% formaldehyde for 30 min at room temperature) for subsequent immunostaining and HCA or immediately analyzed using the MTT and LDH assay kits.

2.4. MTT measurement of viability

The MTT Cell Growth Assay Kit was used to assess viability of chemically treated cells according to the manufacturer’s instructions. Three replicates were performed for all conditions tested. The yellow (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (MTT) substrate is cleaved by metabolically active cells to produce a blue formazan product, which is then dissolved allowing the absorbance to be measured by a spectrophotometer. Prior to the screening phase of this study, pilot MTT experiments were performed with each cell type at the same seeding densities as used during the screening phase, in order to confirm that untreated and treated control cells generated appropriate signal for detection within the sensitivity range of the assay. At the end of the chemical treatment period, cell culture plates containing 200 μL of media volume were removed from the incubator and 100 μL of the media was transferred to a new 96 well plate and set aside for LDH analysis. 10 μL of MTT solution was added to the remaining 100 μL of media in each well of the assay plates and the plates were returned to the incubator for 4 h to facilitate cleavage of MTT. At the end of the incubation period, 100 μL of color development solution was added to each well and mixed thoroughly by repeated pipetting with a multichannel pipette. 150 μL of the reaction was transferred to a new clear-walled 96 well plate and the absorbance was measured on a BioTek Synergy H4 Hybrid reader using Gen5 (1.11) software, at a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.5. LDH measurement of cytotoxicity

Cytotoxicity was measured using the colorimetric Cytotoxicity Detection Kit (LDH) according to the manufacturer’s instructions. Three replicates were performed for all conditions tested. The cytoplasmic enzyme lactate dehydrogenase is released into the cell culture supernatant upon damage of the plasma membrane. The supernatant is incubated with a supplied reaction mixture, resulting in a formazan product which is directly proportional to the LDH activity in the supernatant and can be measured using a spectrophotometer. Prior to the screening phase of this study, pilot LDH release experiments were performed with media and lysates from SH-SY5Y, PC-12 and hN2TM cells at the same seeding densities and under the same culture conditions as used during the screening phase in order to confirm that each cell culture condition generated sufficient LDH signal for detection within the range of the assay. During the chemical screening phase, control untreated wells were seeded in triplicate for each cell type in order to measure LDH activity in the supernatants and cell lysates. These data were used to develop low control (spontaneous LDH release) and high control (maximum possible LDH release) data for each cell type in order to calculate % cytotoxicity in chemical-treated samples. At the end of the chemical treatment period, cell culture assay plates containing 200 μL of media volume were removed from the incubator and 100 μL of the assay supernatant was transferred to a new 96 well plate. 100 μL of the LDH reaction mixture was added to the supernatant and the plates were incubated for 30 min at room temperature, protected from light. At the end of the incubation period, 150 μL of the reaction was transferred to a new, clear-walled 96 well plate and the absorbance was measured on a Biotek Synergy H4 Hybrid plate reader using Gen5 (1.11) software, at a test wavelength of 492 nm and a reference wavelength of 630 nm.

2.6. Cell staining for High Content Imaging

Cell staining reagents and procedures are summarized in Table 2. Three replicates were performed for all conditions tested. Prior to the screening phase of this study, extensive pilot experiments were performed for all HCA detection reagents with each cell type at the same seeding densities as used during the screening phase, in order to determine optimal reagent concentrations and incubation times to provide sufficient signal strength of each stain for automated algorithm detection while ensuring
there was no bleed-through between fluorescent channels. At the end of the assay treatment period, prior to fixation, cell culture plates containing 100 μL of media volume were removed from the incubator and 5 μL of a 5 μM stock of Mitotracker® Red CMXRos was added to the live cells then incubated for 15 min at 37 °C. The plates were then removed from the incubator and the cells immediately fixed with 100 μL of pre-warmed 2 × fixation solution containing 7.4% formaldehyde for 30 min at room temperature. Following fixation, each well was gently washed twice with a cell permeabilization buffer containing 0.25% Triton X-100. A primary antibody working solution containing rabbit anti-BII-Tubulin and mouse anti-pNF-H antibodies was prepared in this buffer and 50 μL applied to each well then incubated for 1 h at room temperature. Following primary antibody incubation, cells were washed three times. A secondary antibody working solution containing FITC-donkey anti-rabbit IgG, Cy5-donkey anti-mouse IgG and Hoechst 33342 Nuclear Stain was prepared and 50 μL applied to each well for 1 h at room temperature. Cells were then washed twice with PBS, and plates were then stored, sealed and protected from light at 4 °C until image acquisition.

2.7. High Content Imaging and Analysis

Plates were imaged with a GE IN Cell Analyzer 1000 high content imaging system, and images were analyzed with GE IN Cell Analyzer 1000 Workstation (3.7) software, utilizing the Multi Target Analysis algorithm to segment cellular features based on size- and fluorescence intensity-related criteria. All images were acquired unbinned, using the excitation/emission filter settings as described: Hoechst 360/460 (wavelength 1), FITC 480/535 (wavelength 2), Mitotracker® Red CMXRos 535/600 (wavelength 3) and Cy5 620/700 (wavelength 4). For SH-SY5Y cells, imaging was performed using a 20× objective lens, acquiring 20 fields of view for each wavelength per well. For PC-12 and hN2TM cells, imaging was performed using a 10× objective lens, acquiring 10 and 12 fields of view, respectively, for each wavelength per well. Scale bars were added to images via use of ImageJ image processing software (http://imagej.nih.gov/ij/) to convert image pixel calibration data (0.322 μm/pixel for 20× images; 0.645 μm/pixel for 10× images) from the GE IN Cell Analyzer to 100 μm measurements.

Image analysis parameters were optimized in GE Workstation (3.7) software for each cell type for most accurate and robust cell and feature segmentation possible, using multiple representative images of treated and untreated cells. Ten parameters across the four wavelengths used were chosen for analysis in this study. Hoechst stained nuclei were detected using Top Hat segmentation, which was used to measure cell count and nuclear area. BII-Tubulin stained soma and neurites were analyzed using Collar and Multiscale Top Hat segmentation, respectively, measuring total neurite count, total neurite area, total neurite length and soma integrated intensity ratio. Mitotracker® staining within and around cell bodies was analyzed for mean stained area and mean staining intensity using Multiscale Top Hat segmentation. pNF-H staining within neurites and nuclei were analyzed using Multiscale Top Hat segmentation, measuring total pNF-H area and pNF-H nuclear intensity.

Automated Image analysis was performed on each well, collecting data on a cell-by-cell basis, which was then averaged within the application and the reported well-by-well summary was used for subsequent analysis.

2.8. Statistical analysis

For MTT, LDH and HCA assays, biological replicates from three separate wells were averaged to obtain the mean, standard deviation and standard error of the mean for each treatment dose. To determine concentration-dependent cellular changes, comparisons were made between each dose and vehicle-only controls. The % change was calculated for all doses and compounds, as well as vehicle controls, and Student’s t-Test (two-tailed distribution, two-sample equal variance, p < 0.05) was used to determine the significance of the response. GraphPad Prism software was used to generate dose-response relationship graphs for HCA, MTT and LDH data. For heat map and cluster analysis of each measurement, lowest effective concentrations (LOEC) were defined as the lowest concentration at which there was a statistically significant change (p < 0.05) of greater than 20% versus vehicle controls. LOEC data were converted to log scale and imported into the R statistical computing environment (http://www.r-project.org/). In order to compare data between assays, cell types and compounds, the LOEC were heat-mapped using the R package pheatmap (http://cran.r-project.org/web/packages/pheatmap/index.html).

Hierarchical clustering was performed across both rows and columns, using default parameters. A color palette ranging from red to black to green was used. Data is displayed with greens corresponding to high LOEC values (less toxic) and reds corresponding to low LOEC (more toxic) values.

3. Results

3.1. Development and feasibility of multiparametric HCA assay

Prior to commencing the chemical screening phase of this study, assay optimization experiments were performed on each cell type (SH-SYSY, PC-12, hN2TM) to determine optimal cell culture conditions to enable robust High Content Analysis. For each cell type, a variety of seeding densities and differentiation conditions were tested. Cell culture conditions which provided for an even distribution of cells in each well and a differentiated neurite network expressing neuronal-specific BII-Tubulin were selected.

Rationale for the panel of detection reagents (Table 2) was as follows; Hoechst stain provides information on cell count and nuclear morphology; BII-Tubulin effectively stains neurites and enables quantitative analysis of important morphological features in neuronal cultures such as neurite length, neurite area, neurite area. Mitotracker® Red CMXRos was chosen as an indicator of mitochondrial membrane potential and mitochondrial mass suitable for HCA-based toxicity studies (Giuliano et al., 2010); mitochondrial dysfunction is a well-known characteristic of many neurotoxic and neurodegenerative conditions and many cell death pathways are characterized by mitochondrial effects. pNF-H was included as a putative neurotoxicity marker for HCA in the reagent set based on reports that loss of pNF-H immunoreactivity in rat DRG tissue correlates with drug-induced neurotoxicity (Jamie-son et al., 2009). Previous studies have also shown that pNF-H is expressed in neuroblastoma (Glass et al., 2002; Sachana et al., 2003).

Fig. 1 shows representative images of each cell type stained with the HCA detection reagents following differentiation and 72 h treatment with DMSO vehicle control. SH-SYSY cells are shown in panels A and D; PC12 in panels 1B and 1E and ArunA hN2TM in panels C and F. These merged images show the morphological features of each culture and the distribution of staining within each cell type. All stains were present in each cell type, and each was readily amenable to image segmentation using image analysis software (Fig. 2; Supplementary Figs. 2 and 3). There are visibly fewer cells and neurites in the hN2TM cultures, this was observed in all hN2TM wells and is attributable to cell loss following thawing of cryopreserved stocks; yield of viable hN2TM cells was consistently lower than expected. Individual images from each wavelength
showing each stain in isolation in each cell type are shown in Supplementary Fig. 1. hN2™ cultures differed from SH-SYSY and PC-12 with regard to Hoechst staining, in that in hN2™ cultures, a large number of Hoechst positive spots not associated with cell bodies or neurites were presented in all fields of view. These likely represent debris from cells which failed to survive and/or differentiate, and were excluded from subsequent image analysis on the basis of fluorescence intensity and size, being significantly brighter and smaller than nuclei of the intact hN2™ cells, enabling them to be filtered out of the image analysis and not counted as cells. Also of note, pNF-H expression appears to be closely colocalized with βIII-Tubulin in SH-SYSY cells, less so in PC-12, with hN2™ cells showing the greatest level of heterogeneity, with a small number of cells expressing either βIII-Tubulin or pNF-H, but not both. Overall the staining reagent panel and cell culture conditions were confirmed as being robust and the staining suitable for all cell types tested in the screening phase of the study.

Supplementary Figs. 1–3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.03.013.

Fig. 2 illustrates the segmentation of acquired images by the automated image analysis algorithm employed in this study. Images from vehicle-treated SH-SYSY cells are shown; segmentation of PC-12 and hN2™ cells are shown in Supplementary Figs. 2 and 3, respectively. All images shown are from a single field of view, with four wavelengths acquired and analyzed. Acquired images are shown on the left side of the figure, the right side of the figure shows the same images overlaid with the image analysis software traces. It can be observed that, when properly optimized for each cell type and feature of interest, the image analysis algorithm very accurately detects Hoechst, βIII-tubulin, MitoTracker® and pNF-staining patterns, enabling accurate quantification of the features of interest within images. Although the stains and features of interest being quantified in each cell type were the same, the image analysis algorithm must be specifically optimized for each cell type; this is because even small differences in nuclear size, neurite length and width etc. between cell types must be taken into account during algorithm optimization in order to avoid inaccurate image segmentation and analysis. For βIII-tubulin and pNF-H staining we chose to segment and measure everything detected within each field of view, however, for MitoTracker® staining, we chose to only segment and measure staining within and around the cell body region. This was because, although MitoTracker® staining was clearly visible along neurites in all three cell types, more accurate segmentation and greater sensitivity to drug-induced changes were made possible by restricting our measurements to non-neurite localized MitoTracker® staining.

Fig. 3 shows the dose-related effects of amoxicillin and vinblastine following 72 h exposure in differentiated PC-12 cells. These data show that multiple quantitative neurotoxicity measurements can be derived from multiparametric HCA assessment, indicating the richness and multiplexity of data available via this methodology. Fig. 3A shows cell count data obtained by analysis of Hoechst nuclear staining. Amoxicillin had no significant effects upon cell count at any dose tested. Vinblastine had no significant effects on cell count at doses ranging from 1 nM to 10 μM, but significantly reduced cell count (p < 0.05) at doses of 100 μM (85% cell loss) and 1 mM (90% cell loss). Fig. 3B shows neurotoxic effects of vinblastine at concentrations much lower than those required to produce cell loss, based on measurement of neurite length via quantitation of βIII-Tubulin staining. A dose-dependent effect of vinblastine upon average neurite length became statistically significant at a concentration of 0.01 μM (neurite length decrease to 33% of control; p < 0.05). Amoxicillin had no significant effects upon neurite length at any dose tested. Fig. 3C shows that vinblastine toxicity in differentiated PC-12 cells has a mitochondrial aspect, with a dose-dependent increase in mitochondrial area becoming statistically significant (p < 0.05) at 0.1 μM. Amoxicillin had no significant effect upon mitochondrial area. Fig. 3D illustrates that pNF-H expression in PC-12 cells is highly sensitive to vinblastine, with a dose-dependent decline in total pNF-H area per cell becoming significant at 1 nM vinblastine, thus pNF-H area was the measurement most sensitive for measurement of neurotoxicity under these conditions. Amoxicillin had no significant effect on this parameter. Acquired and segmented images from the HCA analysis of the effects of vinblastine at the concentration at which the drug effects upon differentiated PC-12 cells became statistically significant as shown in Fig. 3 are shown in Fig. 4. These images provide visual evidence for the decline in cell count, neurite length and pNF-H expression in vinblastine treated cells, along with an increase in mitochondrial staining in the region of the cell body that was a frequently observed characteristic of cells undergoing neurotoxic challenge. The images also highlight another advantage of HCA, namely the

![Merged images showing multiplexed staining of differentiated SH-SYSY, PC-12 and hN2™ cells. (A–C) Differentiated SH-SYSY, PC-12 and hN2™ cells, respectively, stained with Hoechst 33342 nuclear stain, rabbit anti βIII-Tubulin antibody and MitoTracker® Red CMXRos. (D–F) Differentiated SH-SYSY, PC-12 and hN2™ cells, respectively, stained with Hoechst 33342 nuclear stain, rabbit anti βIII-Tubulin antibody and mouse anti-pNF-H antibody, Scale bars represent 100 μm.](image-url)
ability to re-examine the source image data after image analysis in order to visually confirm results.

With regard to vinblastine effects in differentiated PC-12 cells, both the MTT and LDH assays detected toxic effects with high sensitivity, with MTT data (Fig. 3E) in agreement with HCA Mitochondrial Area assessment by indicating a statistically significant (p < 0.05) effect of the drug beginning at a dose of 0.1 μM and LDH (Fig. 3F) detecting a statistically significant effect (p < 0.05) induced by exposure to 0.01 μM vinblastine. Interestingly, in differentiated SH-SYSY and hN2TM cells, vinblastine was also found to be highly neurotoxic by HCA and MTT assessment but not by LDH (Figs. 7–9 and Supplementary Tables 1 and 3), suggesting differences between drug responses across different cell lines, an observation which will be explored in more detail later.

3.2. Comparison of multiparametric HCA with MTT and LDH assays for detection of neurotoxicity in differentiated neuronal cells

The data in Figs. 3 and 4 suggest that it may be possible to use HCA to distinguish between drug effects of cytotoxic endpoints (e.g. cell count) and neurotoxic endpoints (e.g. neurite length) in differentiated PC-12 cells, since vinblastine-induced declines in the neuronal-specific endpoints of neurite length and pNF-H were detected at much lower drug concentrations than those causing cell loss. A major objective of this study was to compare HCA
neurotoxicity assays with the well-established MTT viability and LDH cytotoxicity assays, both of which find widespread use in studies measuring neuronal cell death. Accordingly, test compounds were tested at the same doses and incubation times on cells cultured under the same conditions as for the HCA assay, and MTT and LDH assays performed for each condition tested. Fig. 5 explores this, showing the effects of D-sorbitol (expected to be non-toxic); indomethacin (toxic in other organs but not reported to be neurotoxic); and colchicine (expected to be neurotoxic) in differentiated SH-SYSY, PC-12 and hN2TM cells, as measured by multiparametric HCA, MTT and LDH.

D-Sorbitol was found to have no toxic effects upon either HCA-assessed cell count or neurite length in any of the cell types tested, nor were any toxic effects of this drug observed via the LDH assay (Fig. 5A–C). MTT data for D-sorbitol was in accordance with the HCA data in both PC-12 and hN2TM cells (Fig. 5B and C), with no significant effects of D-sorbitol observed. However, in SH-SYSY cells, MTT values declined in response to the chemical, reaching statistical significance (p < 0.05) at D-sorbitol concentrations of 0.1 μM and 1 μM (Fig. 5E). Taken alone, these data would suggest a cytotoxic effect of D-sorbitol at these doses in differentiated SH-SYSY which is not supported by either HCA or LDH data.

Cellular responses to indomethacin (Fig. 5D–F) were very similar across all assays and cell types: no significant toxic effects were observed except at the highest doses of the drug. For HCA-derived cell count and neurite length measurements, exposure to 1000 μM indomethacin caused a dramatic decline in both cell count and neurite length in all three cell types, but no significant effects at concentrations below 1000 μM (Fig. 5D–F). As neurite length would be expected to decline as a consequence of cell loss, it seems probable that indomethacin induces a broad cytotoxic effect in differentiated neuronal cells at high concentrations, rather than a neurotoxic effect. MTT and LDH data for the effects of indomethacin (Fig. 5D–F) support this with no detectable effects of the drug at lower concentrations. In SH-SYSY cells, MTT data showed a significant effect of indomethacin following 100 μM exposure (Fig. 5D). In PC-12 cells, LDH assessment indicated a significant effect of indomethacin at 100 μM (Fig. 5E). All other
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effects and image analysis software traces for the specified features of interest are shown for untreated and vinblastine treated cells, for the drug doses at which the effects of vinblastine became statistically significant. (A and B) The effects of 0 μM and 100 μM vinblastine, respectively, upon Hoechst-stained cells; (C and D) the segmentation of neurites (blue outline) by the image analysis algorithm. (E and F) The effects of 0 μM and 0.01 μM vinblastine upon MitoTracker® staining; (G and H) The segmentation of mitochondrial areas (yellow outline) around the cell body by the image analysis algorithm. (M and N) The effects of 0 μM and 0.001 μM vinblastine upon pNF-H staining; (O and P) the segmentation of pNF-H positive areas (magenta outline) by the image analysis algorithm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Images of stained and segmented PC-12 following exposure to vinblastine. This figure provides visual evidence for each effect of vinblastine quantified by HCA in Fig. 3. MTT and LDH measurements showed a significant toxic effect of indomethacin at 1000 μM (Fig. 5D–F), in accordance with HCA data for this drug.

The effects of colchicine (Fig. 5G–I) were much more complex, in that differences emerged between the various cell models and between the assays employed. With regard to the HCA endpoints, in differentiated SH-SYS5 and PC-12 cells (Fig. 5G and H), the effects of colchicine were upon cell count and neurite outgrowth were broadly similar: the drug had no significant effect upon cell count at any doses tested in either SH-SYS5 or PC-12, but significantly (p < 0.05) reduced neurite length in a dose-dependent manner in both SH-SYS5 (first significant effect at 0.01 μM) and PC-12 cells (first significant effect at 1 μM). These data indicate that in differentiated SH-SYS5 and PC-12 cells, colchicine is neurotoxic rather than cytotoxic. Reviewing the image data from this experiment (shown in Fig. 6) confirms this; in both SH-SYS5 (Fig. 6A–F) and PC-12 cells (Fig. 6G–L) colchicine has visibly led to an almost complete reduction of neurites while cell count remains unchanged. In contrast to SH-SYS5 and PC-12 cells, hN2™ cells displayed significant dose-dependent declines in both cell count and neurite outgrowth (Fig. 5I), effects which reached statistical significance (p < 0.05) at 0.1 μM colchicine, indicating that differentiated hN2™ cells, unlike differentiated SH-SYS5 and PC-12, are susceptible to cytotoxic injury by colchicine exposure. These observations were confirmed by the imaging data from this experiment as shown in Fig. 6M–R.

MTT data from colchicine treated SH-SYS5 cells (Fig. 5G) suggested that this drug has a dose-dependent cytotoxic effect in these cells, becoming statistically significant at 0.1 μM (p < 0.05) and increasing with the drug dose. This contrasts with HCA data from the same cells (Fig. 5G), which suggested that colchicine was highly toxic to neurites, but did not cause cell loss, an observation supported by reviewing the images from the experiment (Fig. 6A–F). In differentiated PC-12 cells, the MTT data (Fig. 5H) indicated that colchicine had no cytotoxic effect in these cells; in accordance with the corresponding HCA data (Fig. 5I and 6G–L). It is interesting to observe that in the heatmap visualization of the broader effects of colchicine in SH-SYS5 and PC-12 cells presented
in Figs. 7 and 8, colchicine is observed to have a toxic mitochondrial effect in SH-SYSY but not PC-12 cells, which may in part explain the differences in the MTT results between these cell lines. In the case of colchicine effects upon hN2™ cells (Fig. 5I), MTT data aligned with the HCA data for this cell type in showing that the drug was significantly \((p < 0.05)\) toxic to these cells in a dose-dependent manner at doses of 0.01 \(\mu\)M or greater. Taken in isolation, the data in Fig. 5 might suggest that the MTT assay is reliable in reporting presence or absence of cytotoxicity in PC12 and hN2™ cells, but not in SH-SYSY cells. However, data across the entire compound set was not so clear cut, as shown in Figs. 7–9. Additionally, absence of cytotoxicity as assessed by MTT does not indicate that neurotoxic effects are absent, for example see the dramatic effects of colchicine upon the neurite network in PC-12 cells (Fig. 6G–I), which MTT was unable to detect.

With regard to the effects of colchicine as assessed by the LDH assay, LDH data (Fig. 5G–I) recorded no significant cytotoxic effects of this drug in any of the cell lines tested. In these observations, the LDH data was in accordance with HCA data for SH-SYSY and PC-12 cells (Fig. 5G and H), but not for hN2™ (Fig. 5I), and in accordance with MTT data for PC-12 cells (Fig. 5H), but not for SH-SYSY and hN2™ (Fig. 5G and I). Fig. 6 shows representative image data for the effects of each drug in each cell type and provides a striking example of the value of HCA in retaining source image data which can quickly be checked when unexpected or contradictory results are obtained.

3.3. Heatmap and clustering analysis of screening data

Figs. 7–9 contain heatmapped and clustering data showing lowest effect concentration (LOEC) data for all compounds tested upon all parameters in differentiated SH-SYSY (Fig. 7), PC-12 (Fig. 8) and hN2™ cells (Fig. 9). Details of the precise drug concentration data used to generate these heatmaps are provided in Supplementary Tables 1–3. Figs. 7–9 enable visualization of trends across the entire data set, and visually illustrate the large amount of data that can be obtained from individual wells through multiparametric HCA analysis. Each of the 10 HCA parameters selected here was capable of detecting neurotoxic events at low drug concentrations. Heat map and cluster analysis generated a distinct toxicity profile for each of the three cell lines, which revealed similarities and differences in their responses to the chemical test set, suggesting complex and diverse mechanisms of neurotoxicity across different cellular models. The cluster analyses also illustrate the variability between the outcomes reported by each assay endpoint in each of the cell lines.

Supplementary Tables 1–3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.03.013.
Fig. 6. Stained and segmented HCA images show effects of colchicine upon differentiated SH-SY5Y, PC-12 and hN2™ cells. Cells were differentiated then treated for 72 h with 0 μM, 1 μM or 1000 μM colchicine. Images show effects of colchicine upon merged fields of Hoechst nuclear staining (blue) and βIII-Tubulin staining (green) in differentiated SH-SY5Y (A–C), PC-12 (G–I) and hN2™ cells (M–O). Image segmentation of each image is shown for SH-SY5Y (D–F), PC-12 (J–L), and hN2™ (P–R) cells. Nuclear segmentation is represented by red traces, cell bodies by yellow traces, and neurites by cyan traces. Scale bars represent 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 7. Heatmap and dendrogram showing effects of test compound panel on all assay endpoints in differentiated SH-SY5Y cells. Data represent lowest effective concentration (LOEC) of response for each condition. In SH-SYSY LOECs ranged from 0.01 nM to 1 mM. Values >1 mM indicate that no response was detected at any concentration tested.

Fig. 8. Heatmap and dendrogram showing effects of test compound panel on all assay endpoints in differentiated PC-12 cells. Data represent lowest effective concentration (LOEC) of response for each condition. In PC-12 LOECs ranged from 1 nM to 1 mM. Values >1 mM indicate that no response was detected at any concentration tested.
When looking at the HCA data as a whole, heatmap and clustering data suggest that the SH-SY5Y-based assays were most effective at identifying compounds with similar modes of action (Fig. 7; Supplementary Table 1). Vinblastine and vincristine paired together in SH-SY5Y cells, as did colchicine and nocodazole, and together these four compounds formed a cluster, in line with their similar microtubule-disrupting mechanisms of action. Similarly, the two platinum-containing anti-cancer drugs used in this study, namely cisplatin and oxaliplatin paired together due to very exhibiting very similar effects in differentiated SH-SY5Y cells. Paclitaxel, another microtubule-disrupting drug, did not cluster with others in SH-SY5Y cells, primarily because of its weaker effects upon neurite measurements based on βIII-Tubulin staining compared with other microtubule-disrupting drugs tested. Interestingly, the SH-SY5Y endpoints most sensitive to paclitaxel were pNF-H area and mitochondrial area, showing the value of these endpoints in providing additional information beyond that obtained from Hoechst/βIII-Tubulin staining for HCA-based neurotoxicity assessment. SH-SY5Y cells occasionally showed neurotoxic indications in response to compounds expected to be benign. For example, 1 μM metformin evoked a significant decline in neurite length and area in SH-SY5Y cells (Supplementary Fig. 4) which was not observed in the other cell models tested.

Supplementary Fig. 4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2014.03.013.

Heatmap and clustering of HCA data for PC-12 cells (Fig. 8; Supplementary Table 2) indicate that these cells exhibited the fewest toxic events in response to compounds expected to be non-toxic, suggesting greater specificity of the PC-12 assay model employed here versus the greater sensitivity of SH-SY5Y. However, the PC-12 model may be prone to underestimating neurotoxic effects, since 6-hydroxydopamine, vincristine and lidocaine were found to be less toxic in PC-12 than in SH-SY5Y cells (Figs. 7 and 8; Supplementary Tables 1 and 2). Unlike the SH-SY5Y model, compounds known to have similar mechanisms of action did not cluster closely together within the PC-12 dataset (Fig. 8).

Compound clustering results obtained using hN2TM cells (Fig. 9) generally fell between PC-12 and SH-SY5Y, in that hN2TM cells appear to be more susceptible than PC-12 cells to compounds generally considered non-toxic (e.g. nadolol, saccharin), and less sensitive than SH-SY5Y cells to the effects of expected neurotoxins (e.g. 6-hydroxydopamine, U0126). In the hN2TM cell data, vinblastine and vincristine paired together (Fig. 9), showing very similar effects upon multiple endpoints measured, whereas bortezomib, which was profoundly toxic in SH-SY5Y and PC-12 cells clustered together with suramin in hN2TM, a drug which was only weakly toxic in all three cell models employed in this study. It should be noted that due to poorer than expected recovery following cryopreservation of hN2TM cells when setting up the assays, fewer cells were available for study in hN2TM wells than for SH-SY5Y and PC-12, which may have influenced the results from these cells.

Although there were differences across each cell model employed here, some striking similarities were also observed. Notably, according to HCA assessment, lead acetate was profoundly non-toxic in each of the differentiated models employed in this study (Figs. 7–9; Supplementary Tables 1–3). This initially surprising result was confirmed by manual review of the HCA images (Supplementary Fig. 4), and suggests that lead acetate may be significantly less neurotoxic in mature neurons versus developing neurons, since lead acetate is a well-established neurotoxin in developmental neurotoxicity models.

Inclusion of pNF-H and MitoTracker™ measurements within the HCA measurement suite employed here provided several novel insights. In differentiated SH-SY5Y cells, pNF-H area was the HCA endpoint most sensitive to the toxic effects of cisplatin, dipyridamole, oxaliplatin and suramin. Also in SH-SY5Y, mitochondrial area was the HCA endpoint most sensitive to the toxic effects of...
colchicine, lidocaine and vincristine (Fig. 7; Supplementary Table 1). In differentiated PC-12 cells, pNF-H area was the HCA most positive to the effects of methylmercury and vinblastine (Fig. 8; Supplementary Table 2). Both pNF-H and mitochondrial measurements frequently served as an additional confirmation from within the same well that a neurotoxic event occurred. However, clustering of the data from each assay in all three cell types (Figs. 7–9) show that pNF-H area and pNF-H nuclear expression; mitochondrial area and mitochondrial intensity do not consistently pair with other HCA measurements, indicating that additional information about compound effects in each cell model may be obtained by inclusion of these endpoints. For example, pNF-H area measurements are similar to, but not the same as, those obtained by measuring βIII-Tubulin neurite area in each cell type; the unexpected effects of metformin upon neurite length observed in SH-SY5Y cells were not accompanied by changes in pNF-H area in these cells (Fig. 7). In differentiated SH-SY5Y and PC-12 cells, mitochondrial intensity measurements have similarities with cell count data whilst mitochondrial area measurements more closely resemble neurite count data.

For the non-HCA data from MTT and LDH assays, the difficulties with these assays observed on a small scale in Fig. 5 may be seen with a larger number of compounds in Figs. 7–9, and in Supplementary Tables 1–3. Dramatic differences were observed between the results generated by these assays, indicating that these assays are not interchangeable as toxicity assays. This was particularly apparent with differentiated SH-SY5Y cells (Fig. 7; Supplementary Table 1), where the MTT assay reported toxic effects of expected non-toxic celecoxib, D-sorbitol and nadolol, whilst the LDH assay reported no toxic effects at any dose of several compounds found by highly toxic by other endpoints, such as K252a, nocodazole, sodium orthovanadate and vincristine. Cluster analysis revealed that MTT and LDH measurements were closely related to each other in PC-12 cells (Fig. 8), although in these cells too there were dramatic differences in the results from each assay in response to certain compounds such as methylmercury, where MTT reported a toxic effect upon PC-12 cells but LDH did not; and 6-hydroxydopamine, where LDH reported toxic effects whilst MTT reported none (Fig. 8, Supplementary Table 2). In hN2TM cells only three of the compounds initially expected to be neurotoxic were found to be toxic by the LDH assay (Fig. 9; Supplementary Table 3). Interestingly, MTT data did not cluster closely with HCA-based mitochondrial measurements in any of the cell types tested here, clustering more closely with HCA-based neuronal morphology measurements in SH-SY5Y and hN2TM cells, and with HCA-based nuclear measurements in PC-12 cells (Figs. 7–9). LDH assay data in general cluster analysis showed most similarity to HCA-based nuclear area measurements (Figs. 7–9).

3.4. Use of raw image data to verify HCA results

In instances where striking or unexpected results were obtained, it often proved helpful to return to the source image and segmentation data to verify the findings. Occasionally cell seeding errors or lint debris on the assay plate may subsequently be discovered to be the cause of anomalous HCA assay results. With MTT and LDH assays there is no such simple way to re-evaluate experimental results. Supplementary Fig. 5 provides some examples of instances where the heat map data yielded novel and/or surprising results which were then checked against raw image data. Examples included in this figure show the non-toxicity of lead acetate (A–D), where neurite length and cell count were unaffected. The interesting ‘spotting’ of the βIII-Tubulin stain observed in differentiated SH-SY5Y cells exposed to 1000 μM lead acetate was unique to SH-SY5Y cells, and was only observed at this concentration of lead acetate. Also shown in Supplementary Fig. 5 are an unexpected negative effect of metformin upon neurites in SH-SY5Y cells (E–H), sensitivity of pNF-H in PC-12 cells to very low concentrations of troglitazone (1–L), a decline in neurite length in differentiated PC-12 cells exposed to t-retinoic acid (M–P) and effects of 10 nM paclitaxel upon neurites of hN2TM cells (Q–T). In each instance, the raw image and image segmentation visually corresponded with the numerical data generated by the image analysis algorithm presented in the heatmap and clustering data (Figs. 7–9; Supplementary Tables 1–3).

4. Discussion

This study successfully achieved three primary objectives, namely to (1) determine if development of a robust multiparameter HCA assay for neurotoxicity screening in multiple differentiated neuronal cell models is feasible; (2) compare HCA-based neurotoxicity assessment with data from MTT and LDH cytotoxicity assays using a panel of test compounds; (3) compare and contrast the responses of widely used neuronal cell lines with human embryonic stem cell-derived neurons with regard to their responses to a panel of test compounds. Additionally, several pieces of new information were obtained from the data.

HCA has become a well-established neurotoxicity screening tool (Culbrett et al., 2012; Harrill et al., 2013; Krug et al., 2013a; Mundy et al., 2010). However, since the primary focus of most of this research has been on developmental neurotoxicity, most data in the scientific literature refers to neurotoxicity assessments performed by using a starting population of undifferentiated cells and monitoring inhibition of differentiation. In contrast, this study aimed to determine if differentiated neuronal cells, representing more mature neuronal phenotypes, could be effective tools for HCA assessment of neurotoxicity potential of chemicals in mature neurons. The data indicate that differentiated SH-SY5Y, PC-12 and hES-derived hN2TM can be utilized effectively to study the effects of chemical-induced toxicity on neuron-specific endpoints.

This study demonstrates that it is both feasible and useful to develop multiparametric HCA assays which yield large volumes of phenotypic data from a single well in order to assess differentiated neuronal phenotypes. In addition to the previously utilized Hoechst and βIII-Tubulin-based detection reagents (Arastu-Kapur et al., 2011), in this study we added reagents to detect chemical effects upon mitochondria and phosphorylated Neurofilament-H, both of which generated valuable new information about how neurotoxic effects of chemicals are mediated. To the authors knowledge, this study represents the first time that pNF-H has been utilized as a neurotoxicity marker for HCA. Our data both confirm and extend the observations of Jamieson et al. (2009) by showing that decreased pNF-H immunoreactivity corresponds with drug-induced neurotoxicity in differentiated SH-SY5Y, PC-12 and human ES-cell derived hN2TM cells. pNF-H staining was observed in neurites, soma and nuclear regions of each cell type studied here. Although quantitative changes in its staining pattern in the nucleus were commonly observed in response to neurotoxic drugs, its total area of expression in neurites proved to be a more sensitive indicator of neurotoxic challenge. Under many conditions, pNF-H area proved to be the most sensitive indicator of drug-induced neurotoxicity in this study, with declines in expression being seen at lower concentrations than for other endpoints tested, indicating that loss of pNF-H immunoreactivity indicates very early neurotoxic events preceding declines in βIII-tubulin positive neurite area under these conditions. Examples of this included the effects of cisplatin, oxaliplatin and suramin in differentiated SH-SY5Y cells, and the effects of methylmercury and vinblastine in differentiated PC-12 cells. In other instances the pNF-H area data served to provide additional confirmation and clarification for data obtained from βIII-Tubulin-based neurite measurements; thus
pNF-H is a robust, useful and highly sensitive neuron-specific marker for HCA-based neurotoxicity screens and serves as an effective complement to the widely utilized βIII-Tubulin stain. Similarly, quantitative assessment of MitoTracker®-Red CMXRos stained regions revealed that mitochondrial area is a highly sensitive indicator of drug-induced neurotoxicity, notably with regard to the effects of colchicine, lidocaine and vincristine in differentiated SH-SYSY cells. We expect that the panel of detection reagents employed here will be useful in additional cell types, since, although screening of other cell types was beyond the scope of this study, we have tested the same set of detection reagents in primary cultures of rat hippocampal neurons (data not shown), and found that this detection reagent set works effectively in these cells also, indicating wider utility of this assay.

One particular advantage of the HCA assay described here lies in the ability to readily distinguish between neurotoxic and cytotoxic drug effects. By using a starting population of well-differentiated neuronal cells, and having the ability to measure a variety of neuron-specific endpoints, it was possible to visualize changes in neuronal phenotypes, such as loss of neurites and declines in pNF-H immunoreactivity at drug concentrations which have no effect upon cell count. For example, for many drugs tested here, including vinblastine and colchicine, dramatic loss of neurites and pNF-H staining occurred at drug concentrations far below those required to cause cell loss. Other drugs, such as indomethacin, were observed to be more broadly cytotoxic, in that cell loss and neurite loss occurred simultaneously.

Our direct comparison of various differentiated neuronal cell models confirms that the choice of cell line heavily influences the outcome of chemical screening for neurotoxicity, as shown by the distinct toxicity profiles created by heatmap and clustering analysis of the HCA data. It is known that different neuronal cell types do not perform identically in cell-based assays; for example, Mundy et al. (2010) compared several undifferentiated and proliferating neuronal cell lines including SH-SYSY and PC-12, and found that PC-12 was more sensitive to chemically induced proliferation inhibition. Of the cell types tested in this study, our data suggest that differentiated SH-SYSY cells were the most sensitive model for detection of neurotoxic effects, although this cell type may be more prone to produce false positive results from non-toxic drugs than PC-12 or hN2™ models.

Our heatmap and compound clustering data suggested that the SH-SYSY model was the most reliable of those tested in generated results where drugs with similar modes of action clustered together, notably for microtubule inhibitors and platinum-containing anti-cancer drugs. We found no evidence to suggest that the human embryonic stem cell derived hN2™ model is currently superior to the more robust SH-SYSY or PC-12 cellular models at detecting neurotoxicity, although human stem cell-derived neuronal models like hN2™ are rapidly improving and will certainly play an important role in future neurotoxicity test systems. Our observations showing that each cell type responded to the chemical test set quite differently illustrates the difficulties associated with choosing the most appropriate cell model with which to perform neurotoxicity assessment studies. Our data support the observation of Mundy et al. (2010) that no one neuronal cell model is likely able to detect the effects of all neurotoxic chemicals. Perhaps using panels of cell models will ultimately prove more predictive than use of any single cellular model. Much more work remains to be done to more fully explore the potential of improved neuronal cellular models for neurotoxicity assessment. The richness of data available through multiparametric HCA makes this technique highly valuable for performing detailed characterization and comparison of cellular phenotypes.

When assembling a test set of compounds expected to be neurotoxic, we frequently referred to published data from in vitro developmental neurotoxicity studies. This study suggests that it is erroneous to assume that developmental neurotoxins will also prove neurotoxic in differentiated neuronal cell models. A striking example of this came when observing the effects of lead acetate. HCA data from each of the differentiated cell models employed here indicated that this compound was non-neurotoxic, which initially appeared to be a surprising result. Reviewing the source images and checking the image segmentation reaffirmed that no neurotoxic effects were detected. Our observations may be explained in part by the data of Lindahl et al. (1999) who reported that differentiated neuronal cells take up lead much less readily than undifferentiated neuronal cells. We also note that Zurich et al. (2002) have reported that immature brain cells are more sensitive to lead than their differentiated counterparts. In a follow-up experiment looking at the effects of this drug in non-differentiated neuronal cells, we have confirmed that this compound is highly toxic to undifferentiated neuronal cells (data not shown), but not to differentiated neuronal cells. Since glial effects are thought to influence lead-mediated toxicity, a follow-up co-culture study including the cell models used in this study along with glial cells may yield additional information on the precise nature of the effects observed here. Further large scale studies directly comparing the effects of chemicals in undifferentiated and differentiated neuronal cells should also prove helpful in distinguishing the effects of developmental and broader spectrum neurotoxins.

Our lead acetate data also highlighted a key advantage of HCA-based neurotoxicity assessment, namely the ability to return to the source images in order to verify novel and/or unexpected results. The ability to re-verify source images and to check the accuracy of image segmentation is extremely beneficial in determining whether unexpected results represent a true occurrence or experimental artifact. For example, through this approach we were able to visually confirm profound neurotoxic effects of colchicine reported by the image analysis algorithm which the MTT and LDH assays had not detected.

In contrast to our HCA-based observations, we observed that both MTT and LDH assays were frequently unreliable indicators of drug toxicity and/or safety with regard to the chemicals tested in the differentiated neuronal cell models employed here. MTT and LDH data did not prove to be consistent with each other, exhibiting dramatically different results, particularly in SH-SYSY cells. A substantial number of chemicals shown to be neurotoxic via HCA showed no toxic effects in MTT and especially, in LDH assays in each cell type tested here. Additionally, in SH-SYSY cells several non-toxic compounds gave false positive results in the MTT assay. The difficulties we observed in attempting to use MTT and LDH assays to detect neurotoxicity serve to emphasize the need to choose assays and endpoints which are appropriate for the cell types and events being studied. Many factors have the potential to influence the results of the MTT and LDH assays, such as cell type, media components, enzyme stability, and mechanism of cell death and drug sites of action (Twentymon and Luscombe, 1987; Riss and Moravec, 2004). In addition, many therapeutic drugs influence metabolism and mitochondrial proliferation, increasing the risk of false positives and negatives when using MTT to test chemical entities. LDH data can be misleading if the toxic agent only influences intracellular activities and does not affect the plasma membrane, which would lead to false negatives. Because both of these assays are based on enzymatic activity, they may also be adversely influenced by enzyme inhibitor drugs. Finally, while amenable to higher throughput applications, these assays provide very limited information about mechanism of action. Thus, we are in agreement with Forsby et al. (2009) that simple cytotoxicity or cell viability tests using non-specialized cell models are not sufficient and must be complemented by additional tests for the
detection of organ-specific effects. Our data also support recent observations by Krug et al. (2013a), that assays using neuron-specific endpoints improve the specificity of neurotoxicity assays. The likelihood of compound misclassification on the basis of changes to a single assay parameter can be further mitigated by measuring multiple neuron-specific parameters per well, a task for which HCA is especially well-suited.

In summary, this study confirms the feasibility and utility of multiparametric HCA-based approaches for neurotoxicity assessment, indicating that HCA can be used to precisely distinguish between neuron-specific toxicity and general cytotoxicity while simultaneously enabling inclusion of other parameters to detect novel neurotoxic effects of chemicals. The approach is particularly useful for identifying the differences between diverse neuronal models in their cellular responses to drugs. Looking ahead, despite the extensive insights HCA can provide, this technique too frequently relies on single time point assays, with limited information available about the precise kinetics of neurotoxic effects over time, and the possible reversibility of effects upon compound withdrawal and/or re-exposure. A kinetic live-cell neurotoxicity assay with automated control over drug handling synchronized with quantitative imaging would represent a powerful tool for neurotoxicity assessment. It seems likely that such dynamic live cell assays for neurotoxicity may become a reality in the near future through the combination of microfluidics technology with quantitative live cell imaging (Chen et al., 2013; Lee et al., 2011). With the recent emergence of highly promising, complementary, non-imaging based methods for neurotoxicity assessment, such as Multi-Electrode Arrays utilizing Bayesian modeling (Lefew et al., 2013) and transcriptome profiling (Krug et al., 2013b), neurotoxicologists now have a powerful arsenal of tests available which will enable detailed understanding of the neurotoxic potential of chemicals on a large scale.

Conflict of interest statement
All authors were employees of EMD Millipore Corporation when this work was performed.

Transparency document
The Transparency document associated with this article can be found in the online version.

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