Video Article

Real-Time Impedance-based Cell Analyzer as a Tool to Delineate Molecular Pathways Involved in Neurotoxicity and Neuroprotection in a Neuronal Cell Line

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Abstract

Many brain-related disorders have neuronal cell death involved in their pathophysiology. Improved *in vitro* models to study neuroprotective or neurotoxic effects of drugs and downstream pathways involved would help gain insight into the molecular mechanisms of neuroprotection/neurotoxicity and could potentially facilitate drug development. However, many existing *in vitro* toxicity assays have major limitations – most assess neurotoxicity and neuroprotection at a single time point, not allowing to observe the time-course and kinetics of the effect. Furthermore, the opportunity to collect information about downstream signaling pathways involved in neuroprotection in real-time would be of great importance. In the current protocol we describe the use of a real-time impedance-based cell analyzer to determine neuroprotective effects of serotonin 2A (5-HT_{2A}) receptor agonists in a neuronal cell line under label-free and real-time conditions using impedance measurements. Furthermore, we demonstrate that inhibitors of second messenger pathways can be used to delineate downstream molecules involved in the neuroprotective effect. We also describe the utility of this technique to determine whether an effect on cell proliferation contributes to an observed neuroprotective effect. The system utilizes special microelectronic plates referred to as E-Plates which contain alternating gold microelectrode arrays on the bottom surface of the wells, serving as cell sensors. The impedance readout is modified by the number of adherent cells, cell viability, morphology, and adhesion. A dimensionless parameter called Cell Index is derived from the electrical impedance measurements and is used to represent the cell status. Overall, the real-time impedance-based cell analyzer allows for real-time, label-free assessment of neuroprotection and neurotoxicity, and the evaluation of second messenger pathways involvement, contributing to more detailed and high-throughput assessment of potential neuroprotective compounds *in vitro*, for selecting therapeutic candidates.

Video Link

The video component of this article can be found at http://www.jove.com/video/51748/

Introduction

Neuronal cell death plays a critical role in the pathophysiology of many brain-related disorders[1]. The availability of reliable and high-throughput *in vitro* toxicity assays is critical to gain better insight into the mechanisms of neurotoxicity and to help select neuroprotective molecules as therapeutic candidates in drug development[2]. However, there are many limitations to most widely used *in vitro* neurotoxicity assays.They assess neurotoxicity/neuroprotection at a single time-point not allowing kinetic resolution; often use label or probe which can interfere with the signaling pathways and limit additional studies in the same cell population, and are often labor-intensive, and in many cases do not provide mechanistic insight. In the present study we demonstrate the utility of a real-time impedance-based cell analyzer to determine neurotoxicity and neuroprotection in a neuronal cell line in real-time and under label-free conditions and to provide insight into downstream mechanisms through analysis of second messenger pathways involved in the effect.

Previous studies have confirmed the validity of the real-time cell analyzer to determine cytotoxicity as well as effects on cell proliferation in cell lines in comparison with standard techniques[3,4,5,6]. For example, a good correlation was observed between readouts of the standard cell viability WST-1 assay and Cell Index values at several time points under basal proliferation conditions and after two different toxic paradigms in HeLa cells[3]. In A549 and MDA-MB-231 cells proliferation and cytotoxicity provoked with the microtubule stabilizer paclitaxel showed very similar values when assessed by Cell Index measurements and the standardly used sulforhodamine B (SRB) assay[4]. In the neuronal cell line of immortalized hippocampal neurons HT-22 Cell Index measurements were validated for their ability to detect cell proliferation, glutamate cytotoxicity and cytoprotection against the widely used 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium-bromide (MTT) assay[5]. In the same study the MTT assay results and Cell Index measurements also correlated well in measuring neuronal progenitor cells proliferation, cytotoxicity after growth factors deprivation and rescue of cytotoxicity by the pan-caspase inhibitor QVD[5]. Cytotoxicity induced in NIH 3T3 cells by Vandetanib (vascular endothelial growth factor receptor and epidermal growth factor receptor inhibitor) showed similar results measured with Cell Index values or neutral red uptake assay[6].

We have recently used the real-time cell analyzer system to assess neuroprotective effects of the serotonin 2A (5-HT_{2A}) receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) in a neuronal cell line (SK-N-SH cells) and screened for the involvement of second messenger pathways through monitoring the effect of their chemical inhibition on the observed neuroprotection[7]. Interestingly, the 5-HT_{2A} receptor has both hallucinogenic and nonhallucinogenic agonists (like DOI and lisuride, respectively), which may activate both common and distinct second messenger pathways[8].

The advantages of the presented technique are that it allows to collect real-time information on cell survival in the course of days, to delineate second-messenger pathways involved, to assess the possible contribution of proliferation effects to neuroprotection, and to select an optimal time for additional end-point studies on the same cell population. A schematic diagram of the workflow in the current protocol is presented in **Figure 1**.

Protocol

1. Preparation

1. Place the real-time cell analyzer's station in a tissue culture incubator set at 37 °C and with 5% CO_2. Carry out all cell culture handling and pharmacological treatments in a tissue culture hood under sterile conditions.
2. NOTE: Neuronal cell lines requiring different temperature settings compared to standard conditions for culture, should be adjusted accordingly. For weakly adherent cell lines, use coating agents to facilitate the interaction of cells with the gold microelectrodes in the bottom of the wells of the E-Plate 96.
3. Prepare 1,000X stock solutions of the pharmacological compounds for cell culture treatment (neuroprotective agents or second messenger pathways inhibitors in the appropriate solvent – dimethylsulfoxide or sterile water) and store them at -20 °C. Use the solvent as vehicle in the following treatments.
4. Culture human neuroblastoma SK-N-SH cells in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) (proliferation medium) in cell culture flasks with 175 cm^2 surface to density of about 75%. Keep cell passage number within a range (of about 10 passages) to ascertain consistency between experiments in terms of rate of cell proliferation and response to cytotoxicity. Lower passage numbers are preferred.
5. Wash the adherent SK-N-SH cell layer with PBS, and trypsinize with 0.05% trypsin-EDTA solution for 5 min at 37 ˚C. Centrifuge the cells at 170 × g for 5 min to remove trypsin. Resuspend the cells in 10 ml proliferation medium. Count the cells with Scepter cell counter and adjust cell number to 300,000 cells/ml by diluting cells with proliferation medium.

2. Plating and Proliferation of SK-N-SH Cells

1. Add 100 µl proliferation medium to each well of the E-Plate 96 and leave it for 30 min in the tissue culture hood at RT to equilibrate. Insert the E-Plate 96 in the real-time cell analyzer station in the CO_2 incubator at 37 °C.
2. Start the real-time cell analyzer software. On the Layout page select the wells included in the experiment and enter in the edit boxes the information about cell type, cell number, names and concentrations of chemical compounds used for cell treatment.
   NOTE: For the purposes of this protocol at least 4 replicates are recommended for each treatment.
3. On the Schedule software page determine "Steps" included in the experiment, by selecting the number of sweeps measuring Cell Index and the interval between sweeps for each step. Since Step 1 is predetermined for background measurement, select "Add a step" and set Step 2 to measure the Cell Index every 15 min for 96 hr.
   NOTE: For treatments, in which effects with fast kinetics are expected, set sweeps at shorter intervals.
4. Click Start to initiate the automatically predetermined Step 1 and measure the background impedance of the media. This value is automatically subtracted by the software at each data point once the cells are added to the wells.
5. Use the previously prepared in step 1.5) cell suspension of 300,000 cells/ml in proliferation medium. 30,000 cells/well for cell toxicity/neuroprotection studies and 15,000 cells/well for cell proliferation studies. Take out the E-Plate and add 100 µl cell suspension per well for cell toxicity/neuroprotection studies and add 50 µl cell suspension and 50 µl proliferation medium per well for cell proliferation studies. The number of cells plated per well needs to be optimized for each cell line empirically.
6. Gently swirl the E-Plate 96 for even plating of the cells. Leave the E-Plate 96 for 30 min in the tissue culture hood at RT to allow the cells settle evenly to the bottom of the wells.
7. Insert the E-Plate 96 in the real-time cell analyzer station and start Step 2 on the Schedule page. Monitor Cell Index values throughout the experiment by viewing the cell curves on the Plot page and the raw Cell Index data on the Cell Index page of the software.

3. Serum Deprivation

1. 24 hr after plating the cells, pause the experiment, and remove the E-Plate 96 from the real-time cell analyzer station. Dilute second messenger inhibitors from 1,000X stocks with DMEM/F12 serum-free medium – to 200× the final concentration. Treat cells with 1 µl inhibitors of second messenger pathways to be tested for their involvement in neurotoxicity/neuroprotection 30 min before initiating cytotoxicity, to inhibit the respective pathways. Return the E-Plate 96 to the station and resume experimental Cell Index measurements.
2. After 30 min pause the experiment again. Carefully remove proliferation medium fully from the E-Plate 96 wells with a pipette (or multi-channel pipette), taking care not to disrupt the adherent cell layer by directing the edge of the pipette tip to the corner of the well. Add 200 µl/well of serum-free DMEM/F12 medium (serum deprivation medium). Ensure rapid exchange of cell culture medium to minimize mechanical agitation of the cells.
3. Dilute compounds to be tested for their neuroprotective effects from 1,000X stock with serum-free DMEM/F12 medium to 200× the final concentration. Add 1 µl compounds to be tested for their neuroprotective effects and 1 µl inhibitors of second messenger pathways in the individual wells according to the experimental plan.

4. In cells for proliferation studies do not change medium. Dilute compounds to be tested from 1,000X stock to 200× the final concentration in proliferation medium, treat with 1 µl per well and continue to culture in proliferation medium.
5. Resume the experiment to continue with Cell Index measurements every 15 min for 96 hr as previously set.

4. Data Analysis

1. For neurotoxicity/neuroprotection data normalize Cell Index to the last time point before pharmacological treatment or medium change to reduce variation between experiments by selecting “normalized Cell Index” and “normalize Time” on the Plot page.
2. Highlight the wells on the Plot page for the experimental conditions of interest and click “Add” to plot the normalized Cell Index curves. Observe the kinetics of the neurotoxic/neuroprotective effect, or of second messengers inhibition as normalized Cell Index curves as a function of time.
3. Observe the Cell Index curves for the tested drug treatments in proliferation medium as a function of time to assess whether an effect on proliferation is involved in the neuroprotective/neurotoxic effect.
4. Export the experimental info for all Cell Index time points from the Cell Index software page into an Excel file to initiate statistical evaluation of the results
   NOTE: As an initial step in the statistical analysis a set of modified MATLAB programs that use the Welch's t-test, with the p-value plotted semilogarithmically, utilize inverted axis against the time scale (provided as supplementary code files). These programs allow detection of p-values over the time-course of an entire real-time cell analyzer experiment, and thus aid the selection of time points for more detailed statistical analysis. See the supplementary material for detailed information on the programs.
5. For statistical analysis of differences in Cell Index values under different treatment conditions at specific time points, select Cell Index values at respective time points from the exported Excel file. Analyze the Cell Index values for the selected time points with one-way or two-way analysis of variance (ANOVA) followed by a post-hoc test using statistical software.

Representative Results

Serum deprivation leads to decrease in Cell Index values, which can be monitored continuously with the real-time cell analyzer

Neurotoxic stimuli to the cells lead to a decrease in Cell Index values, which can be monitored in real-time with the presented technique and the dynamics of which is dependent on the specific neurotoxic stimulus and the cell type studied. **Figure 2** demonstrates the increase in Cell Index when SK-N-SH cells are grown in proliferation medium until reaching a plateau (green line) and the drop of Cell Index followed by a stabilization at the new lower levels after switching the cells to serum deprivation medium (red line). This drop may represent a change in cellular adhesion due to withdrawal of the serum.

Neuroprotective effects of drug treatment can be monitored continuously with the real-time cell analyzer

The neuroprotective effect of compounds added before (pre-treatment), at the same time (co-treatment) or after neurotoxic stimulus (suggesting cell recovery) can also be followed continuously with the real-time cell analyzer, thus providing information about the rate and duration of the neuroprotective effect. **Figure 3** shows representative results of the dynamics of neuroprotection of two 5-HT_{2A} agonists - 5 µM DOI (**Figure 3A**) and 20 µM lisuride (**Figure 3B**) added at the time of switching cells into serum deprivation medium.

The real-time cell analyzer allows to differentiate between effects on cell survival and cell proliferation

An important question in neuroprotection studies in neuronal cell lines is whether an effect on cell proliferation contributes to the observed compound effect on cell survival. With the real-time cell analyzer the effect on proliferation can be tested in the same E-Plate 96 assessing neurotoxicity. **Figure 4** shows the lack of effect of 5 µM DOI treatment on SK-N-SH cells proliferation, suggesting an effect on proliferation does not contribute to the observed neuroprotective effect.

The real-time cell analyzer allows to determine which second messenger pathways are involved in neuroprotective/neurotoxic effects

Receptor activation initiates a cascade of second messengers effects. The current protocol allows to screen for the involvement of a number of second messengers in a neuroprotective/neurotoxic effect in real-time through pretreatment with their inhibitors. Since the effect of second messengers inhibitors on cell survival can be pronounced, and is in many cases not linear with time, following its kinetics is very informative. **Figure 5** presents the effect of 10 µM phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 on cell survival and DOI neuroprotection in serum-deprived SK-N-SH cells. In the presented graph, 10 µM LY294002 prevented the neuroprotective effect of DOI in serum deprivation, but also had some toxic effect of its own.

Relevant second messenger pathways and inhibitor concentrations for each tested compound can be determined through literature search. Concentrations suitability of second messengers inhibitors has to be verified, and if needed optimized experimentally. The goal of optimization is to select a concentration, which has a minimal effect on Cell Index values by itself, while still inhibiting effectively the respective second messenger. As an example, some second messenger pathways inhibitors, which can be relevant to the 5-HT_{2A} receptor, are presented in Table 1. These inhibitors have been previously used to assess downstream targets involved in serotonergic increase of the extracellular signal-regulated kinase phosphorylation[9]. We have shown their utility to assess downstream pathways involved in neuroprotection by 5-HT_{2A} receptor agonists after confirming and in some cases optimizing their concentrations for our experimental paradigm[7].

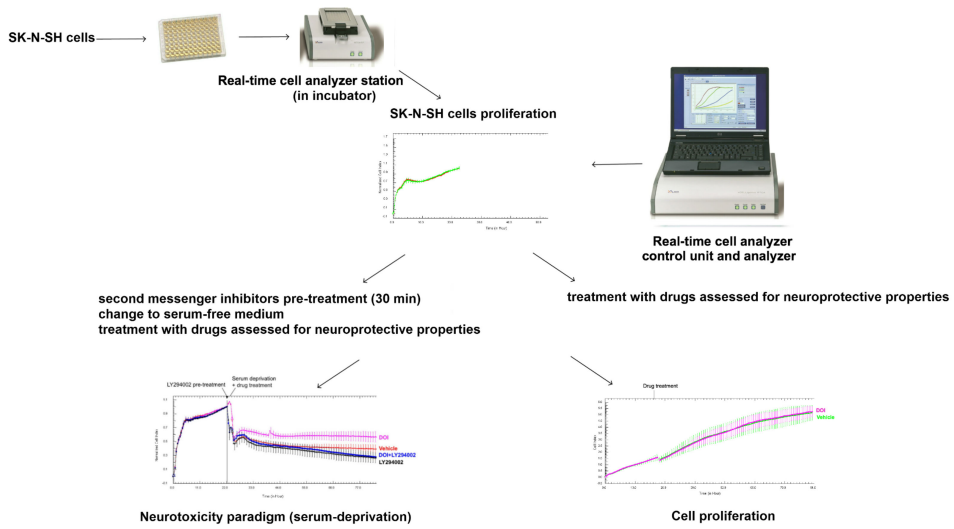

Figure 1. Schematic diagram of the workflow presented in the current protocol. Please click here to view a larger version of this figure.

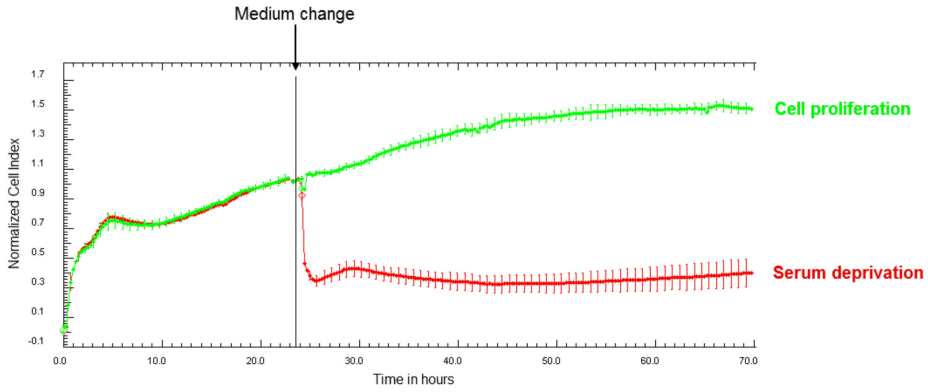

Figure 2. Effect of serum deprivation on Cell Index. Serum deprivation (red line) induces a rapid decrease in Cell Index, compared to the gradual increase in Cell Index values in proliferation medium (green line). Please click here to view a larger version of this figure.

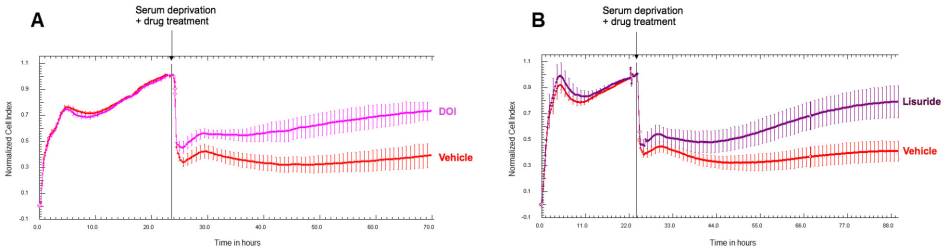

Figure 3. Effect of 5-HT$_{2A}$ agonists on Cell Index after serum deprivation. Treatment with cytoprotective drugs (in this experiment 5-HT$_{2A}$ agonists DOI (5 μM) (**A**) (magenta line) and lisuride (20 μM) (**B**) (dark magenta line) partially restores Cell Index values decreased by serum deprivation (red line). Please click here to view a larger version of this figure.

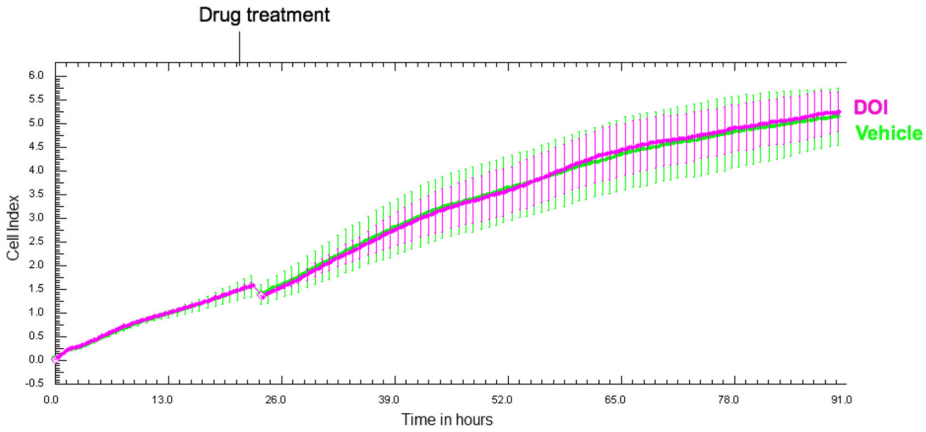

Figure 4. Cell Index values in the course of cell proliferation. Treatment with drugs in proliferation medium (in the current experiment 5 µM DOI) (magenta line) allows to assess the effect on proliferation compared to vehicle treatment (green line). Please click here to view a larger version of this figure.

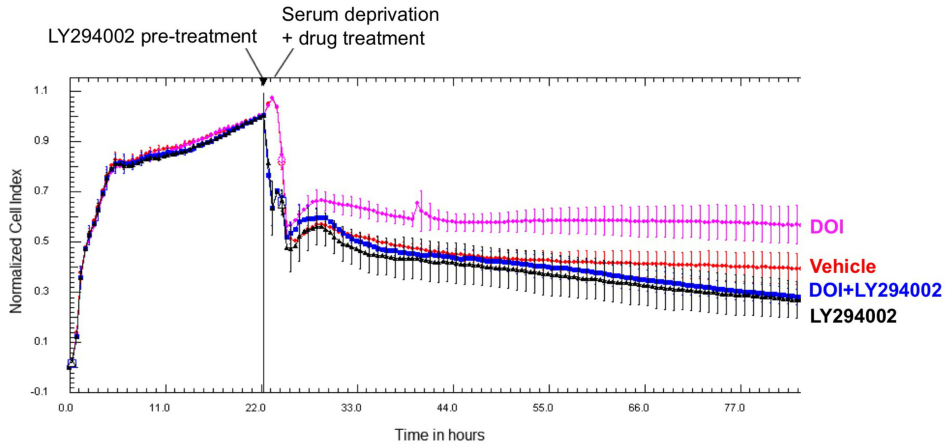

Figure 5. Second messenger inhibitors may affect cell survival on their own and can be used to screen for the effect of second messenger pathways on observed neuroprotection. In this experiment cells were subjected to serum deprivation being treated with vehicle (red line), 1 µM DOI (magenta line), pretreated with 10 µM PI3-K inhibitor LY294002 and treated with 1 µM DOI (blue line) or pretreated with 10 µM LY294002 and treated with vehicle (black line). Please click here to view a larger version of this figure.

Name:	Inhibitor of:	Final concentration:
U73122	Phospholipase C	1 µM
genistein	General tyrosine kinases	50 µM
U0126	Mitogen-activated protein kinase kinase	10 µM
chelerythrine	Protein kinase C	1 µM
LY294002	phosphatidylinositol 3-kinase	10 µM
KN-93	Ca2+/calmodulin-dependent protein kinase II	2 µM
H-89	Protein kinase A	1 µM

Table 1: Examples of second messenger inhibitors, which can be relevant for 5-HT_{2A} receptor downstream pathways and their concentrations used by us in real-time cell analyzer experiments.

Discussion

The current protocol presents the utility of a real-time cell analyzer to assess continuously and under label-free conditions the neuroprotective/ neurotoxic effects of compounds in neuronal cell lines and to gain insight into the second messenger pathways involved in the effect.

Even though the real-time cell analyzer's utility to study cytotoxicity and effects of drugs on cell proliferation is generally recognized, only a few studies have used it in neuronal related cell types. We have previously shown the utility of the real-time cell analyzer to monitor neuroprotection of a 5-HT_{2A} receptor agonist in human neuroblastoma SK-N-SH cells, while Galante *et al.*, have shown it can be used to assess neurotoxicity of beta-amyloid peptides in human neuroblastoma SH-SY5Y cells[7,10]. A recent study has suggested that while the real-time cell analyzer measured reliably cell proliferation and cell death in a neuronal cell line and neuronal precursor cells, its precision in assessing cell death in primary neurons depended on the severity of the insult[5]. Importantly, the alternating current used for the real-time cell analyzer system is of very low magnitude, generating microampere currents. These currents are well below the threshold potential of excitable cells as neurons. The real-time cell analyzer system relies on impedance measurements from the bottom surface of the E-Plate 96 wells, therefore the degree of cell adherence is of critical importance. It is possible that further optimization of coating of the E-Plate wells with extracellular matrix proteins for better adherence may improve results obtained with primary neurons.

The current manuscript demonstrates a novel approach of using the real-time cell analyzer system to delineate second messenger pathways involved in neuroprotection. Our protocol relies on the chemical inhibition of second messengers hypothesized to be involved in the action of a neuroprotective agent. Table 1 lists some commonly used second messenger pathways inhibitors, which can be relevant to the 5-HT_{2A} receptor as an example, and their concentrations suitable for the presented experimental paradigm. This experimental design allows for fast screening of potentially relevant second messenger pathways, to select downstream targets for more detailed studies through complementary approaches.

An important advantage of the real-time cell analyzer is also the ability to assess whether an effect on proliferation is a part of an observed neuroprotective effect. Using neuronal cell lines instead of primary neurons in toxicity assays offers some advantages, as lower cost, easy access and no need for animal experimentation. However, due to the fact that neuronal cell lines in contrast to primary neurons proliferate, potential effect on proliferation needs to be controlled for in neurotoxicity assays. The opportunity the real-time cell analyzer offers to perform this control in the same experimental E-plate 96, facilitates the experimental design.

Several technical parameters need to be considered in experiments with the real-time cell analyzer. The number of cells to be plated per well should be determined empirically through titration experiment for each tested cell type. Cytotoxicity and proliferation treatment are all started after 24 h of initiation of cell culture, to allow full adherence of the cells. Optimal results in cytotoxicity experiments starting when cells have reached 65-70% confluence were obtained in the current protocol. For proliferation experiments lower cell confluence is preferred (20-30%) to follow the effect of a drug on a proliferation curve.

In the current protocol serum deprivation neurotoxicity paradigm is presented due to its robustness, ease of use and utility to approximate trophic deprivation mediated neuronal death, which is important during development, acute brain or nerve trauma, and neurodegeneration[11]. However, a wide range of neurotoxicity paradigms can be used with the real-time cell analyzer system. Here, co-treatment with a cytoprotective agent at the time of cytotoxicity is presented. However, pretreatment or treatment after the toxic event (assessing cell recovery) can also be employed depending on the compounds being tested.

Overall, the current protocol suggests the utility of a real-time cell analyzer system to assess neurotoxicity and neuroprotection in neuronal cell lines continuously and label-free and to gain insight into downstream pathways involved in these effects.

Disclosures

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