xCELLigence System Real-Time Cell Analyzer

Focus Application
Neurotoxicity

Featured Study:
Real-Time Detection of Neuronal Cell Death by Impedance-Based Analysis Using the xCELLigence System

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Introduction

Neuronal apoptosis and necrosis occur in a large number of neurodegenerative diseases. Over the years, several well-accepted cell culture models have been implemented in neuroscience research, allowing the study of cell death mechanisms *in vitro*. Even though progress has been made in understanding the principles of cell death signaling in neurons, technical issues persist, which hamper data collection and interpretation. Among others, real-time detection of neuronal cell death is a major limitation. So far, the majority of available methods to measure cell proliferation, cell survival and cell death are invasive endpoint assays, which often are toxic to cells and even require cell disruption. Time course analyses of neuronal cell death and the kinetics of underlying mechanisms require laborious experiments, covering many time points. Several endpoint assays are available for detecting specific molecular events at certain time points of apoptosis, such as phosphatidylserine translocation to the outer membrane, mitochondrial dysfunction, caspase activation and DNA fragmentation (1). A shortcoming of these endpoint measurements is an inability to identify the appropriate time point for conducting any given assay. To address this issue researchers need to repeatedly inspect cells for morphological signs of cell death.

A non-invasive and label-free way to continuously monitor cellular behavior can now be achieved using the xCELLigence System of Real-Time Cell Analyzers (RTCA) developed by ACEA Biosciences. The xCELLigence System records the electrical impedance of cells grown on sensor microelectrode arrays integrated into the bottom of each well of an E-Plate 96. Impedance measurement, recorded as changes in Cell Index (CI) values, correlates changes in resistance of alternating current circuits (AC) with modifications of cell morphology after cells attach themselves to the electrodes. The xCELLigence System tracks cell viability and cell death by monitoring changes in cell morphology, cell adhesion and cell proliferation. In the present study, we used the xCELLigence System to investigate the response of the neuron-like cell line HT-22 and cultured primary rat cortical neurons to different cell death stimuli. A characteristic of primary neuronal cultures is their dense dendritic network, which may still persist as a remnant on the tissue plate after induction of apoptosis. Apoptotic neurons remain attached to the cell culture plate and may therefore still contribute to the impedance readout regardless of their viability status. How this characteristic affects the monitoring of primary neuronal cultures using the RTCA Instrument was a point of interest in this investigation. Furthermore, we investigated the potential of the xCELLigence System to monitor the phenomenon of neuroprotection. For this purpose, we applied the neuroprotectant BI-6C9, a well established small molecule inhibitor of the BH-3 interacting domain death agonist (BID), a pro-apoptotic member of the Bcl-2 gene family (4, 5).

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Taken together, our findings highlight the versatility of the xCELLigence System for continuously monitoring neuronal cell cultures throughout the entire time course of multi-faceted experiments, such as plating, precultivation, removal of proliferating glial cells, compound administration, and identification of neurotoxic and neuroprotective effects.

Materials and Methods

HT-22 Neuronal-like Cells

HT-22 cells were plated at indicated densities on 96-well E-Plates 96 (ACEA) or on regular 96-well plates (Greiner, Frickenhausen), and grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Karlsruhe, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (PAA Laboratories GmbH, Germany).

Primary Cortical Neuron Culture

Primary cortical neurons were prepared from embryonic rat brains (E16-18), as previously described (4). Briefly, meninges were removed and neurons were separated by mechanical dissociation after mild trypsinization of the isolated cortical tissue. Cells were plated at different densities on polyethylenimine (PEI) pre-coated 96-well E-Plates 96 (ACEA) or standard 96-well plates (Greiner). Neurobasal medium (Invitrogen), supplemented with 5 mM HEPES, 1.2 mM glutamine, 2% (v/v) B27 supplement (Invitrogen) and gentamicin (0.1 mg/ml), was used as a culture medium. After 48 hours, neurons were treated with 1 µM cytosine-arabinofuranoside (CAF) for another 48 hours to inhibit non-neuronal cell growth. Subsequently, medium was completely exchanged, and after 6-7 days of *in vitro* culture, neurons were used for experiments described below. To monitor CI changes in glutamate-treated neurons, it was necessary to add 1 µM CAF to cells on day 0 of cultivation.

Cell Proliferation Assay

Neuronal viability was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay according to the manufacturer's instructions for the Cell Proliferation Kit I (MTT) (Roche). Absorbance for each well was determined using an automated FLUOstar Optima reader (BMG Labtech, Offenburg, Germany) at 570 nm with a reference filter at 630 nm.

Real-Time Cell Analysis

Continuous cell monitoring was carried out in an E-Plate 96 using the xCELLigence RTCA MP Instrument and the RTCA Software 1.2 to measure CI values. Background impedance was determined using 100 µl DMEM supplemented with 10% FCS for HT-22 cultures and 100 µl Neurobasal medium containing 2% B27 supplement for neuronal cultures. Coating with PEI did not interfere with cellular impedance measurements. In contrast, poly-D-lysine or poly-L-lysine-coating was found to interfere significantly with real-time monitoring of primary cortical neurons. HT-22 cells were continuously monitored for 2 days and primary cortical neurons for 12-14 days.

Fluorescence and Light Microscopy – Cortical neurons were grown on PEI-coated IbiTreat µ-Slide 8-well plates (Ibidi, Munich, Germany) for 6 days. Subsequently, they were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) at +4°C. Neurons were permeabilized with 0.2% Triton X-100/PBS and blocked with 10% (v/v) normal goat serum (NGS) and 2% (v/v) bovine serum albumin (BSA) in PBS for one hour at room temperature (RT). Neurons were incubated overnight at +4°C with mouse monoclonal antibodies raised against the neuronal marker microtubule-associated protein 2 (MAP-2) (HM-2, ab11267, Abcam, Cambridge, UK, 1:600 dilution). The next day, cells were incubated with anti-mouse secondary antibodies raised in goat, Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Invitrogen, 1:250 dilution). Fluorescence images were collected using a DMI6000 B inverted microscope (Leica, Germany), and analyzed using LAS AF Software (Leica Application Suite, Advanced Fluorescence 2.2.0, Leica Microsystems, Mannheim, Germany). Light microscopic images were obtained using an Axiovert 200 microscope (Carl Zeiss, Jena, Germany) equipped with a Lumenera Infinity 2 digital camera (Lumenera Corporation, Ottawa, Canada). Light was collected through a 10x 2.5 NA objective (Carl Zeiss, Jena, Germany), and images were captured using phase contrast. Digital image recording and image analysis were performed with the INFINITY ANALYZE Software (Lumenera Corporation).

Results

Cell proliferation of neuronal HT-22 cells

To assess outgrowth and proliferation of the neuronal cell line HT-22, different cell densities (4,500, 8,000 and 20,000 cells per well) were seeded on an E-Plate 96, and monitored for 48 hours using the xCELLigence RTCA MP Instrument. Following a pronounced initial rise in impedance caused by the adherence of the cells to the surface of the well, HT-22 cells showed a constant growth rate over time. The resulting growth curves feature highly similar slopes differing only in the respective absolute CI values, which in turn depend on the initial seeding densities (see Figure 1A and B).

To induce cell death in HT-22 cells different doses of glutamate (3 and 5 mM) were used. In these cells glutamate causes glutathione depletion, enhanced generation of intracellular reactive oxygen species and subsequent mitochondrial damage and cell death (2, 3). No change in CI values could be detected within the first 8-10 hours after glutamate-treatment. Afterwards, CI values decreased rapidly within 4-6 hours. The profile of the impedance curves perfectly reflected the corresponding glutamate-induced cell death in a dose-dependent manner. This finding was supported by MTT assay carried out in the E-Plate 96 after completing the impedance measurements (see Figure 1C). The onset of cell death differed only slightly for the different cell plating densities and glutamate concentrations. CI values show the normalization of xCELLigence-recorded impedance profiles at the time point of compound administration (see Figure 1B). The kinetics of glutamate-induced cell death, as detected by the xCELLigence System, showed good correspondence to the previously reported time course and features for glutamate-induced death in HT-22 cells, including mitochondrial fragmentation and nuclear AIF translocation (2, 3, 4).

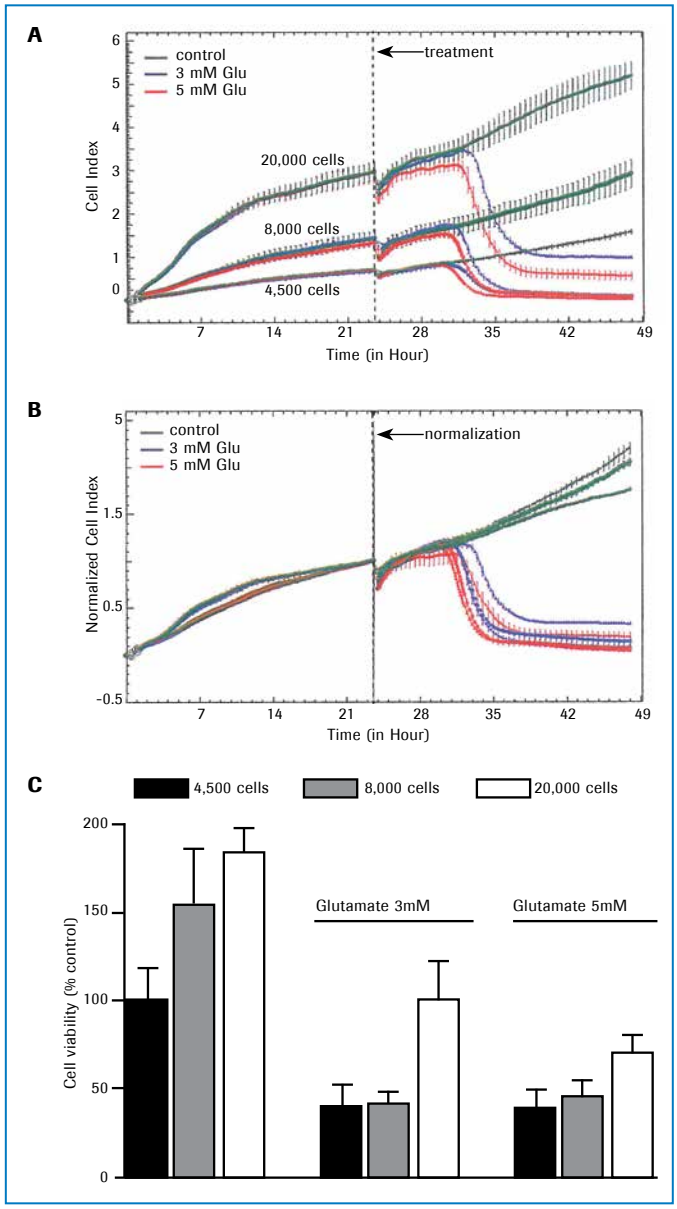

Figure 1: Cell number titration of HT-22 cells in an E-Plate 96 and detection of glutamate toxicity. (**A**) Indicated cell densities of HT-22 cells were seeded in an E-Plate 96 and recorded for 48 hours using the xCELLigence System. Cells were treated with glutamate at 24 hours post-plating. (**B**) Depicted are the HT-22 Cell Index (CI) values, normalized to the time point of glutamate administration. (**C**) Following continuous xCELLigence cell monitoring, cell viability was assessed by performing the MTT endpoint assay in the E-Plate 96.

Detection of neuroprotective effects by the xCELLigence System

To determine whether neuroprotection can be detected using the xCELLigence System, we used the small molecule inhibitor of the pro-apoptotic BH-3 protein BID, BI-6C9, to prevent glutamate toxicity in HT-22 cells. As shown in Figure 2A, BI-6C9 effectively prevented glutamate-induced apoptosis of HT-22 cells exposed to 3 mM or 5 mM glutamate. The continuous increase in CI values, recorded for BI-6C9-treated cells, in the presence and absence of glutamate, suggested that BI-6C9 preserved cell morphology and cell survival. Indeed, BI-6C9-mediated protection could also be verified by cell proliferation (MTT) assays carried out in the E-Plate 96 after terminating the recordings, and in an additional standard 96-well cell culture plate with the same experimental setup (see Figure 2B). The comparability of these results further underscores the applicability of the xCELLigence System to monitor cell viability. The effects of compound treatment on HT-22 cell morphology were documented using light microscopy (see Figure 2C). After glutamate exposure, cells undergo pronounced morphological changes as they become pyknotic and detach from the surface, thereby reducing their impedance-generating capacities. This is well documented by the decline in the corresponding CI values monitored by the xCELLigence System after glutamate treatment. In contrast, administration of BI-6C9, fully protected HT-22 cell morphology and CI values showed the corresponding evidence of neuroprotection (see Figure 2A).

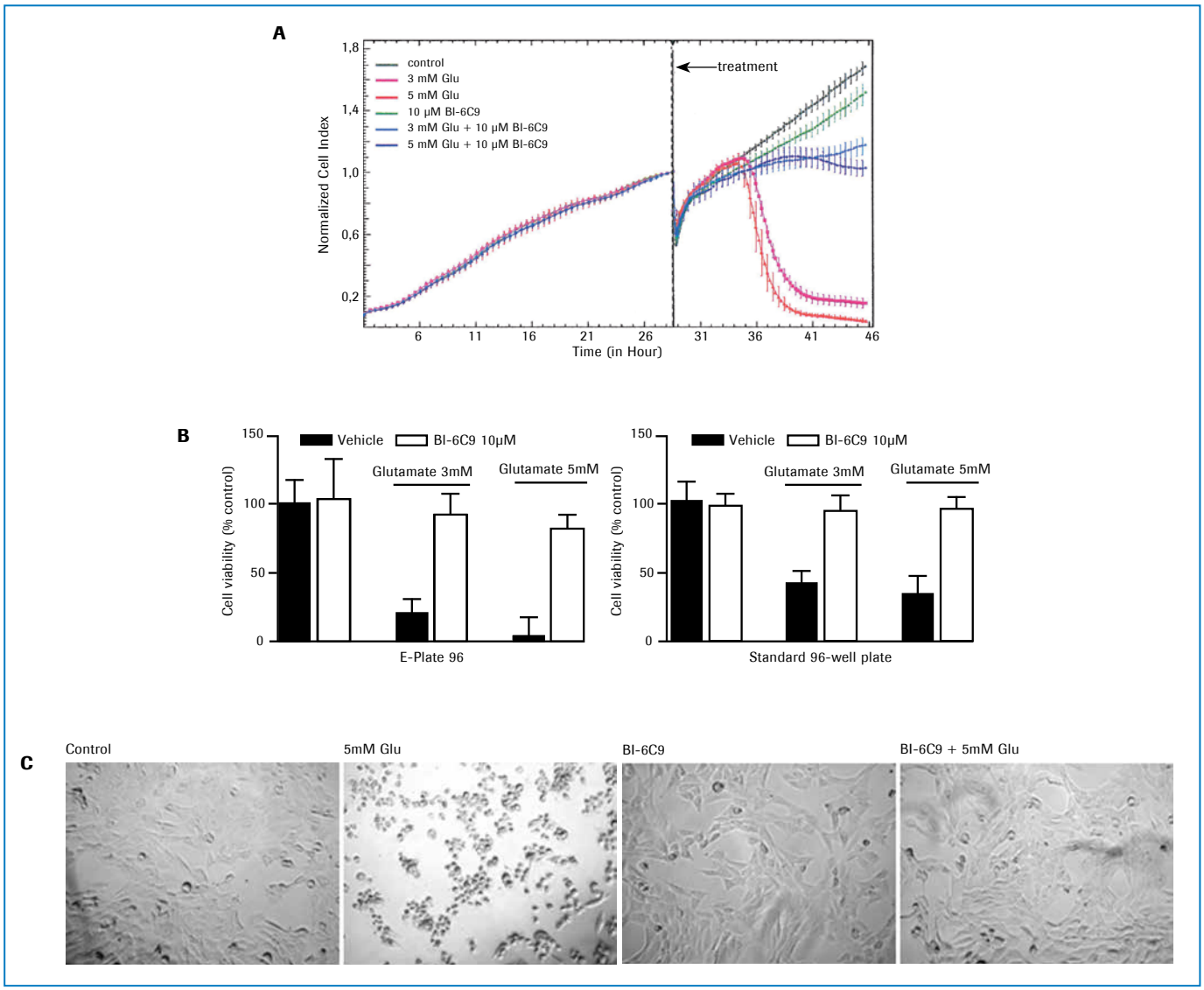

Figure 2: Cell impedance-based detection of neuroprotection in HT-22 cells. (**A**) The neuroprotective effect of the Bid inhibitor BI-6C9 was assessed using the xCELLigence System. HT-22 cells were treated with BI-6C9 and/or glutamate 24 hours after plating and the Cell Index (CI) values were recorded for 24 hours. (**B**) Cell viability was assessed using the MTT assay, carried out in the E-Plate 96 after terminating the CI recording (**left panel**), and in parallel using a standard 96 well cell culture plate (**right panel**). (**C**) The effects of compound treatment on HT-22 cell morphology were documented using light microscopy.

Primary cortical neuronal cultures

Primary rat cortical neurons (PCNs) were isolated and plated on a PEI-coated E-Plate 96 at different cell densities, ranging from 8,000 to 32,000 cells per well. As shown in Figure 3A, coating with PEI did not interfere with cellular impedance measurements. Primary neurons were adapted to cell culture conditions by growing in Neurobasal medium supplemented with 2% B27 for 72 hours. Continuous monitoring using the xCELLigence System revealed an initial increase of the CI values due to cellular attachment to the bottom of the well during the first hours. The neuronal network is best illustrated by microtubule-associated protein 2 (MAP-2) staining of PCNs seeded at different cell densities (see Figure 3B). In order to remove proliferating glial cells from primary cortical cultures, cells were treated with 1 µM CAF at day 3 for another 48 hours. As shown in Figure 3B, CAF-treatment caused a slight decrease in CI values, which most likely reflects the removal of proliferating glial cells (see Figure 3B).

To monitor cell death in primary neuronal cultures (16,000 cells per well), cells were treated with ionomycin or glutamate. Treatment with ionomycin 155 hours post-plating caused a significant drop of CI within 5 to 6 hours (see Figure 4A). Representative pictures of the cellular morphology of primary cultures exposed to ionomycin revealed pyknotic cell bodies in comparison to spindle-shaped flat cells under control conditions (see Figure 4C). As shown in Figure 4B, glutamate treatment induced a steady decline in CI values within 48 to 72 hours. Although glutamate induces a rapid increase in intracellular calcium concentrations through activation of NMDA receptors, the subsequent cell death is significantly delayed compared to ionomycin treatment (compare Figures 4A and 4B). This may be due to a delayed activation of cell death signaling by glutamate compared to the more rapid loss of membrane integrity and necrotic cell death following ionomycin treatment. In line with these findings, microscopic images of glutamate treated neuronal cultures showed fewer changes in cell morphology compared to ionomycin-treated cells (see Figure 4C), consistent with the different kinetics recorded using the xCELLigence System.

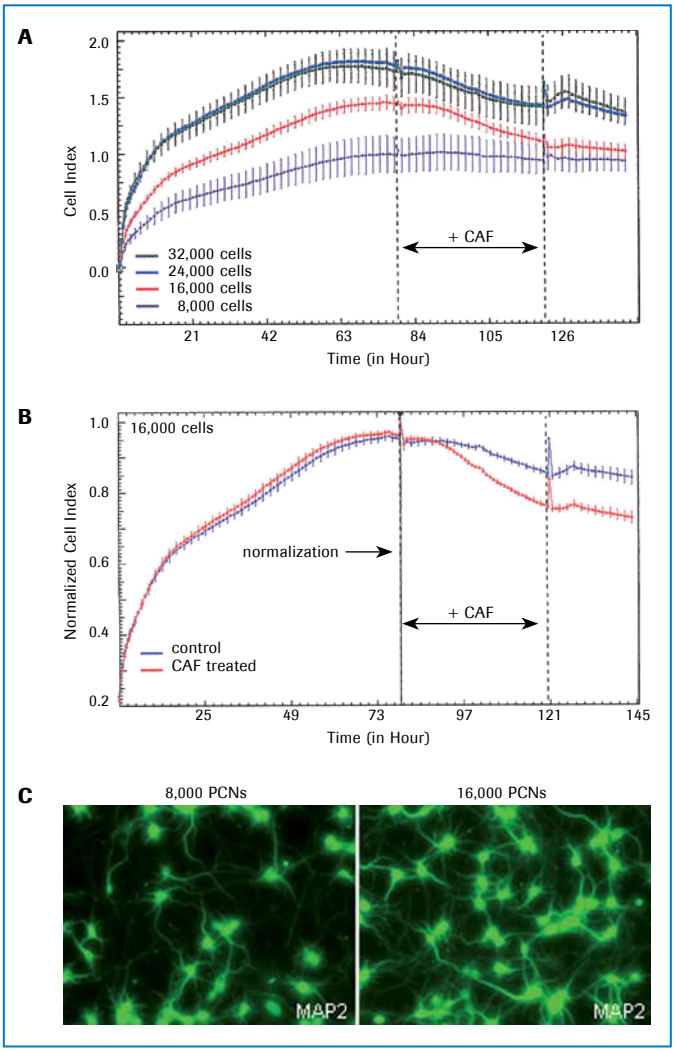

Figure 3: Cell number titration of primary cortical neurons. (**A**) Primary cortical neurons (PCNs) were seeded at the indicated cell densities and recorded continuously over 6 days using the xCELLigence System. After isolation, PCNs were adapted to cell culture conditions for 3 days in an E-Plate 96. Proliferating glial cells were removed by CAF treatment for 48 hours. Subsequently, cells were allowed to recover from CAF-treatment for additional 24 hours. (**B**) xCELLigence recordings reveal the effect of CAF-treatment on PCN cell cultures. CI values were normalized to the time point of CAF administration. (**C**) Representative pictures showing MAP-2 staining of neuronal cultures at different cell densities.

Conclusion

Cellular degeneration and neuronal cell death are frequently linked with acute and chronic degenerative disorders, such as stroke, Parkinson's and Alzheimer's disease. Investigation of the underlying cell death mechanisms in neuronal cells is of major interest to identify the causes of neurodegenerative diseases and develop therapies. In the present study, we tested whether well-established neuronal cell culture models could be used by the xCELLigence System to investigate neurotoxicity and neuroprotection *in vitro*.

Our findings show that neurotoxic effects can be continuously monitored in HT-22 cells and primary cortical neurons using the xCELLigence System. Importantly, glutamate treatment in HT-22 cells was found to cause a rapid onset and progression of neuronal cell death, making it difficult to identify the ideal time point for functional endpoint assays. xCELLigence recording of impedance-based CI values clearly revealed neurotoxic effects in real-time, allowing pinpointing when to perform biochemical endpoint assays in downstream applications addressing questions in proteomics and genomics. In addition, neuroprotective effects could be clearly demonstrated using the Bid inhibitor BI-6C9, highlighting the versatility of the xCELLigence System in neuroscience for performing inhibitor studies. Furthermore, the xCELLigence System allowed us to monitor cell culture conditions of primary cortical neurons throughout the entire time of the experiment, including plating, precultivation, removal of glial cells by CAF treatment, compound administration, and cell death profiling.

In conclusion, the xCELLigence System allowed us to continuously monitor cell culture conditions of the well-established neuronal cell culture models HT-22 cells and primary cortical neurons. We found that this new method adds significant information to the data gained from conventional endpoint assays, while also reducing the time invested in the experiment itself. Furthermore, the xCELLigence System allowed us to determine the optimal time point for additional endpoint analyses, in order to investigate the molecular mechanisms of neuronal cell death in greater detail. The xCELLigence System is thus a very precise and convenient tool for the *in vitro* investigation of neuronal cell death and neuroprotection.

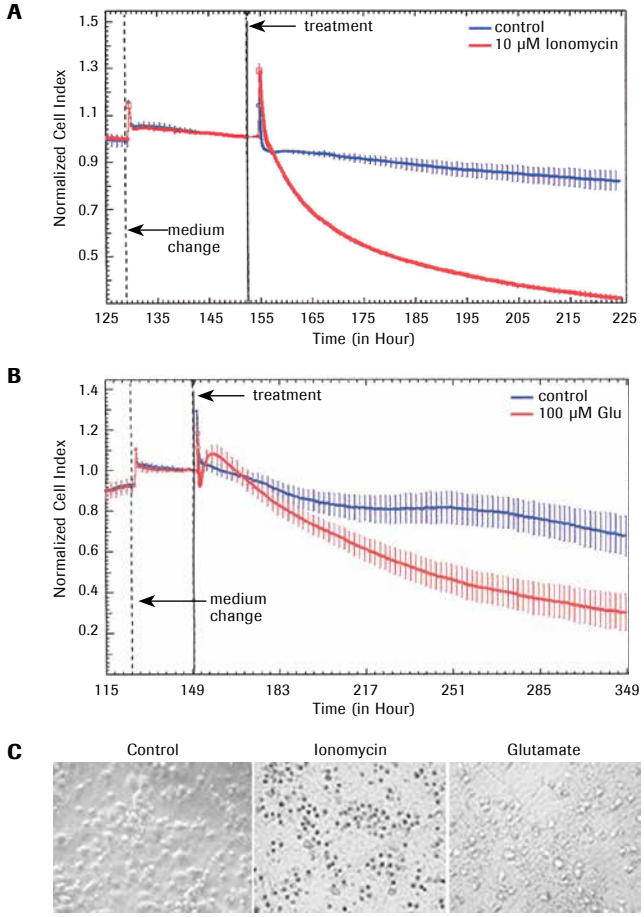

Figure 4: Detection of cell death in primary cortical neurons. (**A**) Primary cortical neurons were treated with ionomycin on day six, and continuously monitored for another 3 days using the xCELLigence System. (**B**) Primary cortical neurons were exposed to glutamate in addition to growth factor withdrawal after six days in culture, and monitored for another 6-8 days. (**C**) The effects of compound treatment on the morphology of primary cortical neurons were documented using light microscopy.

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Key Words:
Neuronal cell death, xCELLigence System, primary cortical neurons, HT-22 cells, cellular impedance, real-time measurements

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