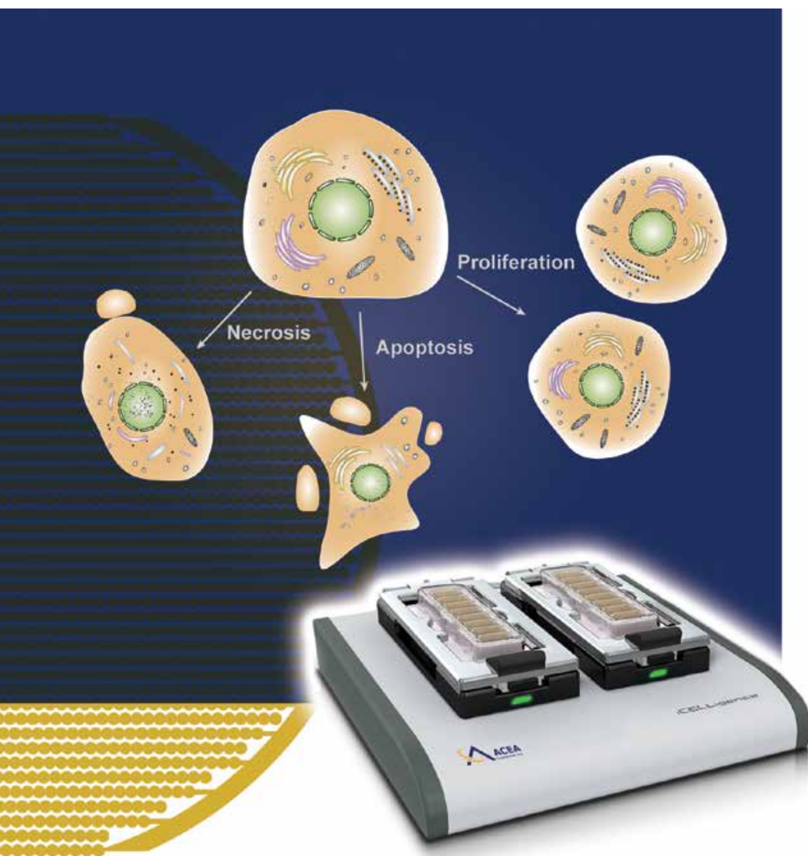
ACEA Biosciences, Inc.

iCELLigence System Application Note No. 2 — December 2012

Quantitative Assessment of Cell Quality, Viability and Proliferation



Introduction

In vitro assessment of cell viability and proliferation is critical to many aspects of basic and biomedical research. A mechanistic understanding of the molecular and biochemical pathways regulating these processes is essential to the development of agents which can modulate pathways leading to the maintenance of cell viability and proliferation. Viability assays are also frequently used to determine the undesired cytotoxic effect of lead compounds on various cell types derived from different organs and tissues of origin.

Traditional viability assays include the Trypan Blue dye exclusion assay to test cell membrane integrity and colony formation assays to test continuous growth of cells. The exclusion of Trypan Blue or propidium iodide dyes is often used as an indication of membrane integrity of living cells, as the dyes cross the compromised cell membrane and stain cellular targets or structures in dead cells. The colony formation assay can document any effect on cell proliferation, including long-term cell cycle arrest, or other anti-proliferative effects. More recently, homogeneous assays for cell viability have been developed which are amenable to automation for high throughput screening (HTS). Viability markers, usually a biochemical event that occurs only in living cells, are often used for HTS assays. Tetrazolium salts, such as MTT, XTT and WST-1, are converted to a formazan product by active mitochondria, which can then be measured as a colorimetric readout. Similarly, non-fluorescent resazurin, e.g. alamarBlue, can be converted to fluorescent resorufin by active mitochondria, which can then be measured by fluorescence. Cellular ATP content is also used as a cellular marker for viability. These assays are conducted at the end of the study to determine the difference in cellular viability between control and toxin-treated samples.

Introduction

While endpoint viability assays can provide an easily discernible parameter for scoring the extent of cell viability, proliferation, or toxicity in response to certain treatments, they are limited in providing in-depth mechanistic information. It is important to note that the extent to which protein targets are modulated by drugs or small molecules is dependent on a number of factors, including expression levels of the target, the effective concentration of the compound, and more importantly, the time needed for the compound to perturb the target. Some compounds can be very fast acting, such as agonists of certain GPCRS or ion channels, which lead to immediate biochemical and cellular changes inside the cell, while others such as cell cycle modulators can have a more prolonged effect. One of the limitations of current end point viability measurements is that typically a single time point after compound addition is chosen to assess the effect of the compound, and therefore the conclusion regarding the mechanism of action of the compound will be based upon the time point at which the samples are processed. It can be envisioned that if the effect of the compound is manifested at any other time, or if a compound has multiple and kinetically distinct effects, these mechanisms can be easily overlooked.

To address this limitation, ACEA Biosciences has developed the iCELLigence System (**Figure 1**) which allows for label-free, dynamic monitoring of cellular events (including proliferation and toxicity) in real-time using cellular impedance as a readout. The key feature of this approach is that the impedance readout is non-invasive, and therefore cellular responses to biologically active compounds and other agents can be continually sampled inside the well throughout the length of the assay (1-2). The kinetic profile offers several distinct advantages: it provides important and quantitative information regarding the quality of the cells between different wells within the same plate and between different experiments; it allows the user to make informed decisions about the timing of certain manipulations such as compound addition; and it provides information regarding the temporal effects of compounds or siRNAs with the cells and can reveal mechanism of action (2-7).

In this application note we will highlight some of the features of the iCELLigence System with regards to assessment of cell quality as well as proliferation and toxicity. Since there are more than 300 publications on the Real-Time Cell Analysis technology, where appropriate, we have provided references to peer reviewed publications.

Figure 1. The iCELLigence System. The station houses two E-Plate L8 devices and transfers data via Wi-Fi to the iCELLigence Control Unit for analysis. The E-Plate L8 devices contain 8 wells each with an electrode grid area at the bottom for impedance-based measurements and a view area for microscopic analysis. The Control Unit of the iCELLigence System controls the experimental setup, displays the data in real time, and serves as a platform to perform data analysis.

Materials and Methods

Cells.

A549 and HeLa cells were obtained from ATCC and maintained in a 37°C incubator with 5% CO_2 saturation. A549 cells were maintained in F12K media (ATCC) containing 10% FBS (Hyclone), 1% penicillin-streptomycin, and HeLa cells were maintained in EMEM media (ATCC) containing 10% FBS, 1% penicillin-streptomycin.

Cell Proliferation Assays.

The indicated number of cells per well was seeded into 480μL of media in E-Plate L8 devices. The attachment, spreading, and proliferation of the cells was monitored every 30 minutes using the iCELLigence System for 48-72 hours, depending on the experiment. Cell sensor impedance was expressed as an arbitrary unit called Cell Index. The Cell Index at each time point is defined as (Rn-Rb)/4.6, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the media alone.

Drug Treatment and Cytotoxicity Assessment.

The proliferation pattern of each cell type was used to determine its optimal cell concentration. The indicated number of cells per well was seeded into 480μL of media in an E-Plate L8. The attachment, spreading, and proliferation of the cells were monitored every 30 minutes using the iCELLigence System. Approximately 24 hours after seeding, when the cells were in the logarithmic growth phase, the cells were treated with 20μL of the indicated compounds dissolved in cell culture media, with a corresponding vehicle control.

Apoptosis Assay and Microscopy.

At desired time points after the addition of compounds, an apoptosis assay was conducted using the Cell Death Detection ELISAPLUS kit following manufacturer recommendations (Roche Applied Sciences). This ELISA-based kit detects histone-complexed DNA fragments, which are indicative of apoptotic cells. Microscopy was conducted at the end of the proliferation and cytotoxicity assay. The media was immediately aspirated from the E-Plate L8 wells and cells in the view area of the E-Plate L8 were photographed under a brightfield microscope using an attached digital camera.

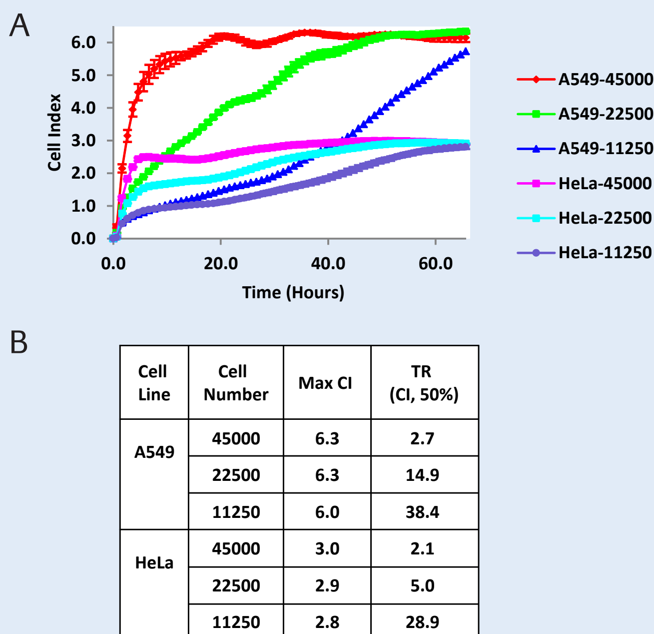
Results and Discussion

Monitoring Dynamic Cell Proliferation in Real-Time Using Impedance Technology

To assess dynamic cell proliferation, human non-small cell lung cancer (NSCL) A549 cells and human cervical cancer HeLa cells were seeded at various densities in E-Plate L8 devices. The cells were monitored every 60 minutes for the indicated period of time (**Figure 2**). Each cell type has its own characteristic kinetic trace (**Figure 2A**), based on the number of cells seeded, their overall size, morphology of the cells, and the degree to which the cells interact with the sensor surface. Also, each of the cell lines can be characterized by its unique adhesion and spreading kinetics, as well as the time at which it enters the log growth and stationary phases. As shown in the summary table (**Figure 2B**), the maximal interaction between each cell line and the sensor surface is represented as CI max. It is different between the two cell lines, yet consistent for the same cell line between different sending densities. However, the time parameter needed to reach mid logarithmic (50% CI max) is different among different seeding densities. Therefore, this parameter offer an excellent internal quality control, as well as a way to standardize and validate stock cultures during different phases of the manufacturing process. In addition, this information can also be utilized to optimize cellular assays as to when to conduct cellular and molecular assays based on cell condition.

Dynamic Monitoring of Cytotoxic Compounds

In order to assess whether the impedance-based system is suitable for analyzing cytotoxicity, it was used to monitor the interaction of cancer cells with cytotoxic agents that have different cell-killing kinetics and mechanisms of action. HeLa cells were treated with two apoptosis inducing agents, MG-132 and 5-FU (**Figure 3**). MG-132 mediated a maximal decrease in Cell Index values during the first 10 hours after compound treatment, while 5-FU required at least 48 hours



Cell Line	Cell Number	Max CI	TR (CI, 50%)
A549	45000	6.3	2.7
	22500	6.3	14.9
	11250	6.0	38.4
HeLa	45000	3.0	2.1
	22500	2.9	5.0
	11250	2.8	28.9

Figure 2. Dynamic monitoring of cell proliferation using the iCELLigence System. (A) A549 and HeLa cells were seeded at a density of 45000, 22500, and 11250 cells per well in duplicates on an E-Plate L8 device. The adhesion, spreading, and proliferation of the cells were dynamically monitored every 60 minutes using the iCELLigence System. (B) Summary table for the proliferation parameters generated with the iCELLigence software, indicating the maximal interaction of each cell line with the sensor surface when culture reaches confluence (Max CI), and the time required to enter mid logarithmic (TR (CI, 50%)) phase.

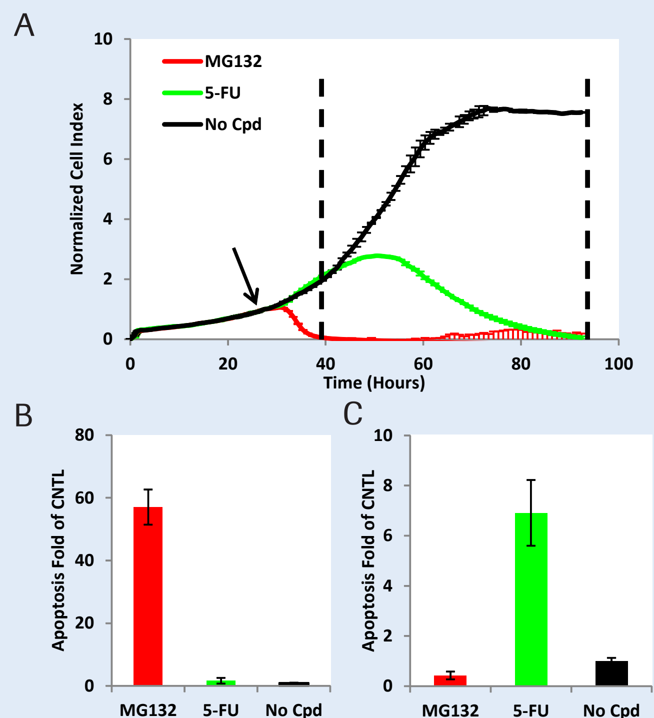
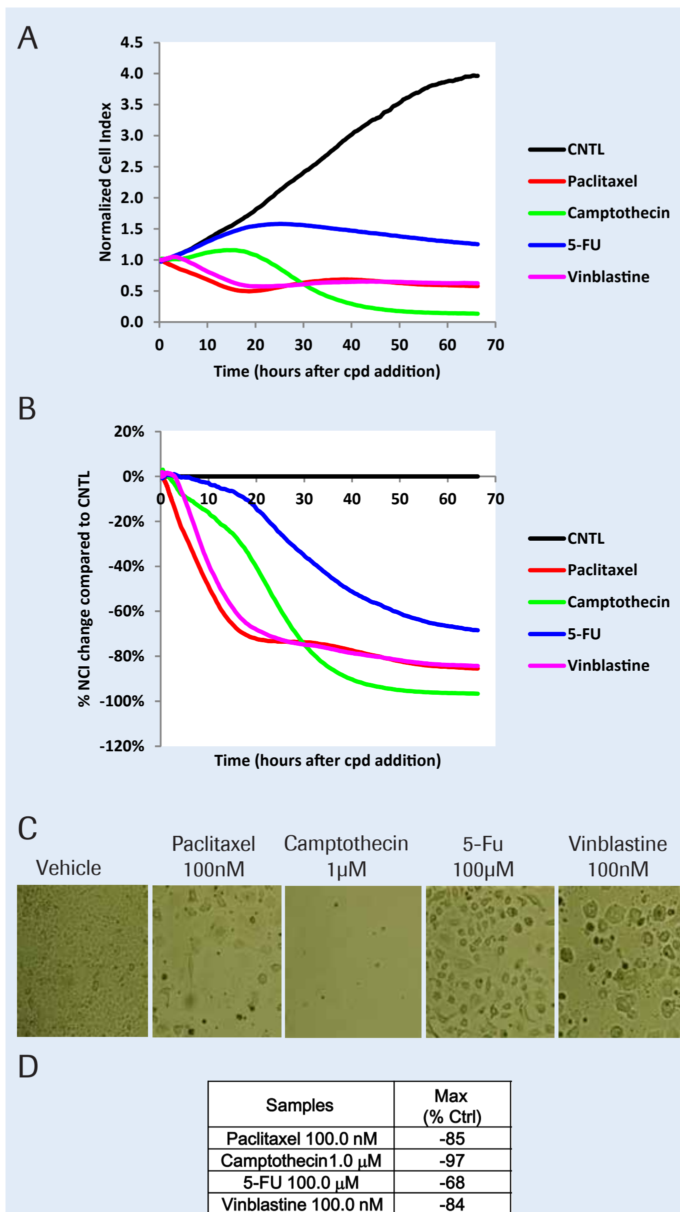


Figure 3. Dynamic monitoring of compounds mediating cytotoxicity and apoptosis. (A) HeLa cells were treated with MG132 (1 µM) and 5-FU (100 µM), and Cell Index was continuously monitored for 64 hours. At 16 hours (B) and 64 hours (C) post compound addition, apoptosis assays were conducted with the cells harvested from the E-Plate L8, fold increase in apoptosis induction was shown for both compounds, and compared to the untreated control.

(**Figure 3A**). To determine whether the kinetic information can be used to select optimal time points for performing functional endpoint assays, apoptosis assays were performed at two different time points: 16 hours post compound addition (**Figure 3B**), when the maximal CI changes were observed for MG-132, and 64 hours post compound addition (**Figure 3C**) when maximal effects were observed for 5-FU. For MG-132, a pronounced apoptotic induction was observed at 16 hours, but not at 64 hours. This finding confirms the transient nature of apoptosis, underscoring the importance of performing the assay at an optimal time point to best capture short-lived (transient) cellular responses. For 5-FU, induction of apoptosis was observed at 64 hours when maximal CI changes were observed, but not at 16 hours, when the CI values for treated cells started to diverge from the control samples. From this data, it is clear that the apoptosis assays should be carried out when the CI profile approaches its lowest value, indicating the lowest level of cell viability. Assaying for apoptosis too early or too late may not provide the best window for assessing the effect of each of the different compounds tested.

It has previously been shown that compounds with similar mechanisms of action often generate similar time-dependent cell response profiles (TCRPs). Once defined, such "signature" TCRPs may be very specific and can be used to identify compounds with desired functional properties and to screen for compounds mediating desired activities such as anti-mitotic effects (2,5). A549 cells were treated with cytotoxic compounds with different mechanisms of action, and monitored with the iCELLigence System (**Figure 4**). Each agent generated characteristic kinetic patterns that corresponded to the mechanism of drug action (**Figure 4A**). Both anti-mitotic compounds, paclitaxel and vinblastine, mediated the signature anti-mitotic response profile; the DNA damaging agent 5-FU mediated a cytostatic pattern, while the topoisomerase inhibitor camptothecin mediated pronounced cytotoxicity. Cell Index values are consistent with bright-field imaging to assess cell status in the E-Plate View area (**Figure 4C**). Furthermore, the growth inhibition kinetics of different compounds may be quantitated as a percentage of the control curves. The time required for different compounds to have similar absolute cytotoxic effects are clearly different (**Figure 4B & 4D**). This provides insightful information that cannot be obtained from a single end-point assay.

In summary, impedance-based technology allows label-free and dynamic monitoring of cell proliferation, viability, and cytotoxicity. This system offers distinct and important advantages over traditional end point assays. It provides a built-in internal quality control to assure consistency and reproducibility between the different assays. By recording the entire course of drug interaction with particular cells, dynamic monitoring allows the user to gain a better understanding of the temporal effects on the cells, therefore pinpointing optimal time points for end-point molecular and cellular assays. Finally, since each compound or drug has its own characteristic profile with respect to its interaction with target cells, impedance- based technology may be useful for determining the mechanism of action of drugs with unknown targets.



Samples	Max (% Ctrl)
Paclitaxel 100.0 nM	-85
Camptothecin1.0 µM	-97
5-FU 100.0 µM	-68
Vinblastine 100.0 nM	-84

Figure 4. Dynamic monitoring of cytotoxic compounds displaying different mechanisms of action. A549 cells were treated with Paclitaxel (100 nM), Camptothecin (1 µM), 5-Fluorouracil (100 µM), Vinblastin (100 nM), and cellular responses were monitored for ~ 3 days post compound addition. (A+B) Curves for Normalized Cell Index (A) and percentage of NCI changes compared to control sample (B) after compound addition are shown; (C) At the end of the experiment, the culture media were removed from the well, and the cells in the view area were imaged using a bright-field microscope. D) Summary table showing the maximal % inhibition for each compound.

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Ordering Information

Product	Cat. No.	Pack Size
RTCA iCELLigence System Bundle	**00380601000**	**1 Instrument and 1 Control Unit**
RTCA iCELLigence Instrument	00380600970	1 Instrument
RTCA iCELLigence Control Unit	00380601020	1 Instrument
E-Plate L8	00300600840 00300600850	1 × 6 Plates 6 × 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)

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