Quantification of Cytotoxicity using the IncuCyte® Cytotoxicity Assay

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Introduction

Assays designed to measure cytotoxicity *in vitro* are used to predict tissue-specific toxicity or to identify and classify leads for anti-cancer therapies. Multiplexed, high-throughput screening (HTS) *in vitro* cytotoxicity assays measuring a variety of different readouts are being employed to assess the cytotoxicity of compounds in early drug development [1]. Commonly used cytotoxicity assays evaluate a range of end-point parameters, such as the release of lactate dehydrogenase (LDH) and glutathione (GSH) following membrane rupture, generation of reactive oxygen species (ROS), cell proliferation, and disruption of mitochondrial trans-membrane potential. Critical factors contributing to the predictive nature of these assays include compound concentration, and more importantly, the time allowed for the compound to elicit an effect [2]. Although these multiplexed assays are able to simultaneously measure multiple indicators of *in vitro* cytotoxicity, they typically assess a single time point and are unable to assess the biological activity over time.

The cellular response to cytotoxic exposure is controlled by complex biochemical pathways, such as necrosis or apoptosis, which results in cell death. In apoptosis, morphological changes include pseudopodia retraction, reduction of cellular volume (pyknosis), nuclear fragmentation (karyorrhexis) and eventually loss of plasma membrane integrity (Figure 1) [3]. Morphological changes that characterize necrosis include cytoplasmic swelling and early rupture of plasma membrane [4]. Compounds that have cytotoxic effects often compromise cell membrane integrity regardless of the pathway.

Methods of measuring cell membrane integrity include the use of propidium iodide and other vital dyes for use in either flow cytometry protocols or fluorescence microscopy [5]. In order to kinetically measure cell membrane integrity, while simultaneously monitoring associated morphological changes, we optimized IncuCyte CytoTox live-cell analysis reagents for use in the IncuCyte® live-cell analysis system. IncuCyte Cytotox reagents are cell impermeant cyanine dimer

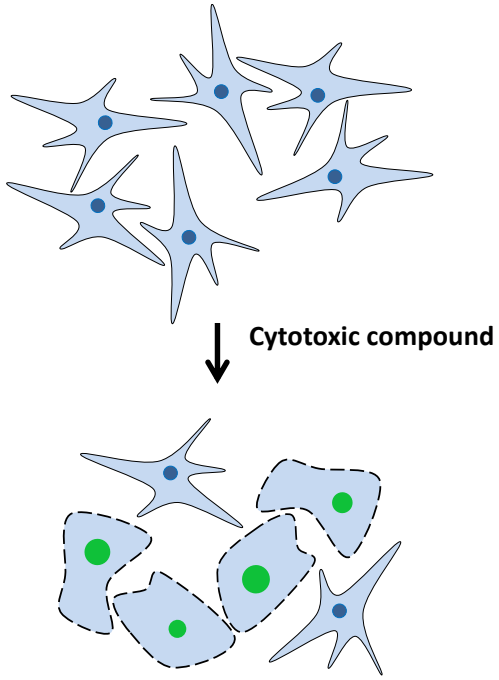


Figure 1. Nuclear staining indicates loss of membrane integrity, a hallmark of cell death. The cell impermeable DNA stain, CytoTox green, stains cell nuclei only when cells have lost membrane integrity following treatment with a cytotoxic compound. Hallmarks of necrotic cell death include cytoplasmic swelling and loss of membrane integrity. Viable cells remain unstained, and their growth unperturbed in the presence of CytoTox green.

nucleic acid stains that bind to dsDNA [5]. When added to the culture medium, CytoTox reagents fluorescently stain the nuclear DNA of cells that have lost plasma membrane integrity. In addition, in this application note, we illustrate how to multiplex the IncuCyte Cytotoxicity Assay with Essen's novel lentivirus based IncuCyte® NucLight reagents that incorporate a red nuclear label. This allows for measurements of proliferation in addition to measurements of cytotoxicity. Additionally, we provide evidence that IncuCyte CytoTox reagents can be used as a live cell reagent that can be added directly to cells in vitro in order enable the kinetic detection of cytotoxicity in a 96-well format. This no-wash, mix-and-read cytotoxicity assay is quantified using live-cell, time-lapse fluorescent images and the IncuCyte fluorescent object counting algorithm. In combination, NucLight reagents and cell lines, when used in conjunction with cell impermeant DNA dyes and

the IncuCyte system, provide the ability to simultaneously measure proliferation and cytotoxicity in a single well. Moreover, phase-contrast images can be used to qualitatively monitor associated morphological changes in the same cells over the same time course.

Approach and Methods

Cell Culture

All cell culture reagents were obtained from Invitrogen unless otherwise noted. Cells (MDA-MB-231, HT-1080, MCF-7 and HeLa) were grown to confluence in either 25 cm^2 or 75 cm^2 tissue culture treated flasks prior to initiation of assays. MDA-MB-231, MCF-7 and HeLa cells were cultured in F12-K supplemented with Pen-Strep, 10% FBS, and 2 mM GlutaMAX. HT-1080 cells were cultured in DMEM supplemented with Pen-Strep, 10% FBS, non-essential amino acids, sodium pyruvate, and 2 mM GlutaMAX.

Stable populations of NucLight Red cells were made by transducing parent cell lines with Essen's IncuCyte NucLight Red (Cat.# 4476, Lent, Ef1α , puromycin) reagent at an MOI of 3 (TU/mL) in the presence of 8 µg/mL polybrene. Populations expressing red fluorescent protein restricted to the nucleus were selected for in complete medium containing 1 µg/mL puromycin for 3-5 days, and then maintained in complete medium containing 0.5 µg/mL puromycin.

Assay Procedure

Prior to beginning the assay, cells were seeded in a 96-well plate at either 2500 or 5000 cells/well and cultured overnight. Staurosporine (SSP), Camptothecin (CMP) and Cycloheximide (CHX) were serially diluted with growth medium containing IncuCyte CytoTox Green at a final concentrations of 0.1 µM, respectively, in complete media, as described above. The concentrations of CytoTox reagent did not affect proliferation or cell morphology of the cell types used in this study relative to identical cells cultured in complete medium (data not shown). Cells were placed in an IncuCyte live-cell analysis system with a 10X objective in a standard cell culture incubator at 37˚C and 6% CO_2. Two images per well were collected every 2-3 hours in both phase-contrast and fluorescence. The assay was considered complete when a maximal response was achieved as determined by image analysis. For assay endpoint, all DNA containing objects were labeled by treating the cells with Triton X-100 to permeabilize the cell membrane. Triton X-100 was diluted in PBS and added directly to the wells at a final concentration of 0.0625%. Cells were incubated at 37˚C for 0.5-1 hours to allow nuclear DNA staining by cell impermeant DNA dyes prior to endpoint imaging. Triton X-100 at 0.0625% did not affect adherence of the cell lines tested (data not shown).

IncuCyte Data Quantification and Analysis

Phase-contrast and green fluorescent images were collected to detect morphological cell changes and plasma membrane permeability (via CytoTox Green DNA staining), indicating *in vitro* cytotoxicity. The integrated object counting algorithm was used to isolate the fluorescent nuclear signal from background. Specifically, images were segmented in order to identify individual objects, counted, and reported on a per-area (mm^2) basis for each time point. To correct for differential proliferation of cells, the total number of DNA containing objects was counted at the final time point using Triton X-100 to permeabilize the cells, allowing CytoTox Green to stain the nuclear DNA. This number was used to calculate the "cytotoxic index", defined as the number of CytoTox Green positive objects divided by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment). In addition, red fluorescent images were acquired to measure kinetic cell proliferation via nuclear counts. Specifically, images were segmented in order to identify individual objects, counted, and reported on a per-area (mm2) basis for each time point.

Results and Discussion

Quantitative Measurement of Cytotoxicity Using IncuCyte CytoTox Green

Staurosporine (SSP) and Camptothecin (CMP) are compounds that are known to cause cell death due to cytotoxicity. SSP is a high affinity, non-selective, ATP-competitive kinase inhibitor and is classically used as a research tool to induce caspase-3 mediated apoptosis [6, 7]. CMP causes cell death by inhibition of the DNA enzyme, topoisomerase I (topo I), resulting in double strand breaks during S-phase and triggering the apoptotic program [8]. These two compounds were used to illustrate the ability of the cell impermeant DNA dye based CellPlayer 96-Well Cytotoxicity Assay to measure cell death over-time using two different cell lines, HT-1080 (human tumor derived fibrosarcoma) and MDA-MB-231 (human tumor derived breast adenocarcinoma). In addition, we also

measured the response of these same two cell types to the protein synthesis inhibitor, Cycloheximide (CHX), a cytostatic compound which was predicted to inhibit cell proliferation while not affecting cell viability (Figure 2). A 7-point concentration curve of each compound clearly illustrated that in both cell types, SSP and CMP induced a concentration- dependent cytotoxic response. Specifically, we observed a statistical induction of cytotoxicity in MDA-MB-231 cells at 16 and 26 hours for SSP and CMP treatments,respectively (Figure 2A). In identically treated HT-1080 cells, we observed a more rapid induction of the cytotoxic responses correlating to 12 and 22 hours for SSP and CMP treatments, respectively, which illustrates a slight cell type dependent difference (Figure 2B). In contrast, no statistical induction of cytotoxicity was observed when either cell type was treated with any of the tested concentrations of CHX (Figure 2A, B).

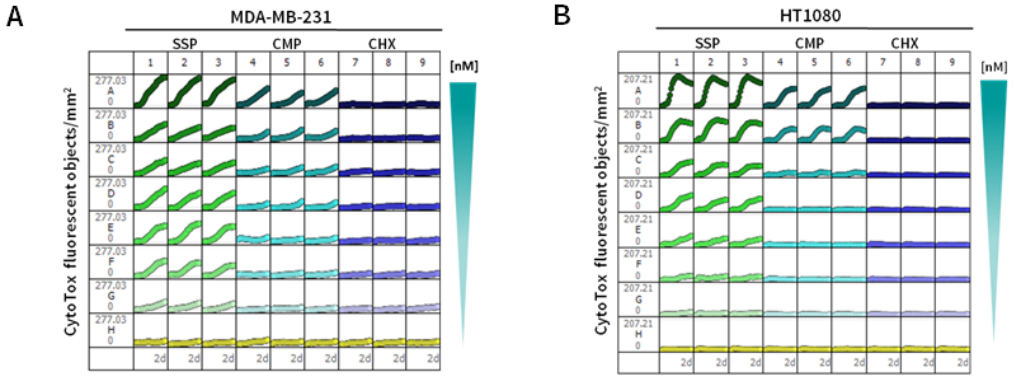


Figure 2. Discrimination of cytotoxic and cytostatic compounds. 96-well microplate graph showing the kinetic measurement of cell death as determined by CytoTox green staining in response to several concentrations of SSP, CMP, and CHX in MDA-MB-231 cells (A) and HT-1080 cells (B).

However, a clear concentration-dependent inhibition of cell proliferation was observed as measured by the NucLight Red fluorescent signal (Figure 3). Moreover, end point normalization, which corrects for differences in proliferation within treatment groups, revealed a concentration-dependent cytotoxic index for both SSP and CMP in MDA-MB-231 and HT-1080 cells, whereas no cytotoxic response was induced by treatment with CHX (Figure 4).

IncuCyte acquired phase-contrast and fluorescent images of HT 1080 cells, under all three compound treatments, confirmed fluorescent object count data as illustrated in Figure 5 (similar results were observed with MDA-MB-231 cells). These data show the potential of this kinetic and morphological approach to the screening, prioritization, and classification of compounds in drug discovery.

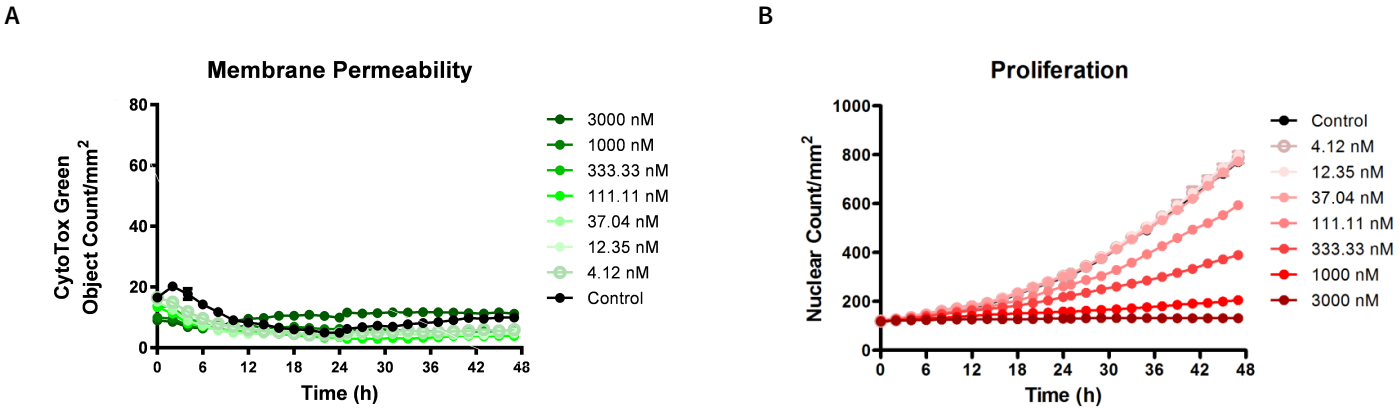


Figure 3. Cytostatic effect of cycloheximide (CHX). NucLight-Red HT-1080 cells were treated with several concentrations of cycloheximide in the presence of CytoTox green. Graphs illustrate no induction of cytotoxicity as measured by CytoTox green staining (A) however, inhibition of cell proliferation (B) as measured by fluorescent nuclear counts is observed. Cell morphology did not significantly differ from untreated cells as illustrated in Figure 5. Each data point represents the mean ± SE in N=3 wells.

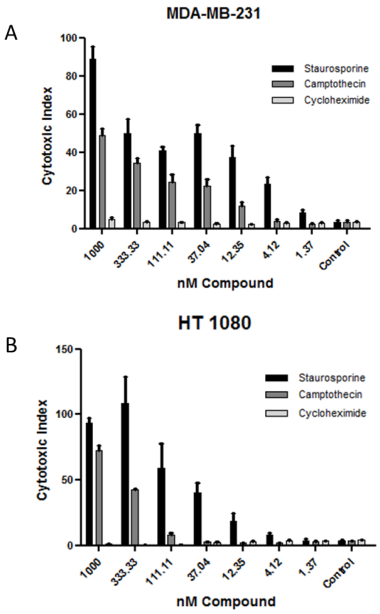


Figure 4. End point normalization of cytotoxic and cytostatic compounds. At the 72-hour end point, Triton X-100 at a final concentration of 0.0625% was added to allow nuclear dsDNA staining by CytoTox green of all cells present/well. The cytotoxic index was calculated by dividing the number of CytoTox green fluorescent objects by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment).

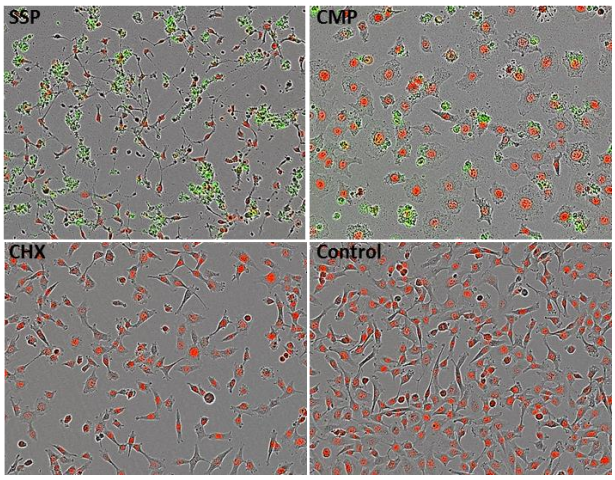


Figure 5. Morphological images. Phase-contrast and fluorescent images of NucLight HT-1080 showing morphological changes in response to SSP, CMP and CHX.

Intra- and Inter- Plate Reproducibility Measurements Highlight the Utility of the IncuCyte Cytotoxicity Assay.

In order to evaluate the accuracy and reproducibility of the IncuCyte Cytotoxicity Assay, we performed a series of experiments using MDA-MB-231 and HT-1080 cell lines and two known cytotoxic compounds (SSP and CMP).

To assess intra-assay reproducibility, we seeded each well of a 96-well plate with 5,000 HT-1080 or MDA-MB-231 cells. Each row of cells was treated with 2-fold decreasing concentrations of CMP (2000 nM to 62.5 nM; N=12 wells per condition) in the presence of CytoTox green and kinetic measurements of fluorescent objects were analyzed. As illustrated in the microplate graph view in Figure 7A, we obtained a highly reproducible kinetic measure of cell viability. In addition, using end point data, we were able to demonstrate an inverse relationship between total DNA object count and cytotoxic index (Figure 7B, C) in both cell lines analyzed.

We calculated assay Z' factors of 0.82 (HT-1080) and 0.64 (MD-MB-231), indicating that this assayplatform is amenable to screening protocols (Figure 8A, B). In addition, using the end point cytotoxic index from the HT-1080 data, we were able to calculate remarkably consistent pEC50 values from each column of the microplate with a total geometric mean of 198 nM (Figure 8C).

In order to determine inter-assay reproducibility and accuracy, HT-1080 cells were seeded in two 96-well plates at a density of 5,000 cells/well. Random wells of eachplate were spiked with independently prepared stocks of

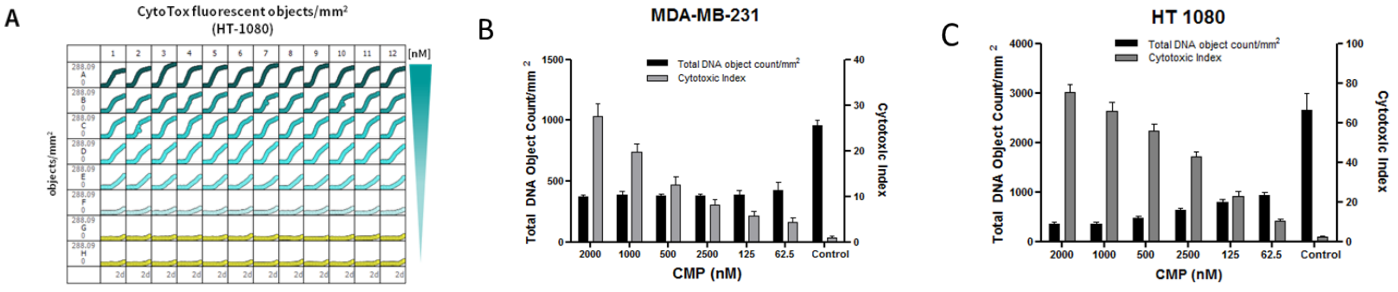


Figure 7. Intra-assay reproducibility of HT-1080 and MDA-MB-231 cells in response to CMP. 96-well microplate graph showing reproducibility of concentration response to CMP (A). After the 48-hour end point, the cytotoxic index was calculated by dividing the number of CytoTox green fluorescent objects by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment) (B and C).

CMP at 1000, 500, 200 and 50 nM (n=6 per concentration), as illustrated in Figure 9A. The data from both plates were then plotted on different axes and analyzed using linear regression (Figure 9B). The resulting R^2 value of 0.9588 demonstrates a strong correlation between identically treated wells on separate plates. Additionally, the Z' factors of 0.72 and 0.62 are again indicative of a high quality assay (Figure 9C).

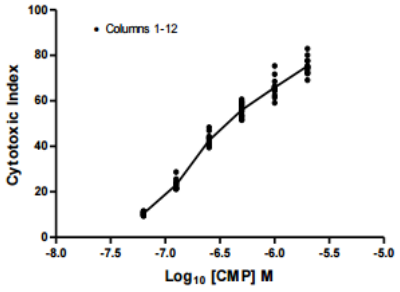
A

[CMP]	Cytotoxic Index (Mean)	SD	
2000 nm	75.280	3.805	z'=0.82
1000 nm	65.895	4.397	
500 nm	55.943	3.293	
250 nm	42.595	2.846	
125 nm	23.063	2.265	
62.5 nm	10.441	0.773	
Control	2.641	0.657	

B

[CMP]	Cytotoxic Index (Mean)	SD	
2000 nm	27.443	2.941	z'=0.64
1000 nm	19.829	1.768	
500 nm	12.636	1.826	
250 nm	8.080	1.234	
125 nm	5.690	1.102	
62.5 nm	4.338	1.078	
Control	0.984	0.264	

C

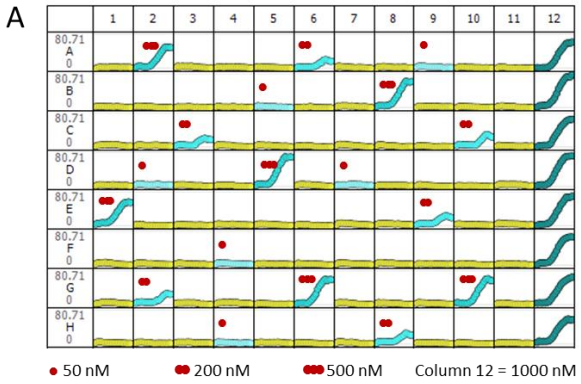


Column	pEC50
1	7.078
2	6.681
3	6.578
4	6.741
5	6.61
6	6.747
7	6.738
8	6.817
9	6.594
10	6.791
11	6.621
12	6.651
MEAN pEC50	6.721
MEAN 95% CI	6.501 to 6.933

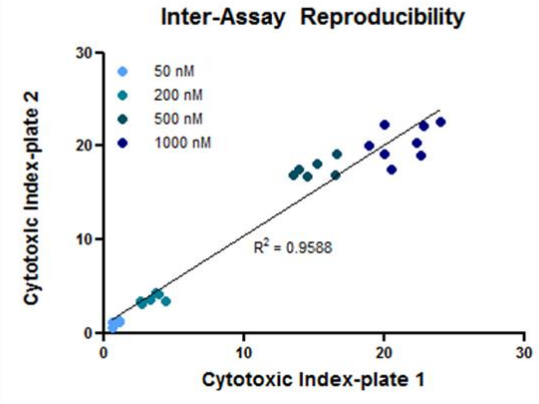
Geometric Mean (antilog converted)	
EC50	190.4 nM
95% CI	116.7 to 315.5 nM

Figure 8. Statistical analysis of intra-assay reproducibility. Calculated cytotoxic index of HT-1080 (A) and MD-MB-231 (B) cells to decreasing concentrations of CMP. (C) EC50 values determined from HT1080 plate described in Figure 7A.

A



B



C

Plate 1 : N = 6 per treatment

CMP	Cytotoxic Index (Mean)	SD	
50 nM	1.09	0.238	z' = 0.72
200 nM	3.65	0.514	
500 nM	17.57	0.936	
1000 nM	20.37	1.829	

Plate 2 : N = 6 per treatment

CMP	Cytotoxic Index (Mean)	SD	
50 nM	0.84	0.224	z' = 0.62
200 nM	3.48	0.703	
500 nM	15.06	1.309	
1000 nM	21.45	1.772	

Figure 9. Inter-assay reproducibility of HT-1080 response to CMP. (A) A 96-well microplate graph showing reproducibility of singe-well responses to various concentrations of CMP on HT-1080 cells. (B) Replicate plates of HT-1080 were spiked with identical concentrations of CMP and results were graphed to show correlation. (C) Statistical measurements from the same plates showing Z' factors exceeding 0.60.

Using NucLight Cell Lines or Reagents with CytoTox Reagents to Differentiate Cytotoxic and Cytostatic Treatments

Using the IncuCyte system, we were able to analyze both cell proliferation and cell permeability to create a multiplexed assay capable of measuring cytotoxicity in addition to cell proliferation. HT-1080 NucLight Red cells were seeded at 5,000 cells/well and treated with serially diluted concentrations of SSP, Camptothecin (CMP), or CHX in the presence of 0.1 µM CytoTox green (all data not shown). The IncuCyte basic analyzer was used to mask the green fluorescent nuclear signal to quantitate cell death (CytoTox green positive cells) as well as the red fluorescent nuclear signal to monitor cell proliferation (NucLight Red). Kinetic dose response curves for both CytoTox positive events as well as nuclear counts of NucLight Red HT-1080 cells were exported to GraphPad Prism. Statistical analysis of the area under the curve (AUC) was calculated for time points within the kinetic curves. Replicate AUC values, at peak response, were used to calculate EC_{50} and IC_{50} values. Figure 10 shows the inverse relationship between cell proliferation (nuclear count) and membrane permeability (CytoTox green positive objects) over time in the presence of SSP. The AUCs were then used to statistically determine at which concentration SSP exhibited purely cytostatic effects. The concentration at which the AUC of CytoTox green objects/mm^2 over time was different from the control group was termed cytotoxic and/or cytostatic. Concentrations where the AUC of CytoTox green objects/mm^2 over time was not different from that of the control, but the AUC of nuclear objects/mm^2 over time was significantly different from the control was termed purely cytostatic. These data show the potential of this kinetic and multi-parametric approach to the classification of compounds in drug discovery.

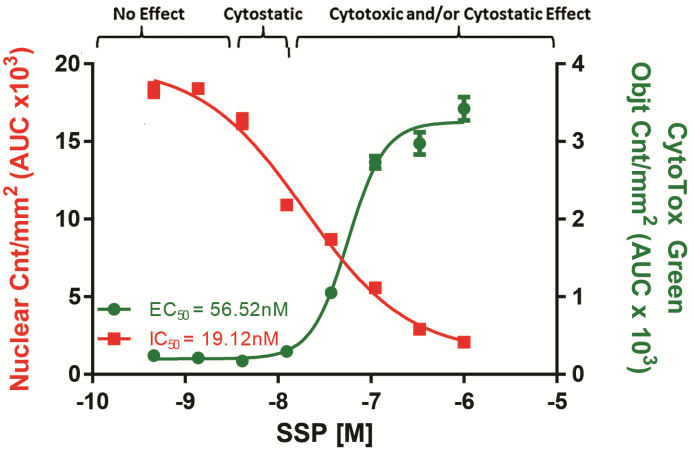


Figure 10. Inter-assay reproducibility of HT-1080 response to CMP. HT1080 cells were treated with varying concentrations SSP. Dual fluorescent images were used to calculate the area under the curve (AUC) of cell death (CytoTox green Object Cnt/mm^2) and cell proliferation (Nuclear Cnt/mm^2) over time. Average AUC values were then used to calculate EC_{50} and IC_{50} values, respectively. Dunnett's Multiple Comparison Test was used to compare the differences between AUCs at each concentration.

Conclusions

Using the IncuCyte live-cell analysis system in conjunction with IncuCyte Cytotox Reagents as a live cell, kinetic assay for the measurement of cytotoxicity has demonstrated quantitative and reproducible detection of cell permeability, a hallmark of cell death. This strategy also gives the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell viability. In addition, NucLight Red reagents or cells lines, when used in conjunction with IncuCyte CytoTox Reagents and the IncuCyte system, provide an additional parameter for measuring cytostatic (anti-proliferative) and cytotoxic events.

Key features demonstrated in the kinetic cytotoxicity assay are:

- Multiplexing: The IncuCyte live-cell analysis system allows for kinetic monitoring of both cell permeability (IncuCyte CytoTx Reagents) and cell proliferation (NucLight Red reagents or cell lines). Multi-parametric cytotoxicity, analysis of proliferation, cell permeability, along with kinetic readouts generates more reliable results and allows for differentiation between cytostatic and cytotoxic effects of treatments.
- Mix, read and monitor: Adding CytoTox green or red as a mix- and-read reagent directly to the cultured cells in complete growth media removes the need for fluid aspiration steps, thus eliminating cell disruption or loss of impaired cells.

- **Kinetic:** Kinetic monitoring of cells allows for the detection of both short-term and long-term alterations in cell viability in physiologically relevant conditions, eliminating the need for determining a universally suitable end point a priori. This feature allows for profiling cell-specific and time-dependent biological activity.
- **Automated data acquisition:** The IncuCyte's software and interface allows for automated data acquisition of phase contrast and fluorescent images that can be used to quantify the amount of cell death and/or proliferation in the culture as well as confirm the fluorescent images and validate the IncuCyte fluorescent object counting algorithm.
- **Track morphology:** The high-contrast phase images acquired using the IncuCyte Live-Cell Imaging System enables the user to qualitatively discriminate between cytotoxic and cytostatic (anti-proliferative) effects by visual inspection of the cells. Unlike many other cytotoxic screens, this assay allows the user to visibly verify assay metrics (other end point assays fail this quality control).

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