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Application Note

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Quantification of Cytotoxicity Using the Incucyte® Cytotoxicity Assay

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Introduction

Cytotoxicity is a general term that describes the detrimental effects of a substance or environmental changes on cell health. Exposure of cells to a cytotoxic stimulus may compromise metabolic activity, inhibit cell growth or ultimately, via complex biological pathways such as necrosis or apoptosis, result in cell death. Regardless of the mechanism, cytotoxic compounds often compromise cell membrane integrity, and once a cell irreversibly loses its membrane integrity, it is destined to die. In apoptosis, morphological changes include pseudopodia retraction, reduction of cellular volume (pyknosis), nuclear fragmentation (karyorrhexis) and eventually loss of plasma membrane integrity.[1] Morphological changes that characterize necrosis include cytoplasmic swelling and early rupture of plasma membrane.[2]

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) cytotoxicity assays measuring a variety of different readouts are being employed to assess the cytotoxicity of compounds in early drug development.[3] Using Incucyte® Cytotoxicity Assays, biocompatibility with Medical Devices at the early R&D Stage can be evaluated via cell-based cytotoxicity tests. Furthermore, commonly used cytotoxicity assays involve a range of end-point metabolic activity measurements, 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. Finally, several cytotoxicity assays involve measurement of membrane integrity, such as the use of propidium iodide and other vital dyes for use in either flow cytometry protocols or fluorescence microscopy.[4] 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.[5] Although these multiplexed assays can 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. To kinetically measure cell membrane integrity, live-cell analysis methods have been developed. However, optimized technology and reagents are required for flexible and accurate assessment. In this application note, we describe the utility of Incucyte® Cytotoxicity Assays, encompassing no-wash, mix-and-read reagents and integrated image-based analysis tools that enable kinetic quantification of cytotoxicity.

Assay Principle

The Incucyte® Cytotoxicity Assay utilizes the Incucyte® Cytotox Dyes in combination with the Incucyte® Live-Cell Analysis System to make real-time measurements of cell death, in response to pharmacological agents or environmental factors, based on cell membrane integrity. The Incucyte® Cytotox Dyes are highly sensitive nucleic acid dyes. Addition of the Incucyte® Cytotox Dye to normal healthy cells is non-perturbing to cell growth or morphology and yields little or no intrinsic fluorescent signal. Once cells become unhealthy, plasma membrane integrity diminishes, allowing entry of Incucyte® Cytotox Dye and yielding a 100- to 1,000-fold increase upon binding to deoxyribonucleic acid (DNA) (Figure 1). Dying cells are identified and quantified over time by the appearance of fluorescently labeled green, red, or near infrared (NIR) nuclei. With the Incucyte® integrated analysis software, fluorescent objects can automatically be quantified, and background fluorescence minimized. Additionally, Incucyte® HD phase-contrast images and movies provide validation of cell death based on morphology (e.g., loss of cytoskeleton structure, loss of motility).

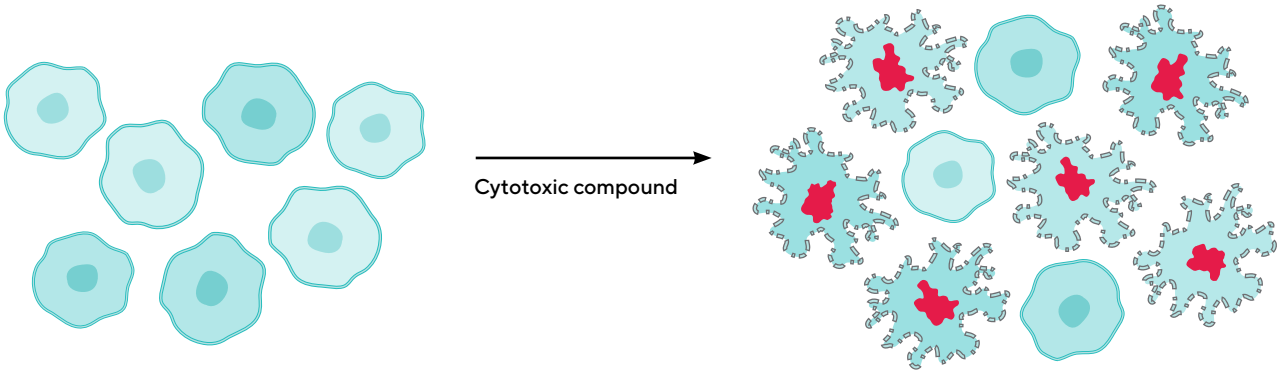


Figure 1
Schematic Demonstrating Assay Principle of Live-Cell Incucyte® Cytotoxicity Assay

Note. Nuclear staining indicates loss of membrane integrity, a hallmark of cell death. The cell impermeable DNA stain, Incucyte® Cytotox, 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 Incucyte® Cytotox Dyes.

Materials and Methods

The Incucyte® Cytotoxicity Assay can be performed using a simple mix-and-read protocol in a high-throughput format as demonstrated in Figure 2. This highly flexible assay can be used with your choice of cells, including adherent or non-adherent cells, and enables real-time quantification of treatment effects non-invasively. The pre-aliquoted Incucyte® Cytotox Dyes can be combined with the Incucyte® confluence metric, Incucyte® Nuclight Reagents for nuclear labeling, Incucyte® Annexin V Dyes, or Incucyte® Caspase-3/7 Dyes for multiplexed measurements of cytotoxicity alongside cell proliferation and apoptosis.

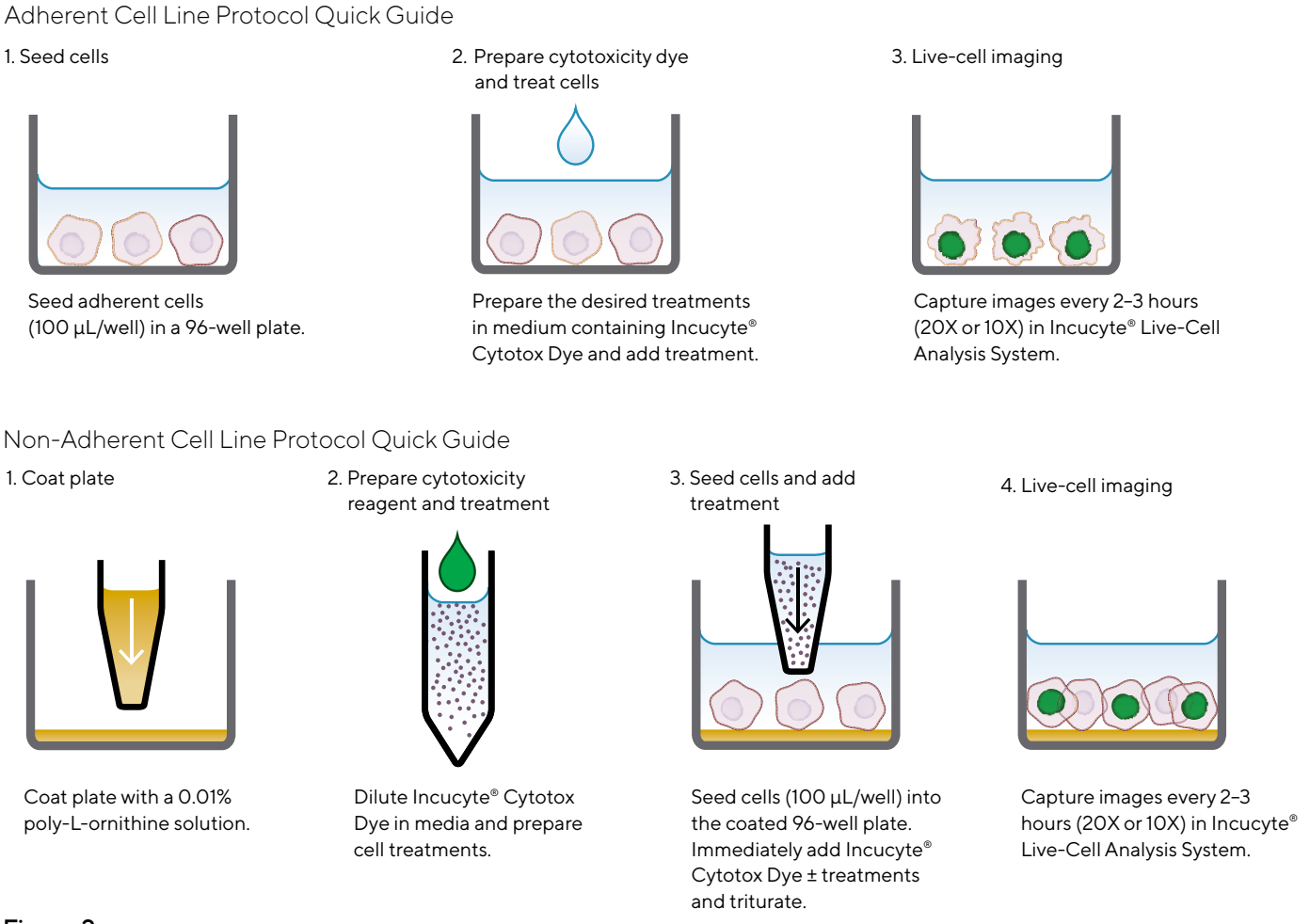


Figure 2

Quick Guide of Incucyte® Cytotoxicity Assay Protocols for Adherent or Non-Adherent Cells

Note. The simple protocols utilize Incucyte® Cytotox Dyes and the Incucyte® Live-Cell Analysis System for image-based fluorescent measurements of cytotoxicity.

Validation Data

Quantification of Cytotoxicity

The Incucyte® Live-Cell Analysis System, in combination with Incucyte® Cytotox Dyes, enable visualization and quantification of cytotoxicity. The integrated image-based analysis tools aid automatic segmentation of nuclear fluorescence over the entire assay time-course and minimizes the impact of background fluorescence. Figure 3 illustrates how the cytotoxic response of a non-adherent lymphoblastic leukemia cell line, Jurkat, to the anti-cancer drug Camptothecin (CMP) can be kinetically quantified. Jurkat cells were treated with 3 µM CMP in the presence of Incucyte® Cytotox NIR Dye. Shown are the acquired phase-contrast and blended fluorescence images (NIR pseudo-colored blue) and the fluorescence segmentation mask (red) used to quantify cytotoxicity following treatment. An increase in nucleic acid binding by Cytotox NIR Dye was observed over 24 h. Additionally, images allow for correlation of membrane integrity with morphological changes associated with cytotoxicity, as demonstrated by the observed characteristic 'halo' effect observed with Jurkat cell death.

This method can robustly be used to quantitatively study cytotoxicity in a high-throughput format. To validate this, we used the Incucyte® Cytotoxicity Assay to examine the cytotoxic response of HT-1080 fibrosarcoma cells to four different compounds. Cells were seeded and treated with 2-fold decreasing concentrations of each compound in

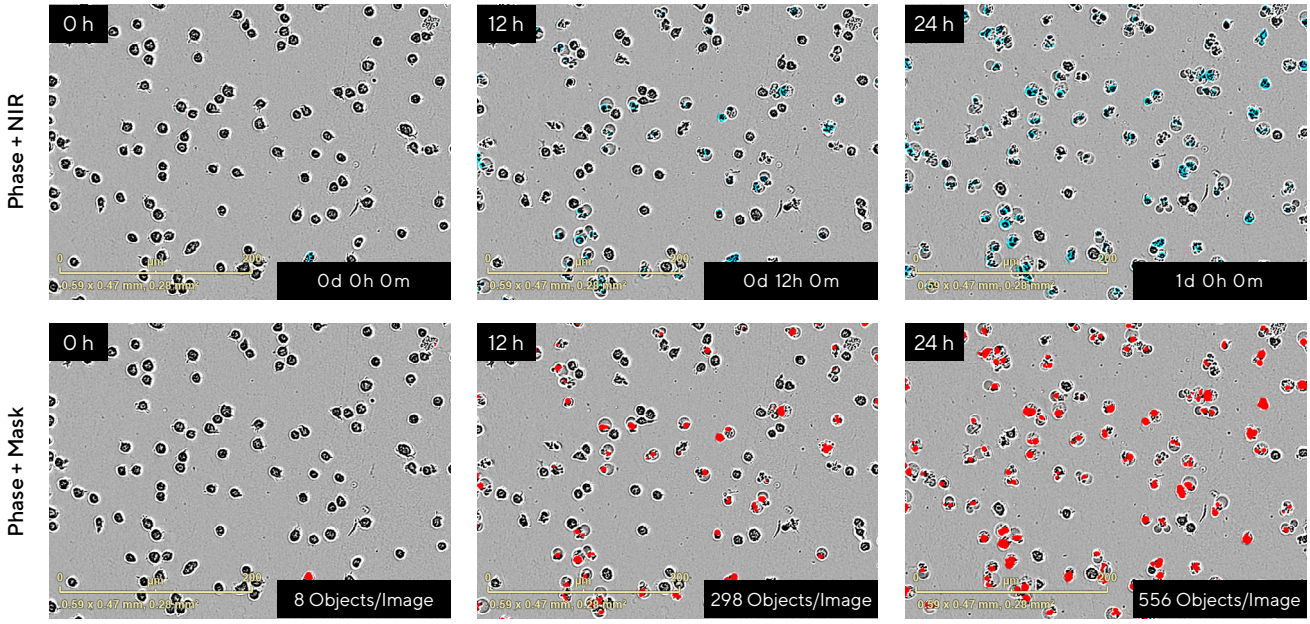


Figure 3

Real-Time Visualization and Quantification of Non-Adherent Jurkat Cell Death in Response to the Anti-Cancer Drug Camptothecin (CMP) Using the Incucyte® Live-Cell Analysis System

Note. Jurkat cells (20,000 cells/well) were treated with 3 µM CMP in the presence of Incucyte® Cytotox NIR Dye. Images were acquired every 2 h at 20X for the duration of the experiment. Representative phase-contrast and blended fluorescence images (NIR pseudo-colored blue; top row) and the segmentation mask generated using Incucyte® integrated image analysis tools (red; bottom row) are shown over time (0–24 h). Observe increase of nucleic acid binding by Incucyte® Cytotox NIR Dye as measured by NIR Object Count (8 at 0 h vs. 556 Objects/Image at 24 h). The fluorescent signal from the Incucyte® Cytotox Dyes can be correlated with morphological changes associated with cell death.

the presence of Incucyte® Cytotox Green Dye and measurements of fluorescent objects were analyzed using integrated software (Figure 4). The microplate view shows a kinetic change in fluorescence for all wells over 72 h, with varying profiles for the different treatments being observed (Figure 4A). Camptothecin (CMP) showed a strong cytotoxic effect at all concentrations tested, while a concentration-dependent effect was observed for Cisplatin (CIS), Staurosporine (SSP), and Nocodazole (NOC). Cisplatin, an alkylating agent, is an established chemotherapeutic used in the treatment of various cancers which cross-links with DNA, activating DNA damage pathways and ultimately resulting in apoptosis.[6,7] The kinetic cytotoxic response of HT-1080 cells to CIS treatment is shown and further quantification revealed a concentration-dependent effect at 48 h (Figure 4B). In control conditions, little to no increase in fluorescence was observed compared to Cisplatin (3.17 ± 0.83 green objects per image for vehicle vs. 214.93 ± 7.61 for 50 µM Cisplatin at 48 h). These data demonstrate the utility of the Incucyte® Cytotoxicity Assay in the visualization and quantification of cytotoxicity and how live-cell analysis is amenable to high-throughput pharmacological investigation.

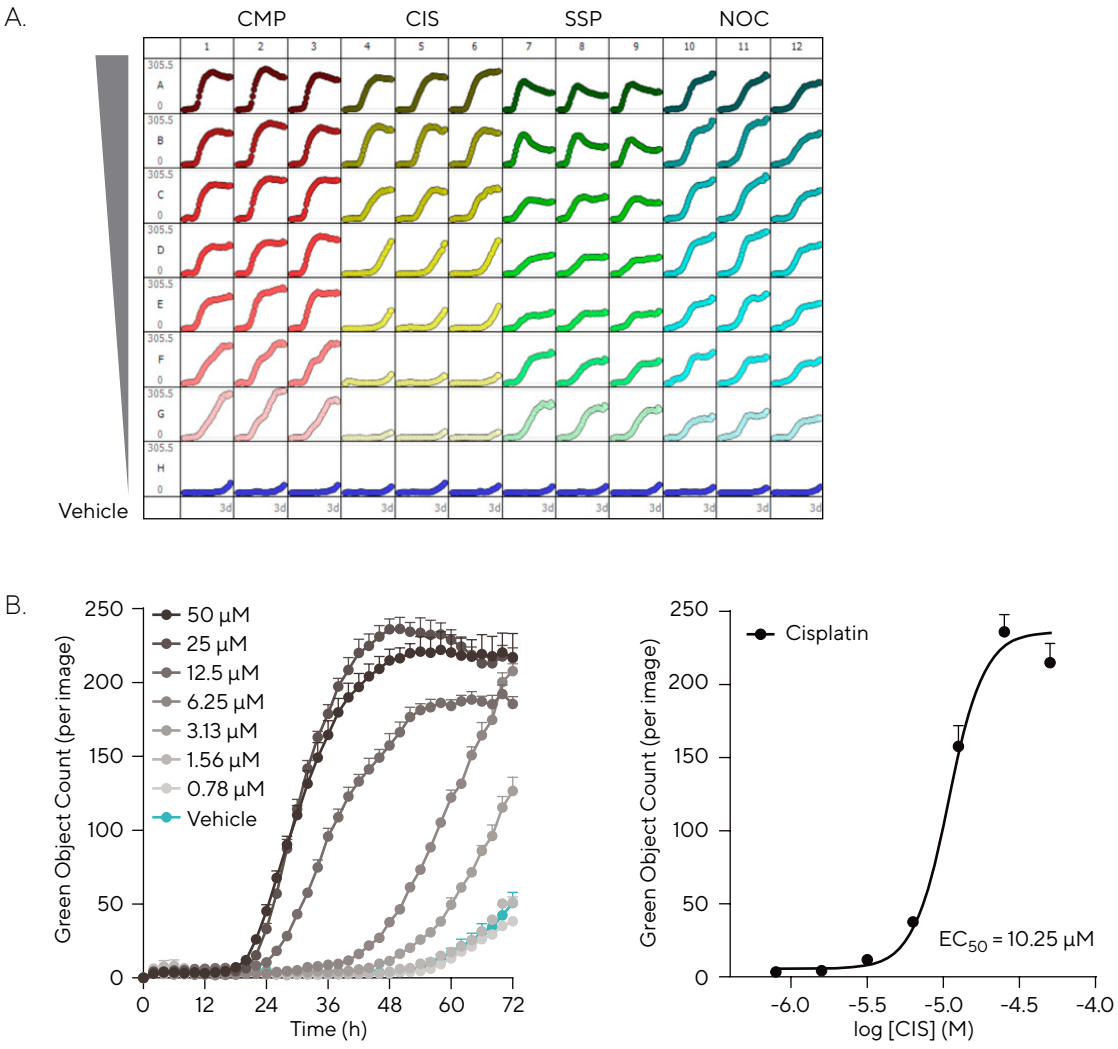


Figure 4

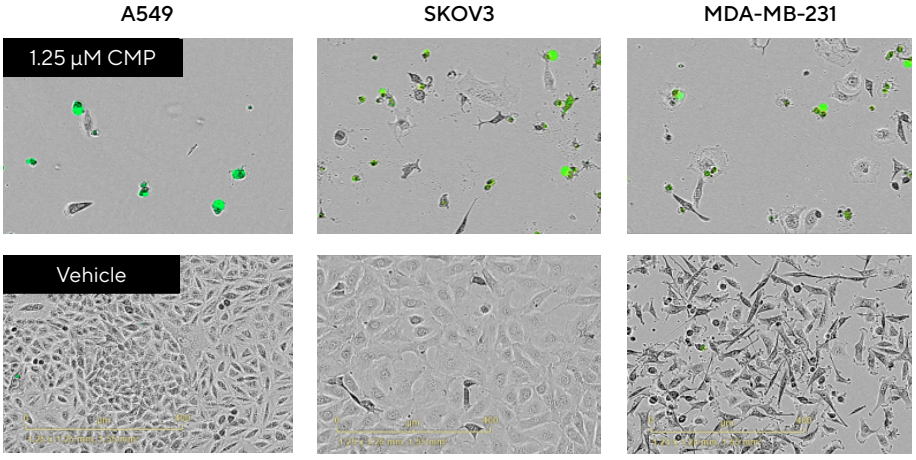
Automatic Analysis of Cytotoxicity in a High-Throughput Manner

Note. HT-1080 fibrosarcoma (2,000 cells/well) were seeded and after 18 h treated with four compounds in the presence of Incucyte® Cytotox Green Dye. (A) Incucyte® Cytotoxicity Assay allows every well of a 96/384-well plate to be imaged and analyzed automatically to provide a microplate readout of cytotoxicity over time. Microplate view shows kinetic change in green fluorescence following treatment with varying profiles over 72 h. (B) Time-course and concentration-response curve at 48 h show the concentration-dependent cytotoxic effect of Cisplatin (CIS; 50–0.78 µM) on HT-1080 cells as measured by Green Object Count. Data presented as Mean ± SEM, n = 3.

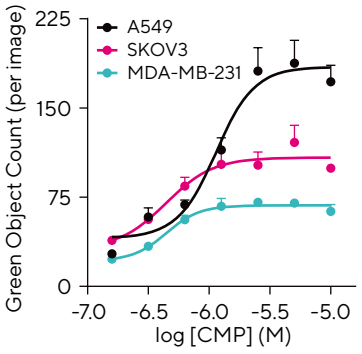
Profiling of Single Compounds Across Multiple Cancer Cell Types

Camptothecin (CMP) is a cytotoxic compound classically used as a research tool to induce cell death by inhibiting the DNA enzyme, topoisomerase I (TOPO I), resulting in double strand breaks during S-phase and triggering the apoptotic program.[8] In a pharmacological study, CMP was used to demonstrate the ability of the Incucyte® Cytotoxicity Assay to assess cytotoxicity over time in three different cancer cell lines, A549 (human tumor derived pulmonary adenocarcinoma), SKOV3 (human tumor derived ovarian carcinoma), and MDA-MB-231 (human tumor derived breast fibrosarcoma). Phase-contrast and blended fluorescent images for 1.25 µM CMP treatment or vehicle for each cell type highlight both the morphological changes and Cytotox fluorescence associated with CMP-induced cell death at 72 h (Figure 5A). Quantification of cytotoxicity over time revealed differential effects of CMP to each cancer cell line with A549 cells showing the strongest cytotoxic response and lower levels of fluorescence being observed for SKOV3 and MDA-MB-231, respectively (Figure 5B). Further analysis allows for direct comparison of cytotoxicity as shown by concentration-response curves at 72 h and derived EC_{50} values (Figure 5C). Our experimental findings substantiate the known cytotoxic effects of CMP, demonstrate the differential sensitives of cancer cell lines, and provide a powerful live-cell assay for investigating novel cancer therapies.

A.



C.



Cell Type	EC_{50} (µM)
A549	1.01
SKOV3	0.66
MDA	0.48

B.

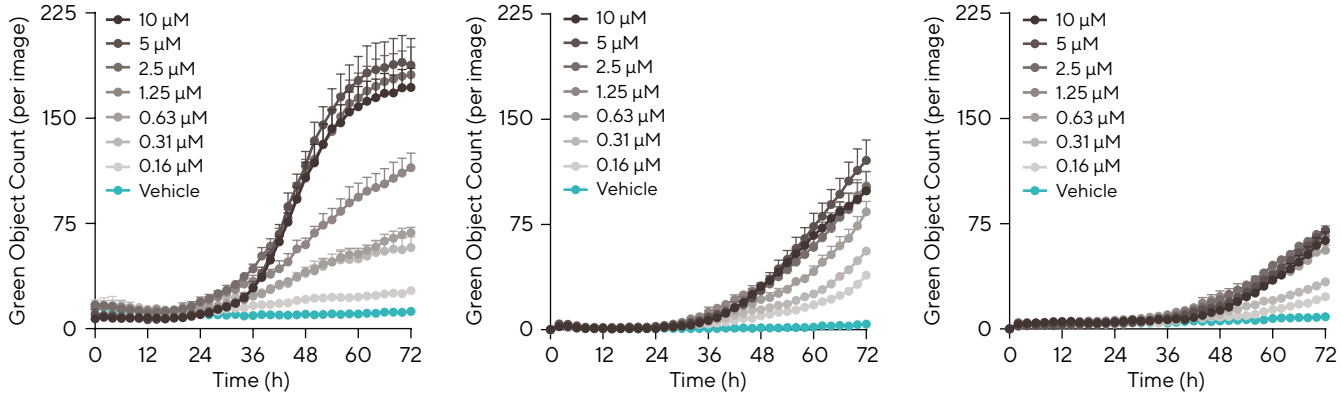


Figure 5

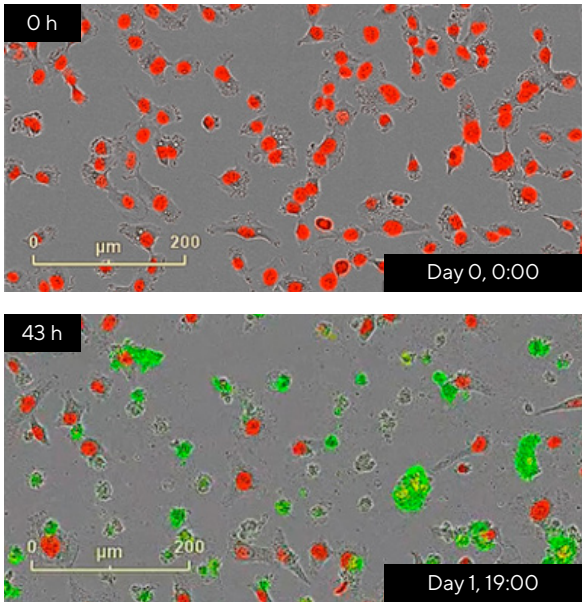
Profiling Treatment Effects in Different Cancer Cell Lines

Note. Cells were seeded (2,000 cells/well), and after 18 h treated with a concentration range of Camptothecin (CMP; 10–0.16 µM) in the presence of Incucyte® Cytotox Green Dye. (A) Phase and fluorescence images (72 h; 20X) show Cytotox fluorescence and morphological changes for CMP (1.25 µM) treated cells (top image) compared to vehicle (bottom image) for three cancer cell types, A549, SKOV3, and MDA-MB-231. (B) Time-courses show differential kinetic profiles of cytotoxic response to CMP as measured by Green Object Count. (C) Transformation of data into concentration-response curves (72 h) enables comparison of pharmacology across the cell lines. Data presented as Mean ± SEM, n = 3.

Multiplexed Measurements of Proliferation and Cytotoxicity

Using the Incucyte® Live-Cell Analysis System, Incucyte® Cytotox Dyes can be combined with Incucyte® Nuclight Reagents for nuclear labeling to conduct multiplexed assays capable of simultaneously measuring cytotoxicity in addition to proliferation. To illustrate this, Incucyte® Nuclight Red HT-1080 fibrosarcoma cells were treated with serially diluted concentrations of CMP in the presence of Incucyte® Cytotox Green Dye (Figure 6). Representative time-lapse images reveal a decrease in red nuclear fluorescence and increase of nucleic acid binding by Incucyte® Cytotox Green Dye following CMP treatment. Incucyte® integrated analysis software was used to mask the green fluorescent signal to quantitate cell death, as well as the red fluorescent nuclear signal to monitor cell proliferation. Quantification showed CMP induced a concentration-dependent inverse relationship between cell proliferation and cytotoxicity with similar derived IC_{50} and EC_{50} values (0.11 µM and 0.49 µM, respectively). These data show the potential of this kinetic and multi-parametric approach to the classification of compounds in drug discovery.

A.



B.

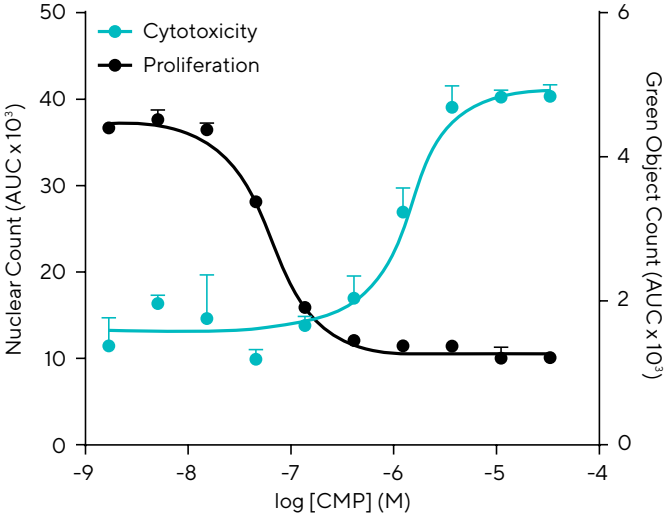


Figure 6

Multiplexed Cytotoxicity Measurements and Live-Cell Counting for Assessment of Compound Effects

Note. HT-1080 fibrosarcoma cells pre-labeled with Incucyte® Nuclight Red Lentivirus (5,000 cells/well) were treated with Camptothecin (CMP; 33.3–0.001 µM) in the presence of Incucyte® Cytotox Green Dye to detect live and dead cells over time. (A) Images show decrease in Nuclight Red signal and increase in Cytotox Green fluorescence following CMP (0.15 µM) addition (0 h vs. 43 h). (B) Statistical analysis of area under the curve (AUC; 0–48 h) revealed a concentration-dependent decrease in proliferation (Red Nuclear count) and concomitant increase in cytotoxicity (Cytotox Green object count) over 48 h (IC_{50} = 0.11 µM and EC_{50} = 0.49 µM, respectively). Data presented as Mean ± SEM , n=3.

Subpopulation Analysis of Cytotoxic and Cytostatic Treatment Effects

The Incucyte® Cell-by-Cell Analysis Software Module enables individual cells in the phase-contrast image to be segmented, and metrics can be extracted per cell relating to fluorescence within the segmented boundary. Using the integrated analysis software, cell populations can be classified based on fluorescence characteristics. To exemplify this, we investigated the effects of two compounds with different mechanisms of action on the total cell population using label-free counting (proliferation) and Cytotox Dye fluorescence classification (cytotoxicity) (Figure 7). The DNA synthesis inhibitor, Camptothecin (CMP), is a cytotoxic compound predicted to induce cell death. The protein synthesis inhibitor, Cycloheximide (CHX), is a cytostatic compound which was predicted to inhibit cell proliferation while not affecting cell viability. HT-1080 fibrosarcoma cells were treated with these two compounds (10–0.004 µM) in the presence of Incucyte® Cytotox Green Dye. Cell subsets were classified based on green fluorescence using Cell-by-Cell Analysis Software, and representative phase and blended fluorescent images for each compound display how cells are classified as live or dead (masking of dead (green) cells shown). Also shown are the classification plots used to identify the dead (green) population. Label-free counting and the calculation of cytotoxic index, as expressed by the percentage of dead cells over total number of cells, within the Incucyte® software revealed differential effects of the two compounds on proliferation and cytotoxicity. We found that CMP induced HT-1080 cell death in a concentration-dependent manner suggestive of a cytotoxic mechanism of action. In contrast, no statistical induction of cytotoxicity was observed for CHX. However, a clear concentration-dependent inhibition of cell proliferation was observed, indicative of cytostatic mechanism of action. Overall, these data show the potential of this dynamic approach to provide insight into drug-induced treatment effects, specifically examining mechanisms of action based on subpopulation studies of cell health.

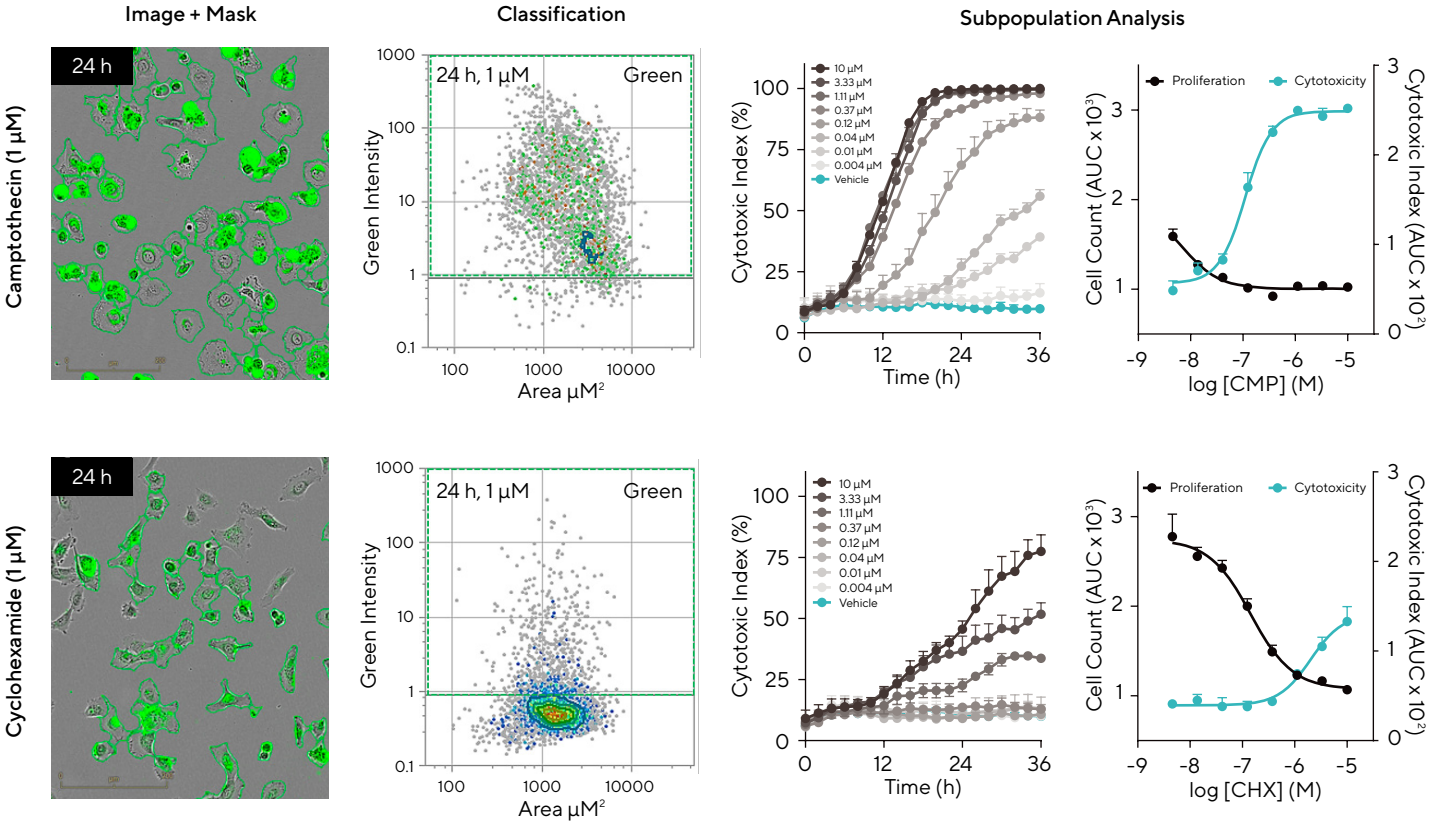


Figure 7

Investigation of Drug Mechanisms of Action in Subpopulations of Cells Using Live-Cell Label Free Counting and Cytotox Readouts

Note. HT-1080 fibrosarcoma cells (5,000 cells/well) were treated with Camptothecin (CMP; Cytotoxic) or Cycloheximide (CHX; Cytostatic) in the presence of the Incucyte® Cytotox Green Dye. Phase and fluorescent images illustrate how Cell-by-Cell Analysis can be utilized to detect live/dead cells at 24 h (masking of dead cells shown). Classification plots to identify green (dead) population (second column), time-course graphs (third column) and concentration-response curves (fourth column) show difference in effect of a cytotoxic and cytostatic compound. Data presented as Mean ± SEM, n = 3.

Summary and Outlook

Cell death is a dynamic process, and the monitoring of cell cytotoxicity is critical in many therapeutic areas, such as oncology. Using the Incucyte® Live-Cell Analysis System in conjunction with Incucyte® Cytotox Dyes 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 approach also provides the ability to monitor morphological changes in parallel with quantification in physiologically relevant conditions. The combination of Incucyte® Cytotox Dyes with Incucyte® Nuclight Reagents or cells lines enables multiplexed measurements of proliferation and cytotoxicity.

Furthermore, Incucyte® Cell-by-Cell Analysis Software provides individual cell segmentation and classification based on fluorescence, allowing for additional parameters, such as the cytotoxic or apoptotic index, to be calculated and facilitating assessment of drug mechanisms of action on cell populations. Overall, the Incucyte® Cytotoxicity Assay is a powerful and flexible tool, which is amenable to pharmacological investigations across a range of assay formats (Table 1) and enables deeper insight into the temporal patterns of cell death.

	Incucyte® Cytotox Dye	Incucyte® Annexin V Dye	Incucyte® Caspase-3/7 Dye
Cell viability	✓		
Apoptosis		✓	✓
2D Monolayer + multiplex	✓	✓	✓
3D Single spheroid	✓	✓	
3D Multi-spheroid + multiplex	✓	✓	
Fluorescence channels	Red, green, near-IR	Red, green, orange, near-IR	Red, green, orange*

*Incucyte® Metabolism

Table 1

Incucyte® Dyes for Live-Cell Imaging and Analysis of Cell Health

Note. Table summarizes Incucyte® Dyes validated for investigation of cell health using the Incucyte® Live-Cell Analysis System across a range of assay formats.

References

1. Keep, O., Galluzzi, L., Lipinski, M., Yuan, J., & Kroemer, G. (2011). Cell death assays for drug discovery. *Nat Rev Drug Discov, 10*(3), 221-237.
2. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W., Nuñez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., & Melino, G. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ, 16*(1), 3-11.
3. Abraham, V. C., Towne, D. L., Waring, J. F., Warrior, U., & Burns, D. J. (2008). Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans. *J Biomol Screen 13*(6), 527-537.
4. Becker, B., Clapper, J., Harkins, K. R., & Olson, J. A. (1994). *In situ* screening assay for cell viability using a dimeric cyanine nucleic acid stain. *Anal Biochem, 221*(1), 78-84.
5. Abassi, Y. A., Xi, B., Zhang, W., Ye, P., Kirstein, S. L., Gaylord, M. R., Feinstein, S. C., Wang, X., & Xu, X. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. *Chem Biol, 16*(7), 712-723.
6. Tanida, S., Mizoshita, T., Ozeki, K., Tsukamoto, H., Kamiya, T., Kataoka, H., Sakamuro, D., & Joh, T. (2012). Mechanisms of Cisplatin-Induced Apoptosis and of Cisplatin Sensitivity: Potential of BIN1 to Act as a Potent Predictor of Cisplatin Sensitivity in Gastric Cancer Treatment. *International Journal of Surgical Oncology*.
7. Dasari, S. & Tchounwou, P. B. (2014). Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol,* 740, 364-378. doi:10.1016/j.ejphar.2014.07.025
8. Ulukan, H. & Swaan, P. W. (2002). Camptothecins: a review of their chemotherapeutic potential. *Drugs, 62*(14), 2039-2057.

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