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Incucyte® Apoptosis Assays for Kinetic Quantification of Apoptosis

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Introduction

Apoptosis, the biological process by which cells undergo programmed cell death, is required for normal tissue maintenance and development. However, aberrations in apoptotic signaling networks are implicated in numerous human diseases including neurodegeneration, autoimmune disease and cancer.[1] Apoptotic pathways are initiated by extrinsic factors that result in activation of pro-apoptotic receptors on the cell surface, or intrinsically by many different stimuli, such as DNA damage, hypoxia, the absence of growth factors, defective cell cycle control, or other types of cellular stress that result in release of cytochrome C from mitochondria.

Induction of apoptosis leads, in most cases, to the activation of a family of proteins called caspases (cysteinyl aspartate proteinases). The activation of caspase-3 or caspase-7 often results in the irreversible commitment of the cell to apoptotic death and is considered a reliable marker for apoptosis.[2] The regulated loss of plasma membrane phosphatidylserine (PS) symmetry is also a classical marker of apoptosis. Dying cells trigger the translocation of the normally inward-facing PS to the cellular surface, allowing for early phagocytic recognition of the dying cell by surrounding phagocytes.[3]

Numerous enzymatic, plate-reader, and flow-cytometric assays have been designed to measure caspase-3/7 activation or PS externalization. Most caspase-3/7 assays involve luciferase, colorimetric, or fluorometric reagent substrates that incorporate a DEVD (Asp-Glu-Val-Asp) peptide motif which is recognized by the enzyme.[4] Annexin V is a recombinant protein with a high affinity and selectivity for PS residues, allowing it to be used for the detection of apoptosis. Apoptosis assays using Annexin V conjugated to a fluoroprobe have been optimized for detection of PS externalization and are most commonly measured by flow-cytometry.[5] The major drawbacks of these common apoptosis assays are (1) they yield a single, (arbitrary) user-defined end-point measurement; (2) they require multiple wash steps or cell lifting that may result in the loss of dying cells or lead to a loss in PS asymmetry; and (3) they are not amenable to long-term measurements due to

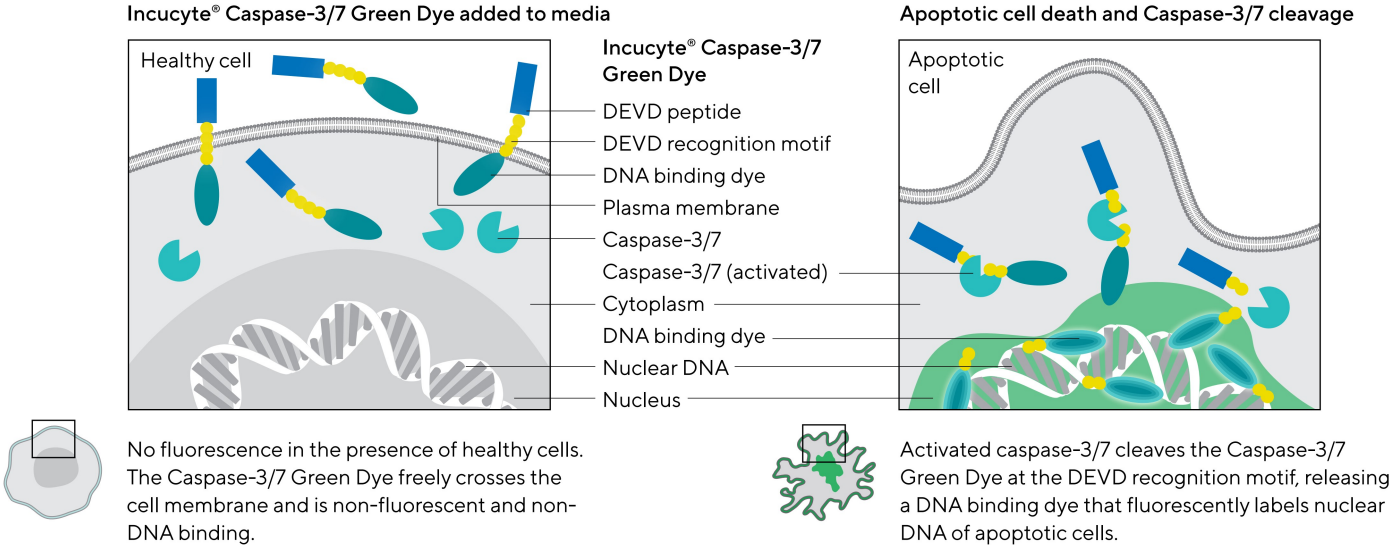
increasing background signal over time. Live-cell imaging and analysis methods have been developed, however optimized technology and reagents are required for flexible and accurate assessment of cell death.[6] In this application note, we describe the value of Incucyte® Apoptosis Assays, encompassing no-wash, mix-and-read reagents and integrated image-based analysis tools optimized for kinetic quantification of apoptosis.

Assay Principle

The Incucyte® Apoptosis Assays utilize Incucyte® Caspase-3/7 and/or Annexin V Dyes in combination with the Incucyte® Live-Cell Analysis System to enable automated measurements of apoptosis in real-time. The Incucyte® Caspase-3/7 Dyes are inert, non-fluorescent (DEVD) substrates that freely cross the cell membrane where they can be cleaved by activated caspase-3/7 to release a green, red, or orange (for metabolism) DNA-binding fluorescent label.[7] Apoptotic cells are identified by the appearance of fluorescently labeled nuclei (Figure 1A).

The Incucyte® Annexin V Dyes are labeled with exceptionally bright and photostable cyanine fluorescent (CF) dyes that emit a green, red, orange, or near-infrared (NIR) signal upon binding to exposed PS in apoptotic cells (Figure 1B). Intuitive integrated analysis software quantifies these fluorescent objects and background signal is minimized. These apoptotic signals can be correlated with Incucyte® high-definition phase-contrast images to provide additional biological insight and morphological validation of apoptotic cell death (e.g., cell shrinkage, membrane blebbing, nuclear condensation, DNA fragmentation).

A. Overview of Incucyte® Caspase-3/7 Dye



B. Overview of Incucyte® Annexin V Dye

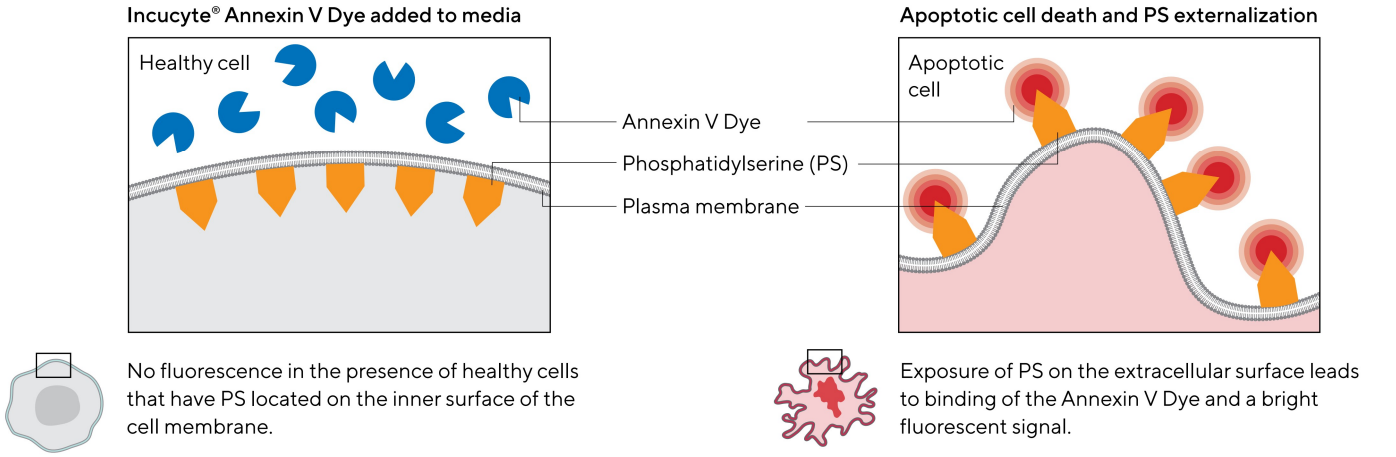
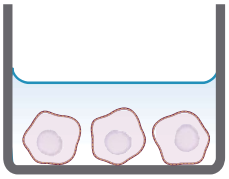


Figure 1
Schematics Demonstrating Principles of Incucyte® Apoptosis Assays

Materials and Method

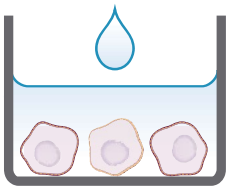
The Incucyte® Apoptosis Assay can be performed using a simple mix-and-read protocol in a high throughput 96/384-well format as demonstrated in Figure 2. This highly flexible assay can be used with your choice of cells, including adherent or non-adherent cells, and enables real-time quantification of treatment effects non-invasively. Readouts of apoptosis can be multiplexed with measurements of proliferation or cytotoxicity by combining with the Incucyte® Confluence metric, Incucyte® Nuclight Reagents for nuclear labeling, or Incucyte® Cytotox Dyes.

1. Seed cells



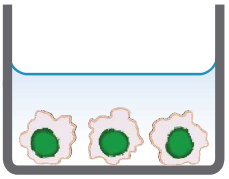
Seed cells (100 µL/well) into a 96-well plate and incubate overnight.

2. Prepare apoptosis reagent and treat cells



Prepare desired treatments at 1X in medium containing Incucyte® Annexin V or Caspase-3/7 Dye and add treatment.

3. Live-cell fluorescent analysis



Capture images every 2–3 hours (20X or 10X) in the Incucyte® Live-Cell Analysis System for 24–120 hours. Analyze using integrated software.

Figure 2

Quick Guide of Incucyte® Apoptosis Assay Protocol for Adherent Cells

Note. The simple protocol utilizes Incucyte® Caspase-3/7 or Annexin V Dyes and the Incucyte® Live-Cell Analysis System for image-based fluorescent measurements of apoptosis.

Validation Data

Quantification of Apoptosis

The Incucyte® Live-Cell Analysis System, in conjunction with apoptosis readouts, enables visualization and quantification of caspase-3/7 activity or PS exposure in response to pharmacological treatment in real-time. To exemplify this, HT-1080 fibrosarcoma cells were treated with the anti-cancer drug Cisplatin (CIS; 12.5 µM) in the presence of Incucyte® Annexin V Red Dye (Figure 3). Phase-contrast and blended fluorescent images were acquired to detect morphological hallmarks of apoptosis and evaluate Annexin V binding. Integrated image-based analysis tools aid automatic segmentation of fluorescence (mask shown in blue) over the entire assay time-course and allow for quantification of apoptosis. CIS treatment induced apoptosis of HT-1080 cells as shown by a kinetic increase in fluorescence due to the binding of Incucyte® Annexin V Red Dye to externalized PS. Additionally, the acquired images allow for correlation of apoptotic readouts with the morphological changes associated with cell death.

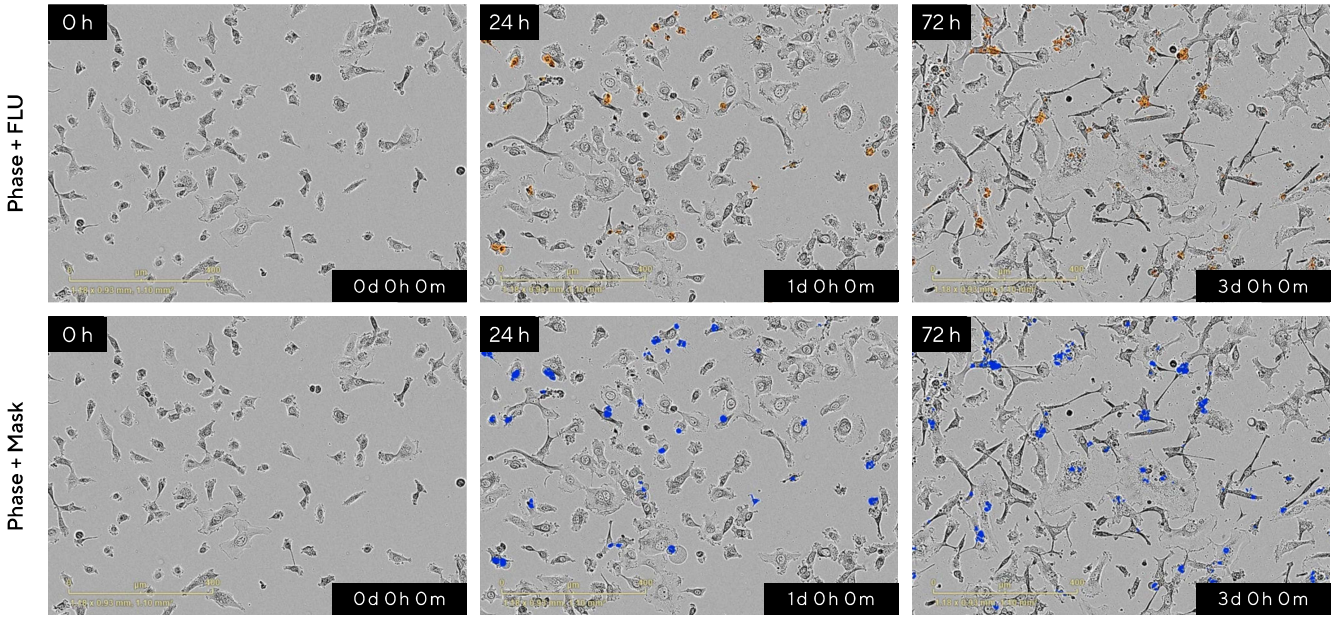


Figure 3

Visualization and Quantification of HT-1080 Fibrosarcoma Cells in Response to the Anti-Cancer Drug Cisplatin (CIS) in Real-Time

Note. HT-1080 cells were seeded (2,000 cells/well) and after 18 h treated with 12.5 µM CIS in the presence of Incucyte® Annexin V Red Dye. High-definition images (20X) were acquired every 2 h for the duration of the experiment using the Incucyte® Live-Cell Analysis System. Representative phase-contrast and blended fluorescence images (top row) and the segmentation mask (blue) generated using integrated analysis software (bottom row) are shown over time (0–72 h). A temporal increase of PS binding by Incucyte® Annexin V Red Dye was observed following drug treatment. The Incucyte® Annexin V Red fluorescent signal can be correlated with morphological changes associated with cell death; note the observed characteristic cell shrinkage and membrane blebbing.

Pharmacological, Kinetic Analysis of Apoptosis

The Incucyte® Apoptosis Assay allows for robust high-throughput investigation of apoptosis in response to compound treatments. To illustrate the amenability of our approach to drug toxicity testing, a pharmacological study was performed in A549 cancer cells. Cells were treated with 2-fold serial dilutions of four different compounds in the presence of Incucyte® Annexin V NIR Dye and fluorescent objects were automatically analyzed using integrated software. A kinetic increase in fluorescence was observed for all compounds as shown in the microplate view with variable profiles being observed for the different compounds (Figure 4A). Concentration-dependent effects were observed for Camptothecin (CMP), Cisplatin (CIS) and Staurosporine (SSP), while Nocodazole showed low-levels of apoptosis across all concentrations tested. The kinetic apoptotic response to CMP, a DNA synthesis inhibitor commonly used as a research tool to induce apoptosis,[8] is shown (Figure 4B). Transformation of the kinetic data at 72 h into a concentration-response curve shows a concentration-dependent effect on A549 cells. In control conditions, no increase in fluorescence was observed. Representative

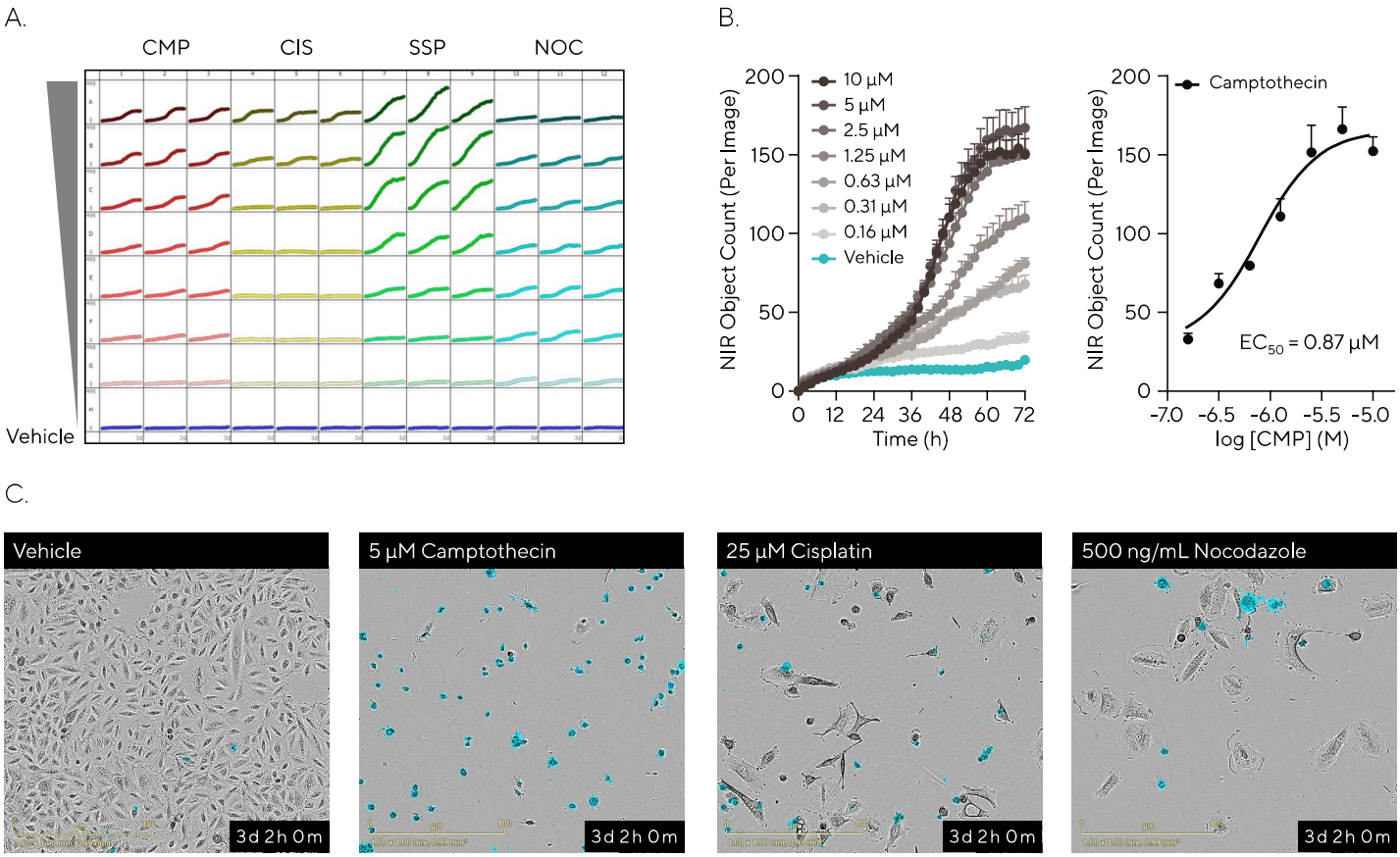


Figure 4

Pharmacological Analysis of Apoptosis in a High-throughput Manner

Note. A549 cells were seeded (2,000 cells/well) and after 18 h treated with a concentration range of four compounds in the presence of Incucyte® Annexin V NIR. (A) Incucyte® Apoptosis Assay allows every well of a 96/384-well plate to be imaged and analyzed to give a microplate readout of apoptosis over-time. Microplate view shows kinetic change in fluorescence following treatment with differential profiles being observed as measured by NIR Object Count. (B) Time course shows concentration-dependent kinetic effect of Camptothecin (CMP; 10–0.16 µM). Further analysis reveals concentration-response curve at 72 h for CMP. (C) Phase and NIR (pseudo-colored blue) fluorescent images (20X; 72 h) enable apoptotic readouts to be correlated with morphological changes. Data represented as Mean ± SEM, n = 3.

phase-contrast and blended fluorescent images reveal the morphological changes associated with apoptosis and allow for the qualitative comparison of different drug treatments to vehicle conditions (Figure 4C). The results demonstrate the utility of the Incucyte® Apoptosis Assay in the visualization and quantification of drug-induced apoptosis and how live-cell analysis is amenable to high-throughput pharmacological investigation.

Multiplexed Measurements of Proliferation and Apoptosis

Incucyte® Apoptosis Assays can be combined with Incucyte® Nuclight Reagents for nuclear labeling, for multiplexed measurements of proliferation and apoptosis using the Incucyte® Live-Cell Analysis System. To demonstrate this approach, Incucyte® Nuclight NIR labeled HT-1080 fibrosarcoma cells were treated with 3-fold serial dilution of CMP in the presence of Incucyte® Caspase-3/7 Dye (Figure 5). Representative phase and blended fluorescence images show a decrease in NIR nuclear fluorescence (pseudo-colored blue) and increase in green fluorescently stained DNA, indicating activation of Caspase-3/7, following CMP treatment. Integrated software was used to automatically mask fluorescence and quantify cell death (green) and cell proliferation (NIR). Quantification showed a kinetic concentration-dependent apoptotic and anti-proliferative effect of CMP on HT-1080 cells. These data illustrate how two-color kinetic assays provide a multi-parametric approach to analyzing the apoptotic and anti-proliferative effects of pharmacological treatments and highlight the potential of investigating novel compounds in drug discovery.

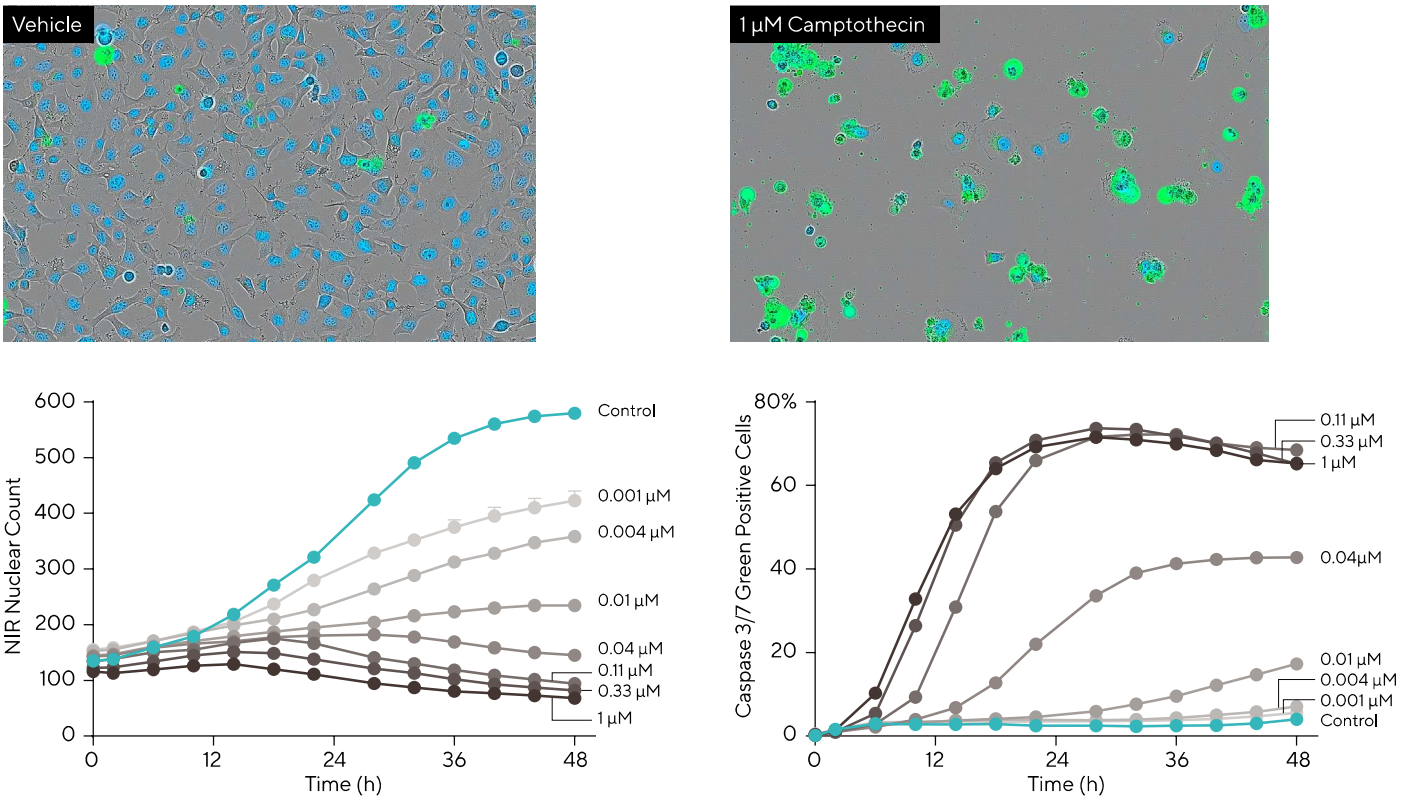


Figure 5

Investigation of Compound Effects Using Multiplexed Measurements of Apoptosis and Proliferation

Note. HT-1080 fibrosarcoma cells stably expressing Incucyte® Nuclight NIR Lentivirus (pseudo-colored blue) were treated with decreasing concentrations of CMP (1–0.001 µM) in the presence of Incucyte® Caspase-3/7 Green. A concentration-dependent decrease in nuclear count and corresponding increase in cell death was observed over 48 h. Representative phase and fluorescent images (10X; 48 h) validate kinetic data of both cell viability and apoptotic death. Data presented as Mean ± SEM, n = 12.

Assessment of Cell Health in Subpopulations of Cells

The Incucyte® Cell-by-Cell Analysis Software Module enables segmentation of individual cells in the phase image, and metrics per cell relating to fluorescence within the segmented boundary can be extracted. Integrated software analysis enables cell populations to be classified based on fluorescence characteristics. This, in combination with multiplexed assays, allows the cell health of sub-populations to be rapidly quantified. To exemplify this, HT-1080 fibrosarcoma cells stably expressing Incucyte® Nuclight Red (nuclear viability marker) were treated with either Camptothecin (CMP; Cytotoxic) or Cyclohexamide (CHX; Cytostatic) in the presence of Incucyte® Caspase-3/7 Green Dye (Figure 6). Images were acquired every 2 h (10X) and cell subsets classified based on red and green fluorescence intensity using Cell-by-Cell Analysis. The representative phase and fluorescence images and classification plots for each compound display how cells are classified as red, red and green, or green. Temporal tracking of these subpopulations revealed that CMP induced a decrease in the red population indicating loss of viable cells, an increase in the red and green population indicating early apoptosis, as well as an increase in the green population indicating late apoptosis after > 24 h. Time courses of the early apoptotic population (red and green) show a concentration-dependent apoptotic response to CMP in HT-1080 cells, consistent with a cytotoxic mechanism of action. In contrast, CHX treatment did not induce apoptosis at any of the concentrations tested but maintained a constant red population (viable) over time, consistent with cytostatic mechanism of action. Overall, data has shown how Incucyte® Cell-by-Cell Analysis in combination with multiplexed assays can be used to gain dynamic insight into drug-induced treatment effects on cell health and is applicable for identifying therapeutic mechanisms of actions on a specific cell type.

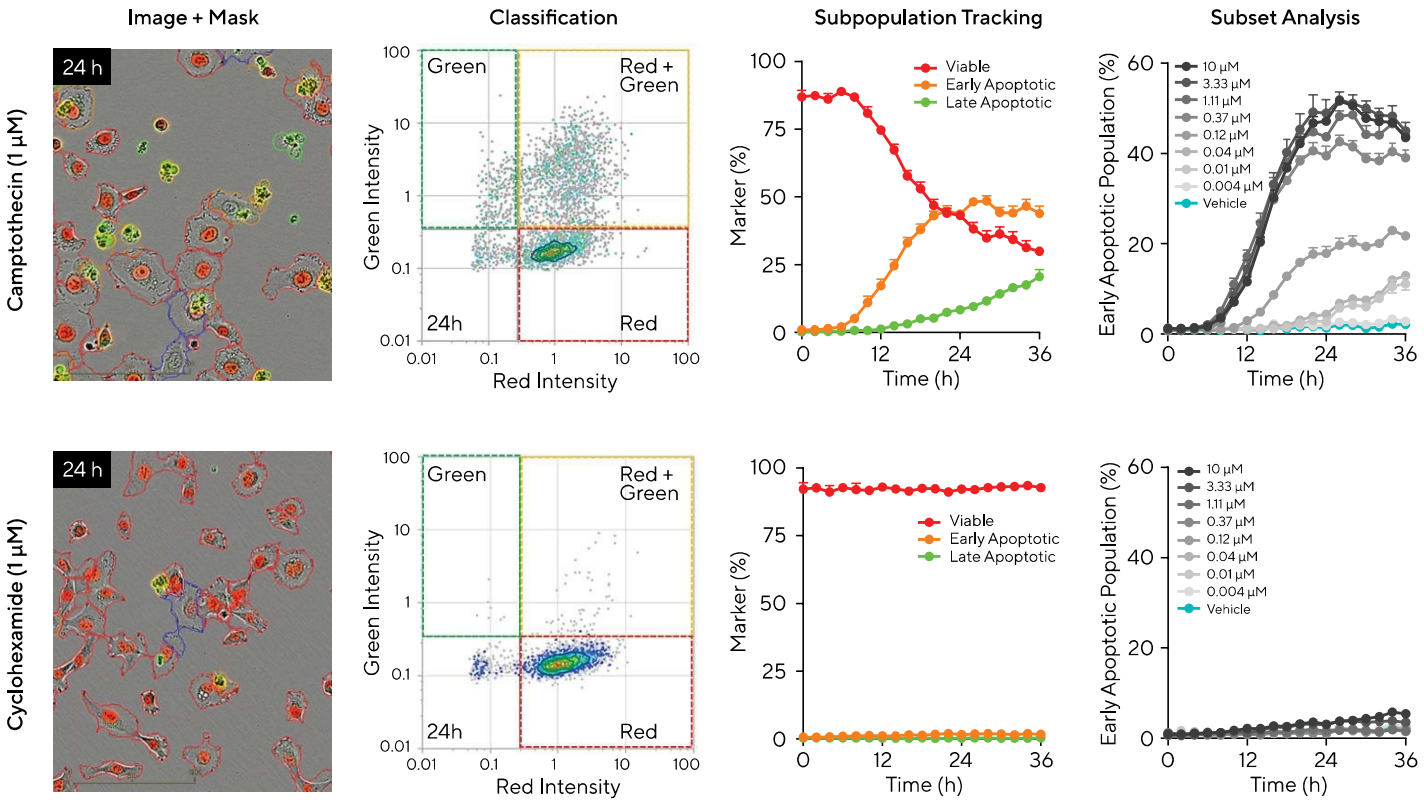


Figure 6

Investigation of Cell Health of Subpopulations of Cells Following Different Compound Treatments

Note. HT-1080 fibrosarcoma cells stably expressing Incucyte® Nuclight Red (nuclear viability marker) were treated with Camptothecin (CMP, cytotoxic) or Cyclohexamide (CHX, cytostatic) in the presence of Incucyte® Caspase-3/7 Green. Cell images show response at 24 h with associated classification masking (first column). Cell subsets were classified based on red and green fluorescence intensity using Incucyte® Cell-by-Cell Analysis Software (second column). After CMP treatment, there was a decrease in the red population (viable cells), increase in red and green population (early apoptosis), and increase in green population (late apoptosis) (third column, top). After CHX treatment, there was a lack of apoptosis (third column, bottom). Concentration-response time courses of the early apoptotic population are shown (percentage of total cells exhibiting red and green fluorescence, fourth column). Data presented as Mean ± SEM, n = 3.

Summary and Outlook

Apoptosis is a complex and dynamic process implicated in several pathologies and a deeper understanding of the mechanisms is vital across a range of therapeutic areas. Combining Incucyte® Caspase-3/7 or Annexin V Dyes with real-time image analysis using the Incucyte® Live-Cell Analysis System enables a quantitative picture of this changing landscape to be achieved. The Incucyte® Apoptosis Assays provide a full kinetic readout of apoptotic signaling, via caspase-3/7 activity or PS exposure, in physiologically relevant conditions. Aside from providing insight into the dynamics and timing of the apoptotic signaling pathway, these assays eliminate the need for determining a single, optimum, assay endpoint; something which can vary considerably for different cell types and for different compound treatment conditions. This approach also enables validation of data using the acquired images and assessment of morphological changes associated with cell death. Further insight can be gained through multiplexing apoptosis readouts with measurements of proliferation, which in combination with Incucyte® Cell-by-Cell Analysis has the added benefit of being able to track the cell health of individual subpopulations of cells. Together, these assays provide a novel and flexible method, amenable to different experimental set-ups (Table 1), enabling apoptotic pathway analysis for both drug discovery as well as basic cell biology research.

	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.

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