AI-Driven Label-Free Quantification of Cell Viability Using Live-Cell Analysis

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Introduction

Live-cell imaging enables acquisition of phase contrast images and provides an ideal platform to study multi-faceted biological paradigms in drug discovery. This is vital to our understanding of human diseases and treatment strategies. The movement of these models towards increasingly complex physiologically relevant ones, including patient-derived cells and induced pluripotent stem cells (iPSCs), has concurrently driven the need for label-free methods that are non-perturbing to deliver deeper biological insights.[1,2] The elimination of fluorescent reporters reduces workflow time, ensures that experimental outcomes are not attributed to the label, or labeling process itself, and is non-perturbing for when fluorescent labeling is not possible, such as when using rare or sensitive cell types.[3,4]

Incorporating artificial intelligence (AI) into image analysis workflows has enabled powerful quantification of a wide range of cellular models, allowing researchers to make data-driven decisions and understand disease at a more granular level.[5] These leading-edge technologies, based on neural-network algorithms, are much more complex than traditional image analysis and facilitate more robust segmentation of heterogenous cell morphologies whilst minimizing user-introduced bias.[2] However, there exists several barriers to the widespread use of AI in image analysis, including hardware requirements, knowledge of training and testing methods, access to robust datasets for training, pre- and post-processing image analysis pipelines with a vast amount of data, and the general applicability of algorithms across cell types.[6]

Find out more: www.sartorius.com/incucyte-ai-cell-health-software

In this application note, we describe an automated, robust solution for label-free cell segmentation and live/dead classification of individual cells using integrated AI-based software. The Incucyte® AI Cell Health Analysis Software Module, driven by trained convolutional neural networks (CNN), allows us to reliably monitor cell viability in a non-perturbing unbiased manner with minimal user input.

Here, we show validation of the analysis software across a wide range of live and dead adherent and non-adherent cell types and exemplify how this approach can provide high-throughput, physiologically relevant insights into cell health through accurately predicting cell death across multiple treatments.

Assay Principle

The Incucyte® AI Cell Health Analysis Software Module enables label-free quantification of live or dead cells. The analysis module uses trained CNNs, which automatically analyze images to segment individual cells and classify them as live or dead, all in one step. This streamlined workflow (Figure 1) requires little user input, providing unbiased results which can be directly compared across assays.

Phase contrast images are acquired using AI Scan acquisition with 10x or 20x objectives. These images can be analyzed using Incucyte® AI Cell Health Analysis Software Module which provides metrics such as Total Cell Count (All Objects), as well as the number and percentage of live and dead cells. In cases where optional fluorescence images are acquired, the Mean and Total Integrated Intensity within all cells, as well as the live or dead subpopulations, will be provided. Fluorescence classification can be performed as an additional analysis, again providing metrics describing the count and percentage of high vs low fluorescence within total cells, and within live or dead subpopulations.
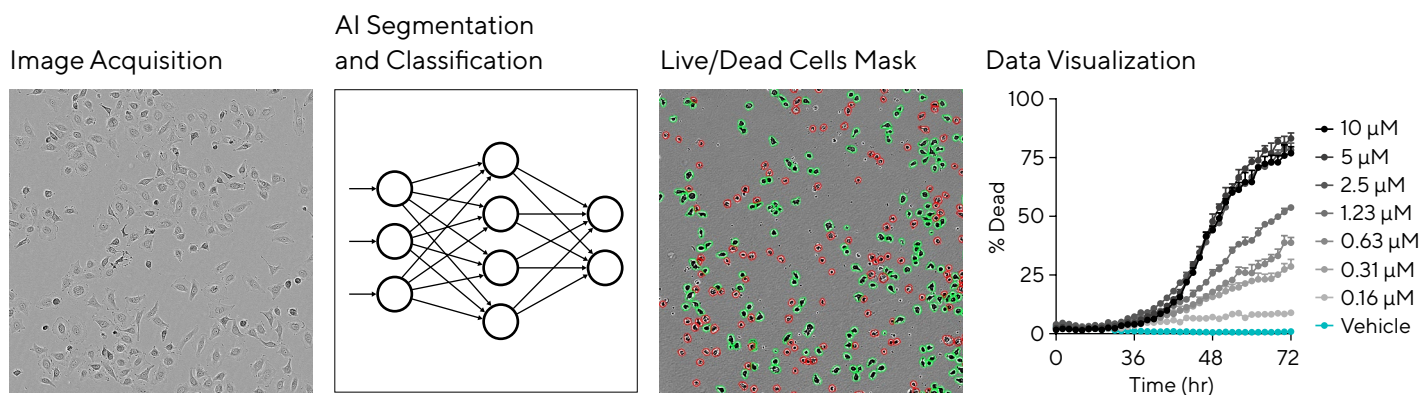
Image Acquisition | AI Segmentation and Classification | Live/Dead Cells Mask | Data Visualization

Figure 1: *Incucyte® AI Cell Health Analysis Workflow.*

Phase Contrast Images Are Acquired and Processed Using Neural Networks (CNN), to Automatically Segment and Classify Cells as Live or Dead.

Precise segmentation provides accurate cell count data even at high cell confluence (up to 99% depending on cell morphology), yielding reliable proliferation data. Label-free classification of cells as live or dead enables quantification of cell viability within a physiologically relevant and non-perturbing environment. Combining this label-free analysis with optional fluorescence readouts from the live or dead subpopulations provides additional insight into mechanisms of cell death.

AI-Driven Cell Segmentation

The AI Cell Health segmentation model was trained using phase contrast images which were manually annotated to identify the boundary of individual cells. A wide diversity of over 2 million individual cells were annotated including adherent and non-adherent cell types at a range of confluences, both healthy and apoptotic; adherent cell examples covered a wide range of morphologies. This broad spectrum of cells ensures that the final trained model is highly versatile, competently segmenting a multitude of cell types - even those which were not included within the training and validation datasets.[7]

The resulting segmentation is highly accurate even in confluent images and adapts to a multitude of cell morphologies – even where these are present within the same image. Figure 2 shows the AI segmentation applied to a highly clustering breast cancer cell line (MCF-7), an invasive epithelial-like cell line (MDA-MB-231), flat and transparent primary cells (primary rat astrocyte), and a non-adherent B cell line (Ramos). In addition, dead cells are accurately delineated (HMC3 cells treated with camptothecin, 1.1 µM), as are monocytes in the presence of pHrodo® Bioparticles® for Incucyte® (RAW 264.7 in the presence of *E. coli* bioparticles, 10 µg per well).

MCF-7
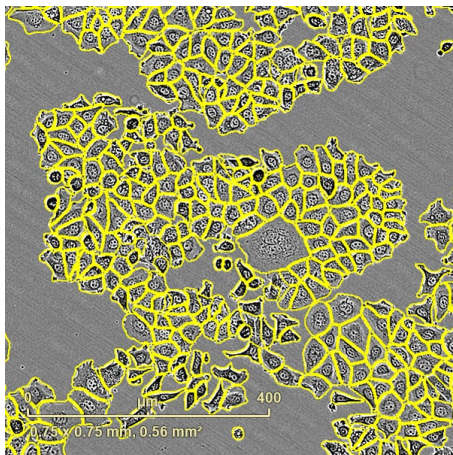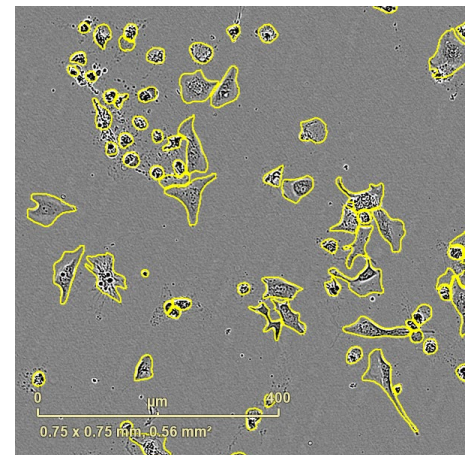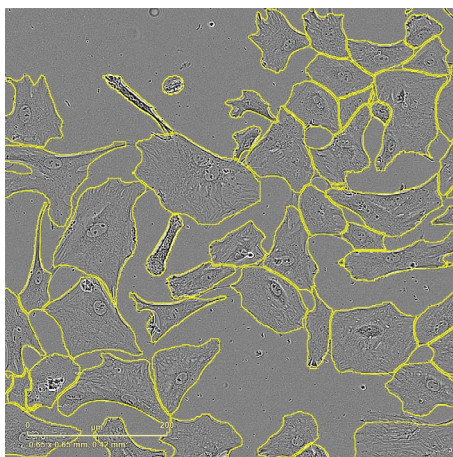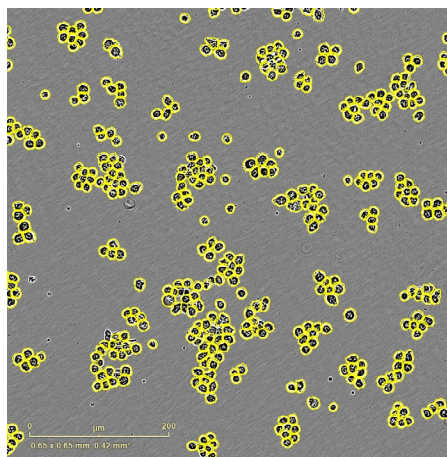
MDA-MB-231
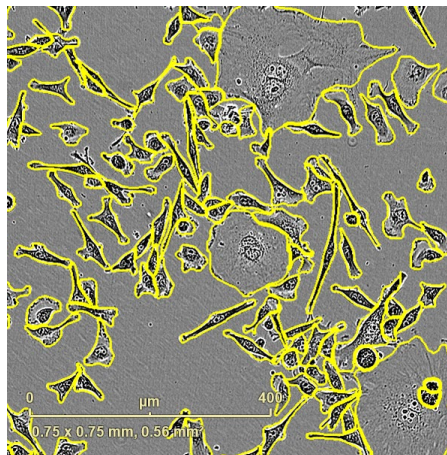
HMC3 + Camptothecin

Primary rat astrocyte

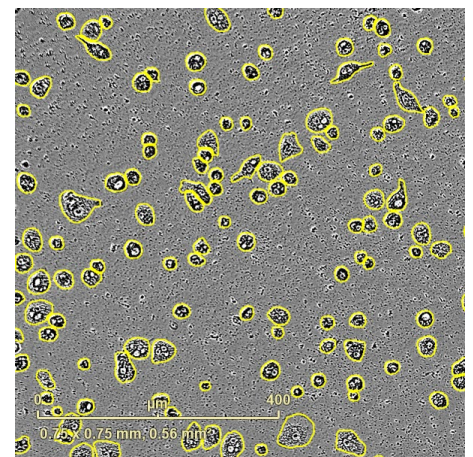**Ramos**

RAW 264.7 + *E. coli*

Figure 2: *AI Cell Health Analysis Accurately Segments a Wide Range of Cell Types With Diverse Morphologies, Including Apoptotic Cells and Cells in the Presence of Bioparticles.*

The segmentation model is trained specifically to detect cells and therefore ignores most non-cell objects. Cell segmentation is thus possible even in the presence of debris, compound precipitate, or bioparticles. Figure 3 demonstrates this advantage, showing primary macrophages accurately segmented in the presence of pHrodo® E. coli Bioparticles® for Incucyte® (10 µg/mL, 24 hr post treatment). As the bioparticles are engulfed, they are processed into acidic lysosomes, and the low pH causes the pHrodo® label to increase in fluorescence intensity. Quantification of fluorescence within the AI-masked cell boundary shows increasing intensity over time as the bioparticles are engulfed (Figure 3B), and the total intensity is dependent on the densities of both bioparticles and cells (Figure 3C). Fluorescence classification of cells as high or low intensity enables identification of cells which are phagocytic (Figure 3D). At 30 µg/mL bioparticles, around 40% of the cells are phagocytic; this percentage decreases as the amount of bioparticles decreases. Interestingly, the percentage of phagocytic cells is independent of the cell seeding density but increases with higher bioparticle density, suggesting the bioparticles themselves have an activating effect.
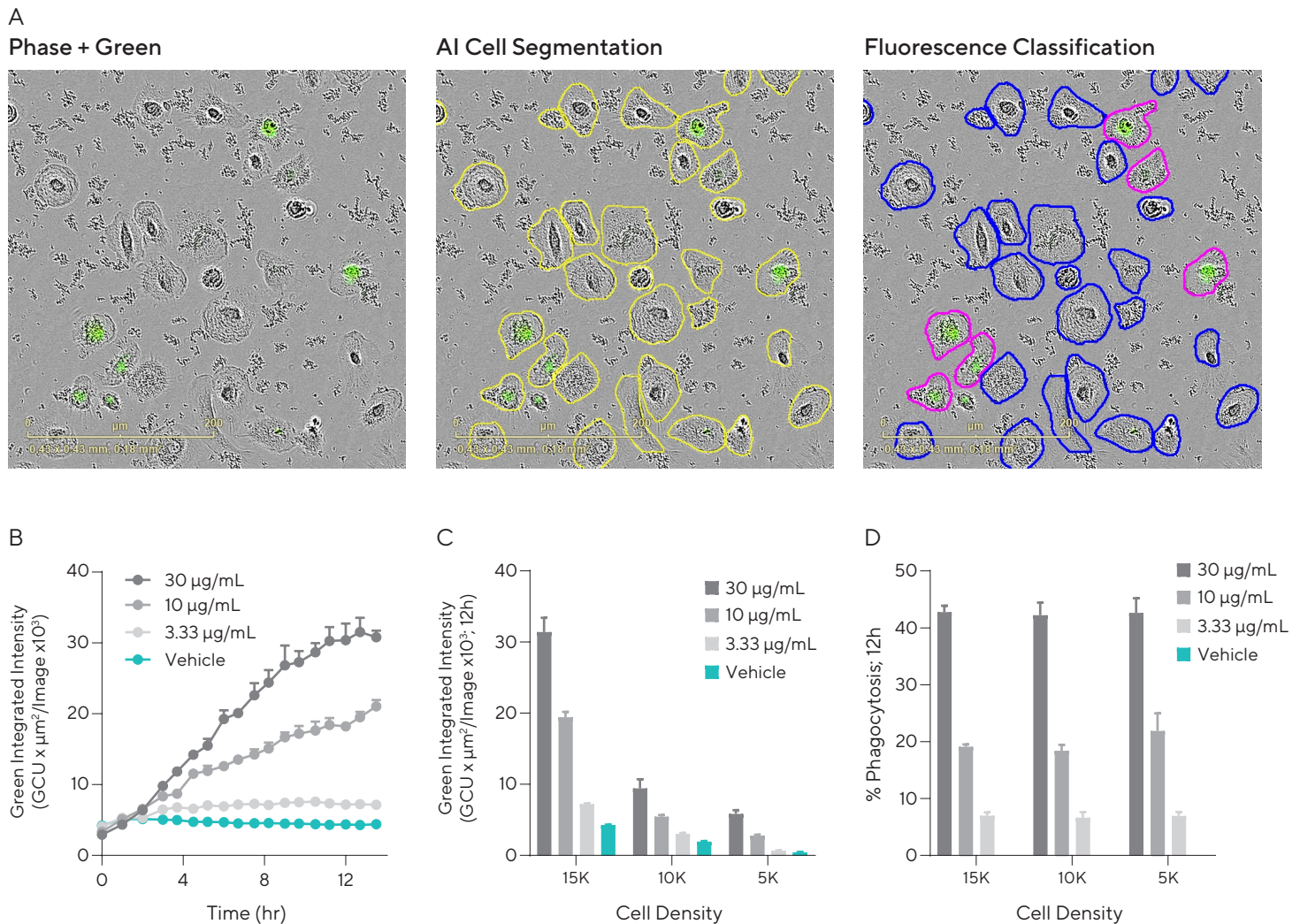

Figure 3: *Robust Cell Segmentation Enables Accurate Quantification of Phagocytic Cells.*

Images show primary macrophages 12 hr post treatment with pHrodo® E. coli Bioparticles® for Incucyte® (A). Non-engulfed bioparticles are visible in the phase contrast image while engulfed bioparticles fluoresce (green). AI Cell Segmentation shows accurate masking of cells alone, and fluorescence classification indicates phagocytic (magenta outline, high fluorescence) and non-phagocytic (blue outline, low fluorescence) cells. Time course demonstrates increasing fluorescence intensity within the segmented cell boundary over 12 hr (B). The increase in fluorescence is both cell- and bioparticle-dependent (C), while the percentage of phagocytic cells is dependent on bioparticle concentration but not cell density (D).

AI-Driven Live/Dead Classification

The AI model for classification was trained using pairs of phase contrast and fluorescence images of cells treated with cytotoxic compounds in the presence of Incucyte® Cytotox Dye. These paired images enable the neural network to infer cell death responses from the phase contrast image alone. These two AI models were combined to form the Incucyte® AI Cell Health Analysis Software Module, which was validated on multiple cell types using fluorescent markers for comparison. During validation studies, cells were treated with cytotoxic compounds in the presence of Incucyte® Cytotox Dye which enters non-viable cells, increasing their fluorescence intensity. Quantification of cell death was performed using both AI Cell Health Live | Dead classification (AI-driven, label-free analysis) and fluorescence classification of Cytotox positive cells. Cell image classification, evaluation of cell death time courses, and concentration response curves determining compound efficacy were used to confirm that cells exhibiting high fluorescence of Incucyte® Cytotox Dye are also being classed as dead by the Incucyte® AI Cell Health Analysis Software Module (Figure 4).

Images of HeLa cells treated with camptothecin (1.1 µM, 24 hr) with applied live (top image) and dead (bottom image) classification masks (Figure 4A) indicate a high correlation between cells which were analyzed using both label-free analysis (outline class mask) and fluorescence analysis (filled class mask). Time-course analyses of % dead cells induced by increasing concentrations of cisplatin (top graph, label-free analysis; bottom graph, fluorescence analysis) display similar time and concentration dependent responses (Figure 4B). The concentration response curves compare % death at 72 hr for 4 cytotoxic compounds calculated using label-free and fluorescence analysis (Figure 4C).

This validation was performed across a wide range of adherent and non-adherent cell lines in monoculture and confirmed that the label-free Incucyte® AI Cell Health Analysis accurately identifies cell death induced by compounds with different efficacies and mechanisms of action.
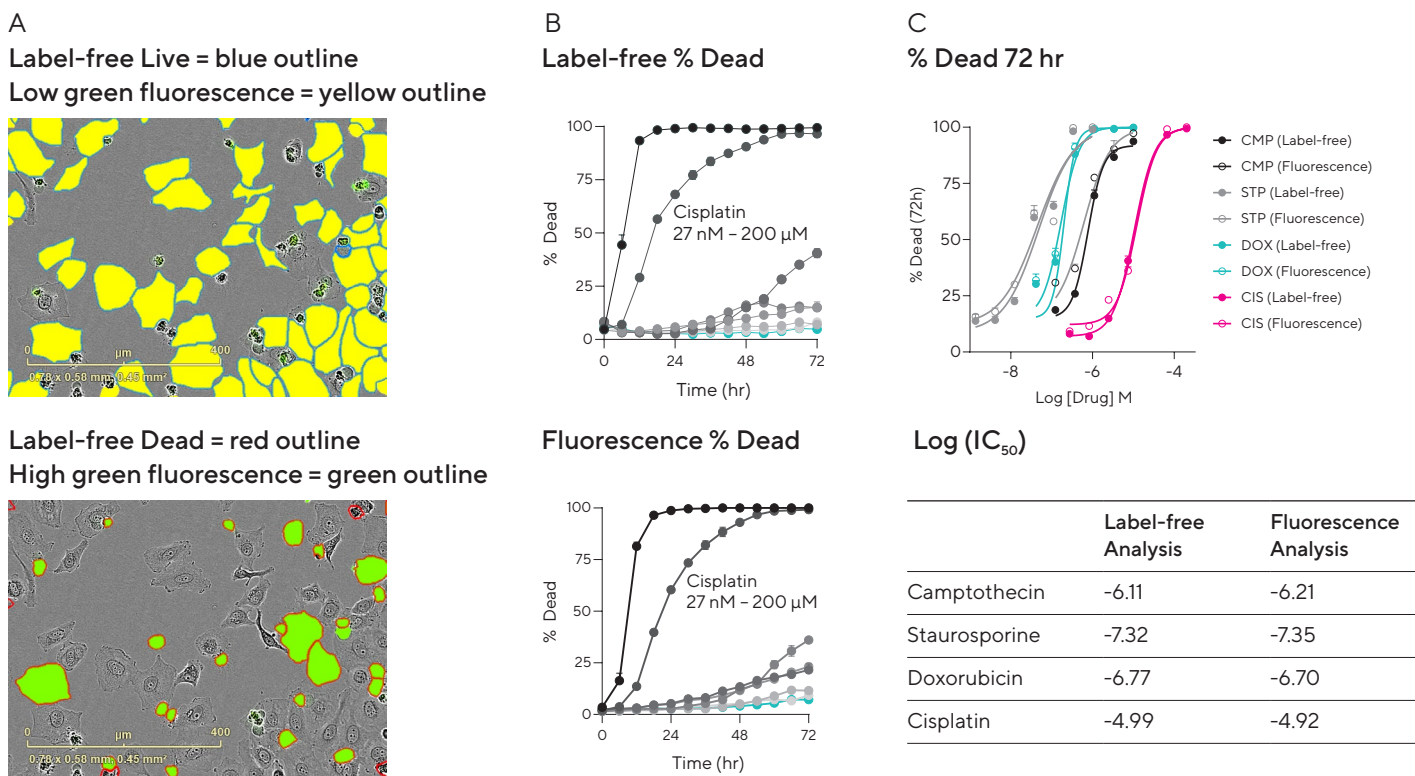
A
Label-free Live = blue outline
Low green fluorescence = yellow outline

Label-free Dead = red outline
High green fluorescence = green outline

B
Label-free % Dead

Fluorescence % Dead

C
% Dead 72 hr

Log (IC$_{50}$)

	Label-free Analysis	Fluorescence Analysis
Camptothecin	-6.11	-6.21
Staurosporine	-7.32	-7.35
Doxorubicin	-6.77	-6.70
Cisplatin	-4.99	-4.92

Figure 4: *AI Cell Health Analysis Generates Comparable Cytotoxicity Data to Standard Fluorescence Methods.*

HeLa cells were treated with concentration ranges of 4 different cytotoxic compounds in the presence of Incucyte® Cytotox Dye. Images show HeLa cells 24 hr post treatment with camptothecin. Top image (A) shows classification of live cells using Incucyte® AI Cell Health label-free analysis (blue outline) and fluorescence analysis of Cytotox Negative cells (yellow fill). Bottom image shows classification of dead cells (red outline) and cytotox positive cells (green fill) in the same image. Time courses (B) show the percentage of dead cells over time using label-free analysis (top row) or fluorescence classification (bottom row) of HeLa cells treated with increasing concentrations of cisplatin. Concentration response curves (C) plot cell death at 72 hr post treatment of camptothecin (CMP, black), staurosporine (STP, grey), doxorubicin (DOX, teal) and cisplatin (CIS, magenta). The table indicating log IC$_{50}$ values confirms that across compound with different mechanisms of action the efficacy values as calculated by label-free and fluorescence methods are comparable.

Label-Free Analysis of Cytotoxicity in Microplate Throughput

Incucyte® AI Cell Health Analysis is conducive to compound screen experiments in 96 and 384- well microplates. The highly adaptable analysis can be applied to cells with a wide range of morphologies providing directly comparable datasets, and visualization of the % dead cells per well in microplate view (Figure 5A) enables rapid and simple identification of cytotoxic compounds or conditions. End-point analysis at 48 hr post-treatment (Figure 5B) was used to confirm assay window and identify mechanisms of action between the vehicle (teal point) and positive control (high camptothecin, magenta point).

Vehicle control and similar compounds induced no inhibitory effect on cell growth (% confluence) and cell viability (% live) – these cluster in the top right of the scatter plot. Cytostatic compounds typically reduce cell growth but do not reduce cell viability – these compounds cluster in the top middle. In contrast, cytotoxic compounds reduce both cell growth and viability, clustering in the bottom left part of the plot.

A
Jurkat cells, % Dead 0 – 72 hr
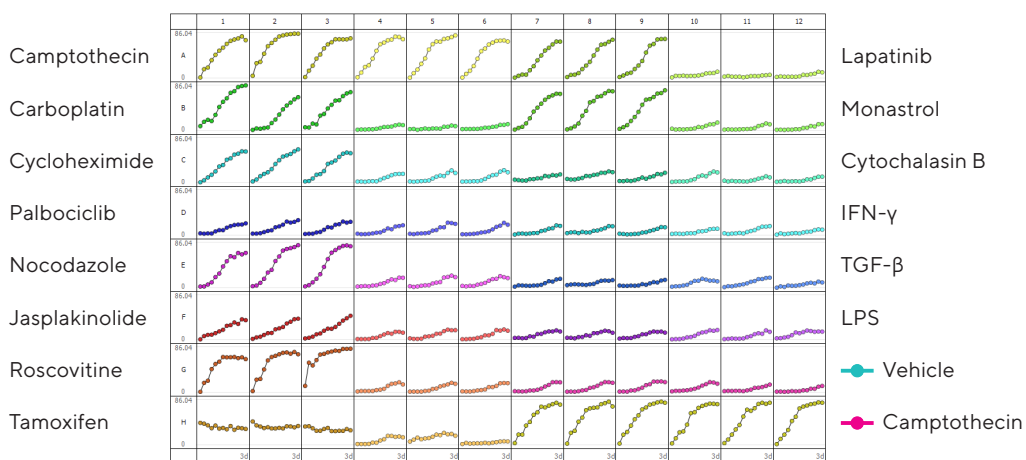
B
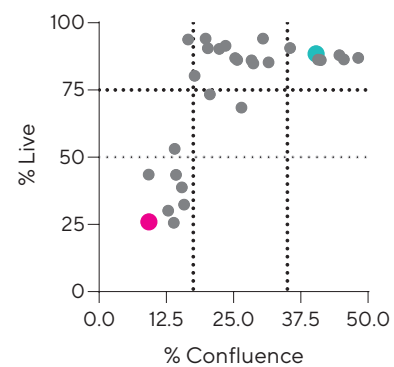**Jurkat cells, % Live vs % Confluence, 48 hr**

Figure 5: *Analysis of Compound Effects in Non-adherent Cells.*

Jurkat cells were seeded into 96-well plates coated with poly-L-ornithine and briefly centrifuged to lightly adhere to the plate surface. Cells were treated with 14 compounds in triplicate wells with high and low concentrations of each and placed into the Incucyte® to acquire phase contrast images every 2 hr for 3 days. Incucyte® AI Cell Health Analysis was used to quantify the % dead cells in each image over time (A). Mechanism of action was examined by correlating cell viability (AI Cell Health % live) with cell growth (AI Confluence) at 48 hr post treatment. Vehicle (teal) conditions displayed high viability and high growth; cytostatic compounds exhibit reduced cell growth but viability remains high; cytotoxic compounds including positive control camptothecin (10 µM, magenta) reduce both viability and growth.

Combined Label-Free and Fluorescence Analyses Yield Additional Insight Into Mechanism of Apoptosis

Incucyte® AI Cell Health Analysis Software Module provides label-free analysis of live and dead cells, however label-free analysis also enables deeper insight into cell behavior when combined with optional fluorescence measurements. Staurosporine is known to induce cell death via both caspase-dependent and caspase-independent mechanisms. To examine these pathways, MDA-MB-231 cells were treated with staurosporine (1 μM) in the presence of pan-caspase inhibitor Z-VAD-FMK (3 – 250 μM). To measure caspase activation, Incucyte® Caspase 3/7

Apoptosis Dye was included. Phase contrast and fluorescence images were acquired every 2 hr for 3 days. Total cell death was quantified using Incucyte® AI Cell Health Analysis and indicated that staurosporine induced rapid cell death in the presence of all concentrations of Z-VAD-FMK (Figure 6A). Within the dead cell population, caspase activation was measured using fluorescence classification. Time course (Figure 6B) shows that the number of caspase positive dead cells decreased as the concentration of Z-VAD-FMK increased with efficacy log IC_{50} = -4.3 M (Figure 6C).
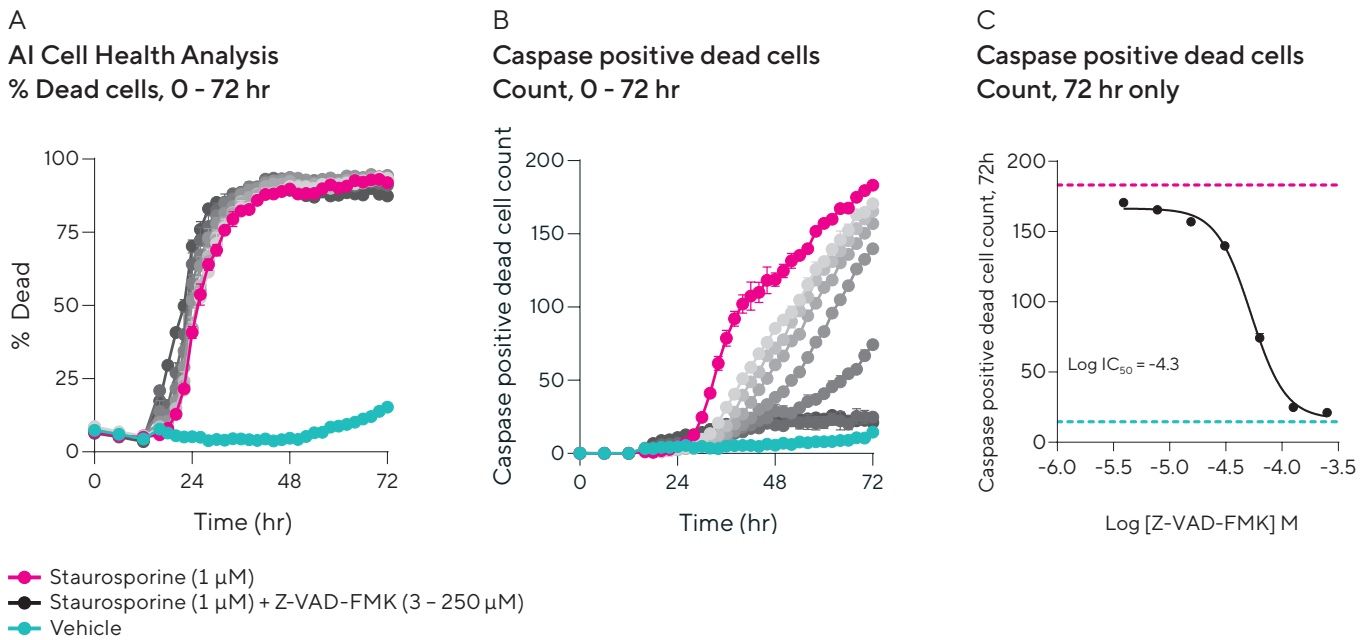
A
AI Cell Health Analysis
% Dead cells, 0 - 72 hr

B
Caspase positive dead cells
Count, 0 - 72 hr

C
Caspase positive dead cells
Count, 72 hr only

- Staurosporine (1 μM)
- Staurosporine (1 μM) + Z-VAD-FMK (3 – 250 μM)
- Vehicle

Figure 6: *Label-Free Analysis With Additional Fluorescence Information Reveals Mechanisms of Apoptosis.*

MDA-MB-231 cells were treated with staurosporine (1 μM, magenta) in the presence of caspase inhibitor Z-VAD-FMK (3 - 250 μM, grey) and Incucyte® Caspase 3/7 Apoptosis Dye. Total cell death was quantified using AI Cell Health label-free classification, and time course indicates that staurosporine induces cell death in the presence of all concentrations of Z-VAD-FMK (A). Fluorescence classification of caspase activity within the dead cell population reveals that Z-VAD-FMK reduces the number of caspase positive dead cells in a time-and concentration-dependent manner (B, C).

Summary & Conclusion

Incucyte® AI Cell Health Analysis Software Module enables accurate cell segmentation and live/dead classification. Using trained neural networks integrated into the Incucyte® live-cell analysis workflow, we have enabled user-friendly deployment of AI models for cell analysis which provide label-free quantification of cell death over time. Label-free analysis yields non-perturbing quantification of cytotoxicity which is increasingly vital when using precious patient-derived cell types. However by combining the label-free analysis with optional fluorescence readouts, additional information on the mechanisms of apoptosis can also be revealed.

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