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Label-Free Imaging and Kinetic Analysis in Live Cells

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Introduction

Cells are the fundamental units of life and the place where scientists look for answers to questions about a range of biological events. To that end, monitoring cell growth, morphology and behavior is vital to both our understanding of human diseases, and the development of treatment strategies. However, cell biology is not a static process; it is a complex series of events that unfold over days, and weeks. Capturing accurate, high-quality information requires round-the-clock observation using technologies that are not disruptive to cells, or to the phenomena under study.

As the use of complex, physiologically relevant cell models becomes more common, there is a growing need for label-free, non-perturbing solutions that deliver deep insights (Figure 1). Fluorescent labeling prolongs workflows and complicates analysis by introducing an additional variable that can influence study outcomes. Eliminating fluorescent reporters altogether ensures that experimental observations are not attributed to the label, or to the labeling process itself.

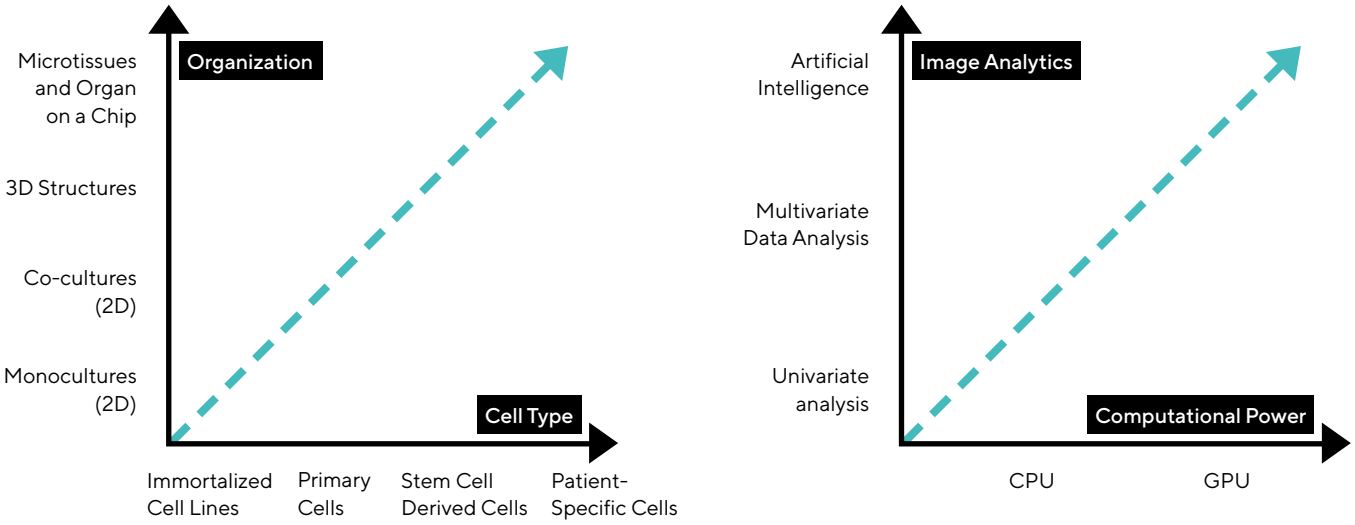
Label-free analysis is a non-invasive and non-perturbing method for studying the activity of cell populations when fluorescent labeling is not feasible, such as with rare cell types. Rapid advancements in live-cell analysis and computational power in recent years has provided more solutions for real-time, label-free analysis of cell behavior and function (Figure 1). Biologists can use these advanced tools to continuously observe and quantify cell behavior in complex cultures, completely label free.

Find out more: www.sartorius.com/incucyte-proliferation

The Incucyte® Live-Cell Analysis System is well known in the industry for performing real-time, high throughput quantification studies in living cells. This system comprises a microscope that sits inside a standard tissue culture incubator, enabling round-the-clock time-lapse imaging of cells in their natural environment. The integrated image analysis and seamless data visualization for cell-based experiments provides rich insights without the risk of missing important biological events.

In this white paper, we describe recent developments in software tools for label-free analysis on the Incucyte® Live-Cell Analysis System, namely the Incucyte® Cell-by-Cell Analysis Software Module for uni-variate analysis, Incucyte® Advanced Label-Free Classification Software Module for multi-variate analysis, and Incucyte® AI Confluence Analysis, which is our first software module to use a neural network, a type of deep learning associated with artificial intelligence (AI). We demonstrate how these tools can be applied for label-free kinetic analysis in routine culture assays.

Figure 1
Label-Free Cell Analysis



Note. Drug discovery is moving towards complex and more relevant cell models. Advancements in real-time, label-free imaging technologies and computational power provide deeper insights about cell behavior and morphology, in a non-perturbing way with less user input.

Solutions for Label-Free Analysis

The Incucyte® Live-Cell Analysis System offers a suite of powerful computational tools for label-free analysis of live-cells, using time-lapse high definition (HD) phase-contrast images.

- Incucyte® AI Confluence Analysis (now offered within the Incucyte® Base Analysis Software) measures confluence, which is routinely used in normal tissue development, regeneration and renewal.[1] Confluence measures the percent area of each image that is occupied by cells and uses this information to calculate cell proliferation. The original Classic Confluence Analysis uses traditional computer vision methods to separate cells from background in every image and requires user input for accurate segmentation. The AI Confluence Analysis Module uses a neural network to automatically identify and separate cells from unwanted objects, with little user input. This software was trained using a wide range of cell type examples, allowing a single analysis to be applied to most cell types.
- Incucyte® Cell-by-Cell Analysis provides an actual count of cell number in each image, which is different than the approach used in Incucyte® AI Confluence Analysis. The module first segments cells as objects that are different than background, and then groups cells into subsets based on either fluorescence, area or eccentricity (shape). Using fluorescence, area, or eccentricity allows for single-metric (uni-variate) label-free classification of adherent or non-adherent cells.

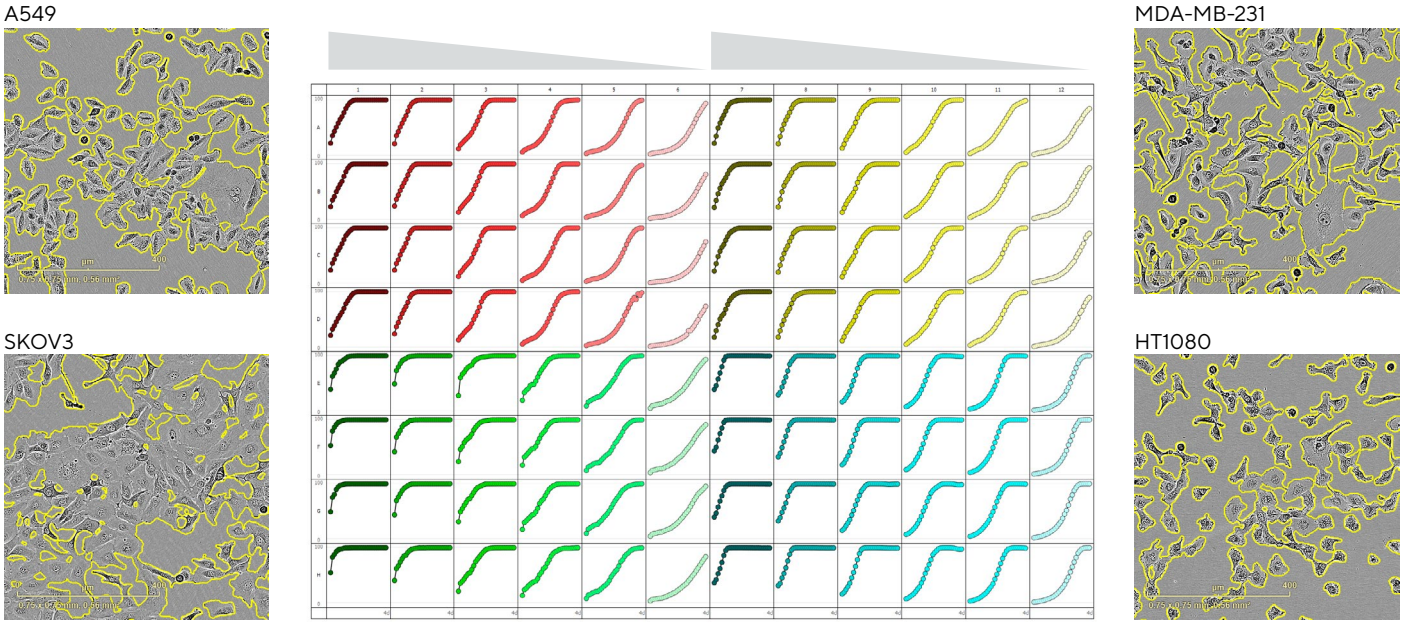
- Incucyte® Advanced Label-Free Classification is an add-on to the Incucyte® Cell-by-Cell Analysis Software Module that automatically identifies more complex changes in cell morphology using label-free segmentation and multi-variate analysis. Cells are first segmented using the Incucyte® Cell-by-Cell Analysis algorithm, and then processed through a trained classifier that simultaneously analyzes each cell based on 25 different shape metrics under the symmetry, area, texture, circularity, perimeter length and solidity categories. This information is then used to classify cells into two user-defined groups based on morphology and quantify the percent of each cell sub-group over time. Incucyte® Advanced Label-Free Classification Analysis can be used to monitor processes involving a change in morphology, such as cell death, differentiation, and mitosis.

Cell Growth (Confluence)

Cell growth is a routine measurement in cell culture for basic research and clinical studies, like drug safety screening. We used Incucyte® AI Confluence Analysis to measure the growth of four different cell types, A549, SKOV3, MDA-MB-231, and HT-1080, in real time (Figure 2). We seeded cells at increasing densities and used Incucyte® AI Confluence Analysis to measure confluence in images acquired every 2 hours, over 4 days, directly from the culture plate. The plate view provides easy visualization of cell growth across the entire 96-well plate, showing faster growth rates correlated with higher seed densities. All cells reached 100% confluence by Day 4.

The Incucyte® AI Confluence Analysis also successfully segmented different cell morphologies in a single analysis job. Transparent SKOV3 cells and the MDA-MB-231 cells with characteristic long thin protrusions typically require additional optimization using the Classic Confluence Module. In contrast, Incucyte® AI Confluence Analysis easily masked all cell types without user optimization. This data demonstrates that Incucyte® AI Confluence Analysis can be used to measure cell growth in a non-perturbing way, providing fast and easy analysis across different cell morphologies.

Figure 2
Incucyte® AI Confluence Analysis of Cell Growth



Note. A549, MDA-MB-231, SKOV3 and HT-1080 cells were seeded into a 96-well plate each at a range of densities (250–8,000 cells/well), indicated from high to low seed density by gray triangles. Cells were allowed to settle and placed into an Incucyte® Live-Cell Analysis System. High-definition (HD) phase-contrast images were acquired every 2 hours for 4 days and images were analyzed using Incucyte® AI Confluence Analysis. Analysis was automatically performed after every image acquisition enabling real-time, kinetic measurement of cell confluence. Images show each cell type (seeded at 250 cells/well, image at 96 hr). Plate view displays % Confluence over time for the full 96-well plate.

Phagocytosis

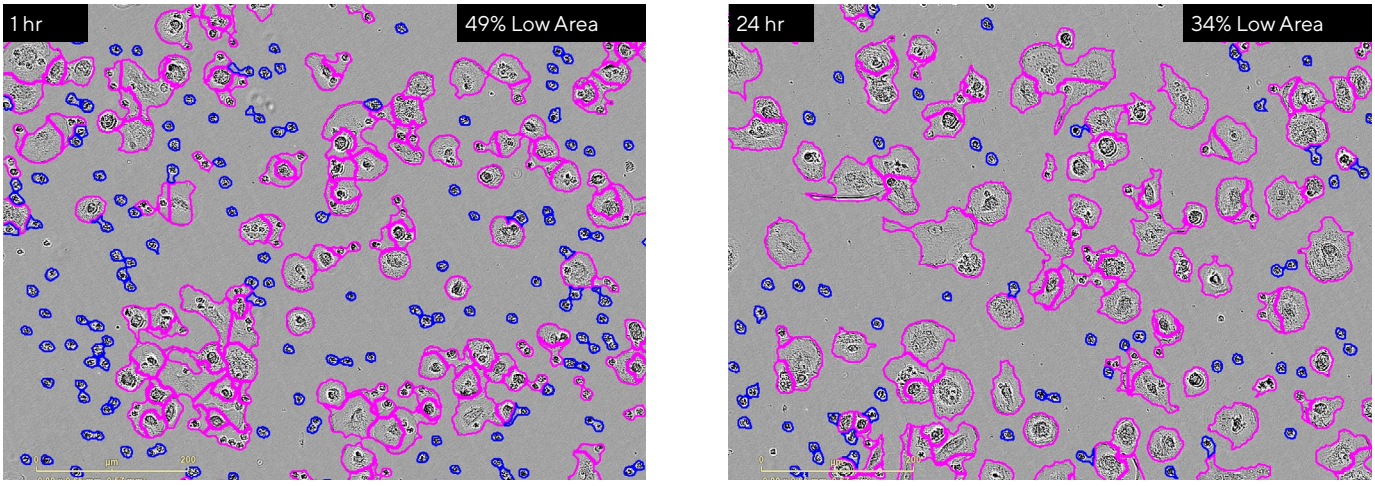
The immune system uses the process of phagocytosis to clear pathogens or infected cells. During this process, macrophages engulf apoptotic cells. Phagocytosis is traditionally quantified using fluorescent labeling of target cells, but we applied Incucyte® Cell-by-Cell Segmentation followed by a uni-variate area classification to quantify apoptosis in a non-perturbing, label-free way (Figure 3).

We added apoptotic Ramos target cells to primary macrophages and acquired images over the course of 24 hours on the Incucyte® Live-Cell Analysis System. After 1 hour, 49% percent of low-area target Ramos cells remained (3A-B, left panels). This number is reduced to 34% after 24 hours (3A-B, right panels), showing continued phagocytosis. This type of analysis is not possible using the Incucyte® AI Confluence Analysis as it does not count individual cells.

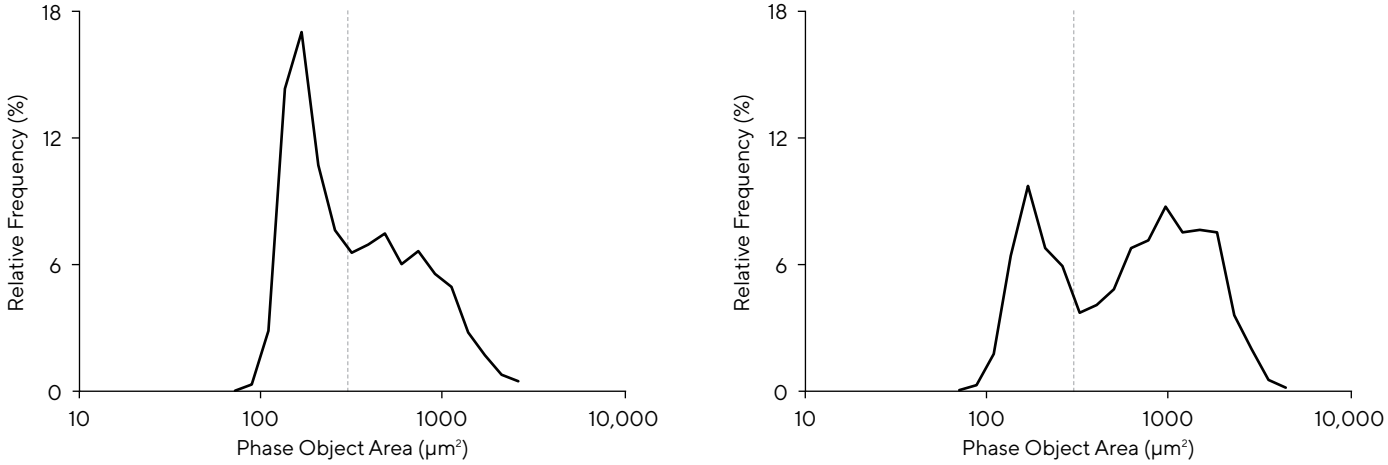
Figure 3

Quantifying Cell Phagocytosis using Incucyte® Cell-by-Cell Analysis Software Module

A.



B.



Note. Apoptotic Ramos target cells were added to primary macrophages. Images were acquired in the Incucyte® Live-Cell Analysis System and Incucyte® Cell-by-Cell Analysis Software Module was used to quantify phagocytosis of target cells overtime. (A) Images show classification of cells by phase object area into two populations, high-area effector cells (pink) or low-area target Ramos cells (blue), at 1 hr and 24 hr. (B) Histograms display frequency distribution of cells in co-culture at both timepoints with the gate value used for classification shown (dashed line; 300 µm²).

Differentiation

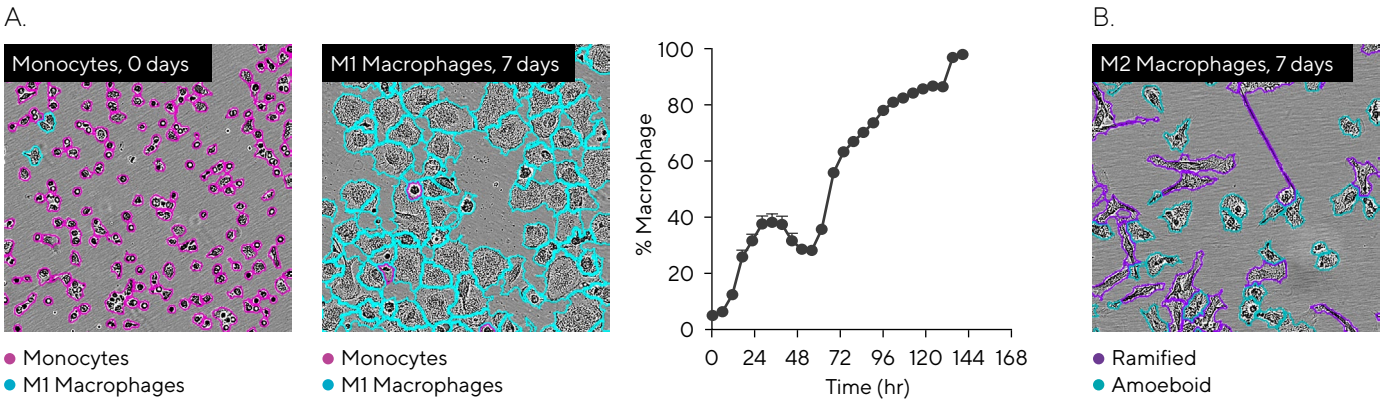
Studying the dynamics of cell morphology reveals valuable insights into cell health, but traditional approaches for this type of analysis involve labeling schemes and multi-step workflows. We used the Incucyte® Live-Cell Analysis System and Incucyte® Advanced Label-Free Classification Software Module to monitor monocyte differentiation into M1 and M2 macrophages, using multi-variate analysis (Figure 4).

We extracted primary monocytes from fresh blood and induced differentiation into M1 or M2 macrophages. Using the Incucyte® Live-Cell Analysis System, we acquired images every 6 hours, for 7 days. Cells were segmented using the integrated Incucyte® Cell-by-Cell Analysis Software Module and the add-on Incucyte® Advanced Label-Free Classification Software Module, which enables cell classification into two groups based on morphology.

The data revealed differentiation from a predominantly monocyte cell population into M1 and M2 cell populations. The monocyte to M1 macrophage classification showed how cells can change morphology over time (Figure 4A), while the M2 classification into ramified and amoeboid sub-populations showed that within a heterogeneous population of cells, two distinct classes can be identified (Figure 4B). This study demonstrates that label-free multivariate analysis on the Incucyte® Live-Cell Analysis System can be used to successfully separate cells based on morphology.

Figure 4

Incucyte® Advanced Label-Free Classification Software Module for Quantification of Differentiation



Note. Primary monocytes were extracted from fresh blood and differentiated into M1 or M2 macrophages. Images were acquired using Incucyte® Live-Cell Analysis System every 6 hours for 7 days. Cells were segmented using integrated Incucyte® Cell-by-Cell Analysis Software Module and Incucyte® Advanced Label-Free Classification Software Module. Analysis was performed to identify (A) monocytes and M1 macrophages; and (B) ramified and amoeboid subpopulations within M2 macrophages. Images show monocytes, M1 and M2 macrophages. Pink and teal outlines indicate cell classification as either monocytes (pink) or M1 macrophages (teal); and ramified (purple) and amoeboid (blue) macrophages. Time-course shows % macrophage cells over 7 days.

Compound-Induced Effects

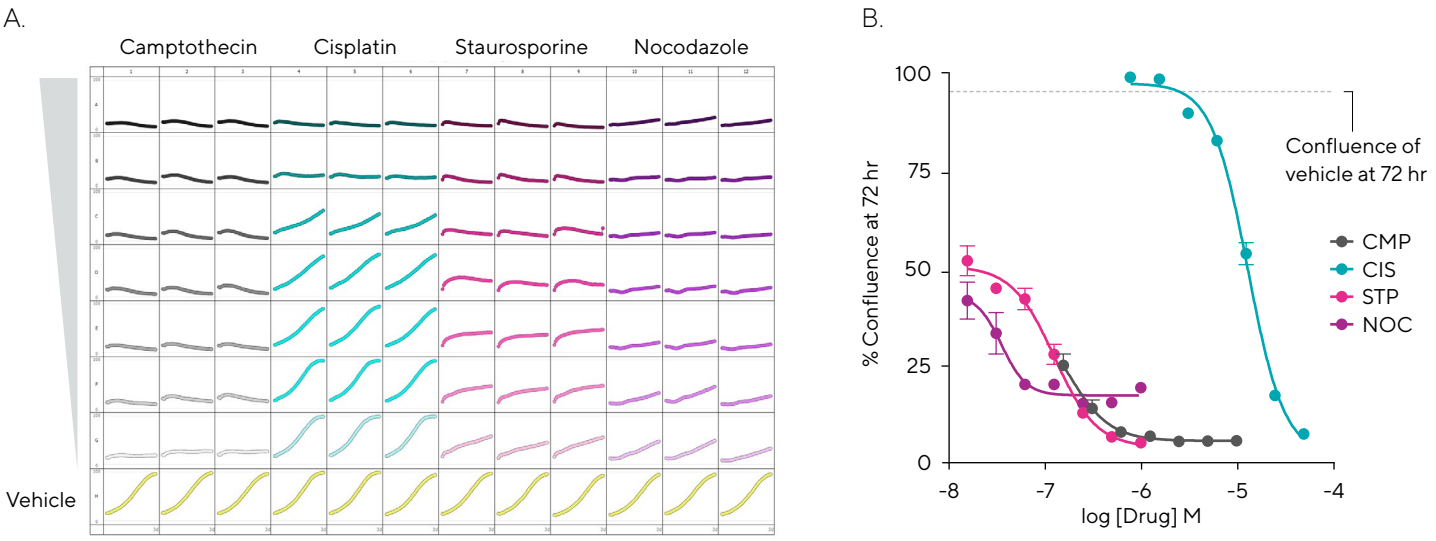
Cell health assays are a critical step when evaluating new therapeutic drug candidates to treat cancer and other diseases. The Incucyte® Live-Cell Analysis System and the integrated software for high-throughput label-free analysis help accelerate workflows, while generating data that is reproducible, reliable, and free of bias.

We used Incucyte® AI Confluence Analysis to monitor and quantify cell growth in the presence of four compounds known to affect cell proliferation with different mechanisms of action (Figure 5). We seeded A549 cells into a 96-well plate, and after a period of growth, added a different compound to each well, at increasing concentrations.

Images were acquired every 2 hours, for 3 days, and the percent confluence was quantified using Incucyte® AI Confluence Analysis. The plate-level view shows both time- and concentration-dependent inhibition of cell growth in the presence of all compounds (Figure 5A). The chemotherapy drug cisplatin showed nice dose-response behavior, while the topoisomerase inhibitor, camptothecin, had the strongest effect on confluence, even at the lowest concentration tested.[2,3]

Figure 5

High Throughput, AI-Driven Label-Free Analysis of Cell Proliferation

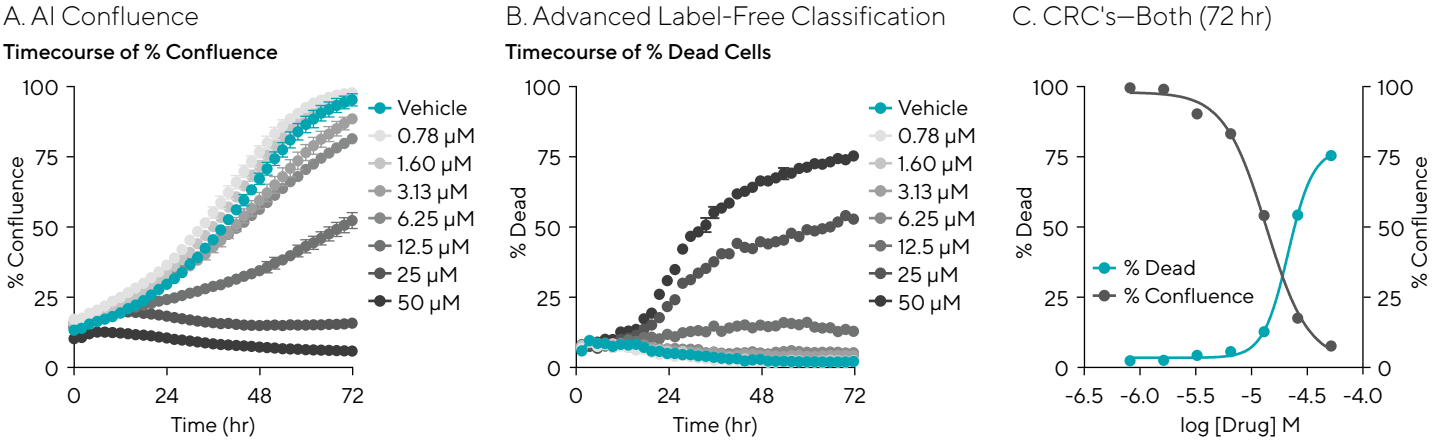


Note. A549 cells were seeded into a 96-well plate at 2,000 cells/well. After 18 hours, cells were treated with concentration ranges of camptothecin (0.2–10 µM), cisplatin (0.8–50 µM), staurosporine (16 nM–1 µM) and nocodazole (16 ng/mL–1 µg/mL). Images were acquired every 2 hours for 3 days, and % Confluence was quantified using Incucyte® AI Confluence Analysis. (A) Plate view shows % confluence over 3 days for the 96-well plate and demonstrates time- and concentration-dependent inhibition of cell growth. Experiments were performed in triplicate; gray triangle indicates decreasing compound concentration. (B) Concentration response curves show % confluence at 72 hours in the presence of each compound; line indicates % confluence of vehicle at 72 hours. Camptothecin, staurosporine and nocodazole inhibit cell growth at all concentrations.

Next, we expanded on the cisplatin study with more detailed analysis. We set up the experiment following the protocol used in the previous study, and quantified the images using both Incucyte® AI Confluence Analysis and Incucyte® Advanced Label-Free Classification Analyses. Overlay of the AI Confluence time-course data shows concentration-dependent inhibition, as observed in the previous experiment (Figure 6A). In order to classify live and dead cells during the same time course, we used Incucyte® Advanced Label-Free Classification Software Module to classify cells based on multiple shape parameters. We observed similar efficacy to the Incucyte® AI Confluence Analysis results (Figure 6B). The two top concentrations of cisplatin were associated with the most cell death.

Figure 6

Label-Free Analysis Provides Insight Into Effect of Cisplatin



Note. A549 cells were seeded into a 96-well plate at 2,000 cells/well. After 18 hours, cells were treated with a concentration range of cisplatin (0.8–50 µM). HD phase-contrast images were acquired every 2 hours for 3 days and images were quantified using both Incucyte® AI Confluence (A) and Advanced Label-free Classification Analyses (B). Time-course of % Confluence (A) shows a time- and concentration-dependent inhibition of cell proliferation, while time-course of % dead cells (B) indicate that the two highest concentrations of cisplatin induce high levels of cell death over 3 days. Concentration response curves (C) demonstrate a similar inhibition of cisplatin on cell proliferation (EC_{50} = 13.1 µM) and cytotoxicity (EC_{50} = 19.9 µM).

Multi-Compound Screening

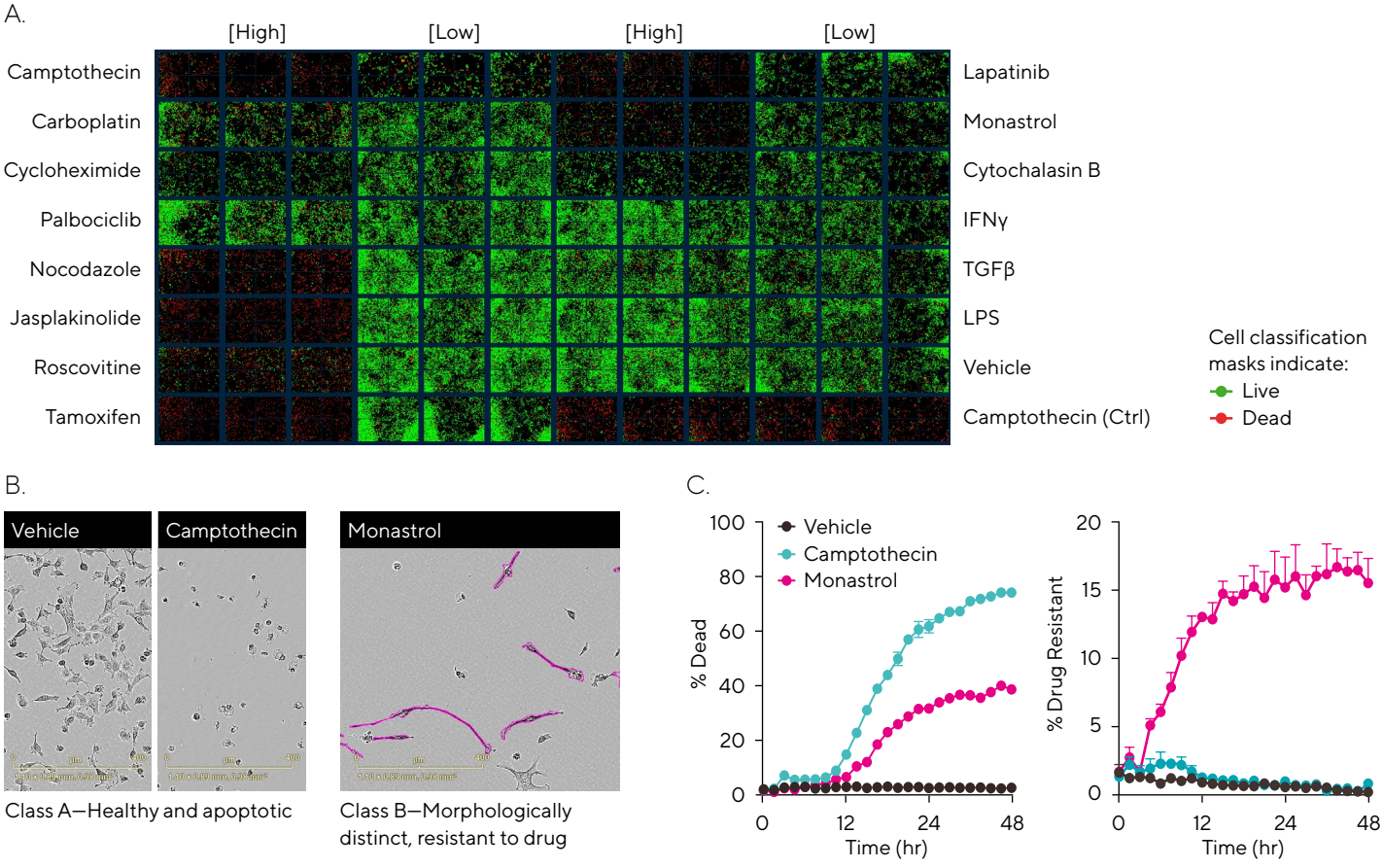
The Incucyte® Live-Cell Analysis System with associated Software Modules provide easy analysis and visualization of complex datasets to facilitate quick identification of cytotoxic compounds in high throughput. We performed a screen of 14 compounds and two controls using Incucyte® Advanced Label-Free Classification Software Module to classify cells by morphology (Figure 7).

We seeded HT-1080 cells into a 96-well plate, and, after a period of growth, added two concentrations of 14 compounds in triplicate wells. A deep plate view shows a heatmap of live (green) and dead (red) cells, allowing high-level visualization of cytotoxicity (Figure 7A). Using Incucyte® Advanced Label-Free Classification, we were able to isolate and quantify different cell morphologies in wells associated with two of the most cytotoxic compounds, camptothecin and monastrol, a kinesin inhibitor (Figure 7B). Time-course analysis showed 80% cell death with camptothecin and 30% cell death with monastrol, 48 hours post treatment (Figure 7C, left plot).

Cells treated with camptothecin displayed apoptotic morphology that was distinct from control wells. Interestingly, the cells treated with monastrol displayed a mixture of live and dead cells. The live, drug-resistant cells were morphologically distinct long, thin cells (pink classification mask). A second Incucyte® Advanced Label-Free Classification Analysis was performed on the drug-resistant pool and showed that they made up 18% of the total cells in the monastrol-treated population (Figure 7C, right plot). This type of analysis is only possible with label-free analysis and cannot be achieved using a fluorescent cell death reporter.

Figure 7

High-Throughput Compound Screen



Note. HT-1080 cells were seeded into a 96-well plate at 2,000 cells/well, and treated after 18 hours with two concentrations each of 14 compounds with different mechanisms of action. Experiments were performed in triplicate. All six positive-control wells (bottom right) contain camptothecin at 10 µM. Images were acquired every 2 hours for 3 days, and cells were segmented using integrated Incucyte® Adherent Cell-by-Cell Analysis. Incucyte® Advanced Label-Free Classification Software Module was used to identify live and dead cells. (A) Deep view shows an overview of the classification masks for each well at 48 hours, where green masks indicate live cells and red masks indicate dead cells. (B) Images of cells at 48 hours treated with vehicle, camptothecin (left) and monastrol (right), showing morphologically distinct long, thin cells (pink classification mask). (C, left plot) Time-course of % dead cells in the presence of camptothecin (teal line), monastrol (pink line), or vehicle (black line). (C, right plot) Time-course of drug-resistant cell morphology over the course of treatment with camptothecin (teal line), monastrol (pink line), or vehicle (black line).

Conclusion

The experiments described here demonstrate how the Incucyte® Live-Cell Analysis System coupled with integrated software for label-free analysis allow scientists to achieve precise, robust and unbiased measurement of complex cell behavior and morphology, in a non-perturbing way. The range of purpose-built software tools offer users simple cell health assessment in high-throughput drug screening campaigns, to more complex analysis of heterogeneous cell models using cell segmentation and multi-variate analysis. Streamlined workflows and automated segmentation aided by AI-driven approach, provide users with actionable insights with less user input and help accelerate discovery, improve reproducibility and productivity of their workflows.

Further Reading

Application Note: Kinetic Quantification of Cell Proliferation Using Live-Cell Analysis

Proliferation Assays for Live-Cell Analysis

Incucyte® Cell-by-Cell and Advanced Label-Free Morphological Analysis

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