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Application Note

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Advanced Label-Free Classification of Cell Morphology Subpopulations

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Introduction

The morphology of a cell contains vast amounts of information on cell health and differentiation state, and yields insight into cell phenotype. Biologists use this information daily to drive decisions around cell culture conditions and responses during assay development. Traditionally this important information has been described qualitatively or via the use of single metrics as a surrogate for total cell shape. However, these methods are subjective evaluations and can lead to loss of data and a lack of robustness and reproducibility within cell-based assays.

Objective quantification of morphology enables researchers to make data-driven decisions for successful cell culture propagation and experimentation. Morphological data can be used as a kinetic readout to determine compound effects—for example, counting the number of cells with viable versus apoptotic morphology yields a direct measure of cytotoxicity. Furthermore, using label-free image analysis to derive these measurements has the advantage of being completely non-perturbing to cell cultures, ensuring that the data generated is not an artifact of the detection method. This can be vital when using highly sensitive or rare cell types.

Find out more: www.sartorius.com/live-cell-analysis-software

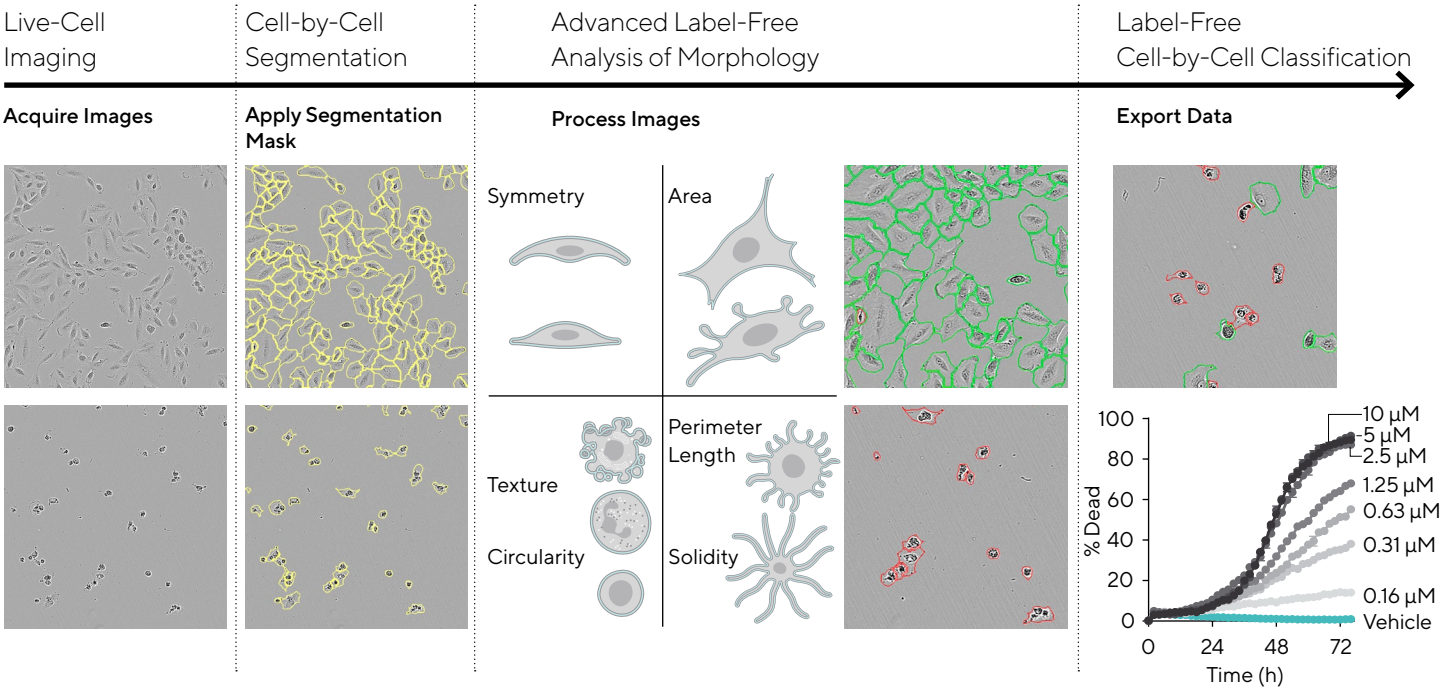
The Incucyte® Advanced Label-Free Classification Software Module enables automated quantification of cell morphology by employing multivariate analysis to identify multiple morphological features such as cell area, texture, brightness, and symmetry. These parameters are then used to create an unbiased, meaningful score value that enables cell subpopulations to be classified into two user-identified groups.

Overview of Incucyte® Advanced Label-Free Classification Analysis

The Incucyte® Advanced Label-Free Classification Analysis Software Module is an add-on to the Incucyte® Cell-by-Cell Analysis Software Module. It enables two classes of cells to be identified by their morphology and quantified over time in kinetic assays.

This workflow is summarized in Figure 1. Images of cells are acquired using the Incucyte® Adherent Cell-by-Cell scan setting, and individual cells are segmented using the integrated software. Advanced classification can then be applied, where the classifier is trained using control images of the two classes of interest. For example, to perform a label-free live | dead assay, the live class is represented using healthy, growing cells at a range of confluence values and the dead class is represented by images of dead cells when a cytotoxic compound has taken effect. Once the classifier has been trained to detect these two morphological classes, it can be applied to any other images containing the same biological model. Integrated software automatically classifies individual cells and the percentage of cells in each class over time can be visualized.

Figure 1
The Incucyte® Advanced Label-Free Classification Analysis Workflow



Note. This workflow can be applied to any use case in which two subpopulations of cells have distinct morphology. For example, mitotic cells can be identified within a culture; undifferentiated monocytes can be distinguished from macrophages.

Method

Mammalian cells have a wide range of different morphologies, which can be characterized in several ways—they vary in size (area, outer perimeter length), shape (aspect ratio, solidity) and texture. Incorporating all these features within the Incucyte® Advanced Label-Free Classification Analysis Software Module, we have employed multivariate analysis that uses over 20 metrics describing different cell attributes. For every cell these metrics are distilled onto a single axis, resulting in a score value between 0 and 1. Dead cells will have a score close to 0 whereas live cells will have a score nearer to 1. A threshold is then applied to group the cells into one of two classes. Where the threshold is set at 0.5, all cells with scores < 0.5 will be classed as 'dead' and those > 0.5 will be classed as 'live.'

Figure 2 demonstrates this classification process. A549 cells were treated with a concentration range of camptothecin to induce cell death in the presence of Incucyte® Annexin V reagent for the purpose of comparing the label-free multivariate response to that of a known apoptosis detection reagent.

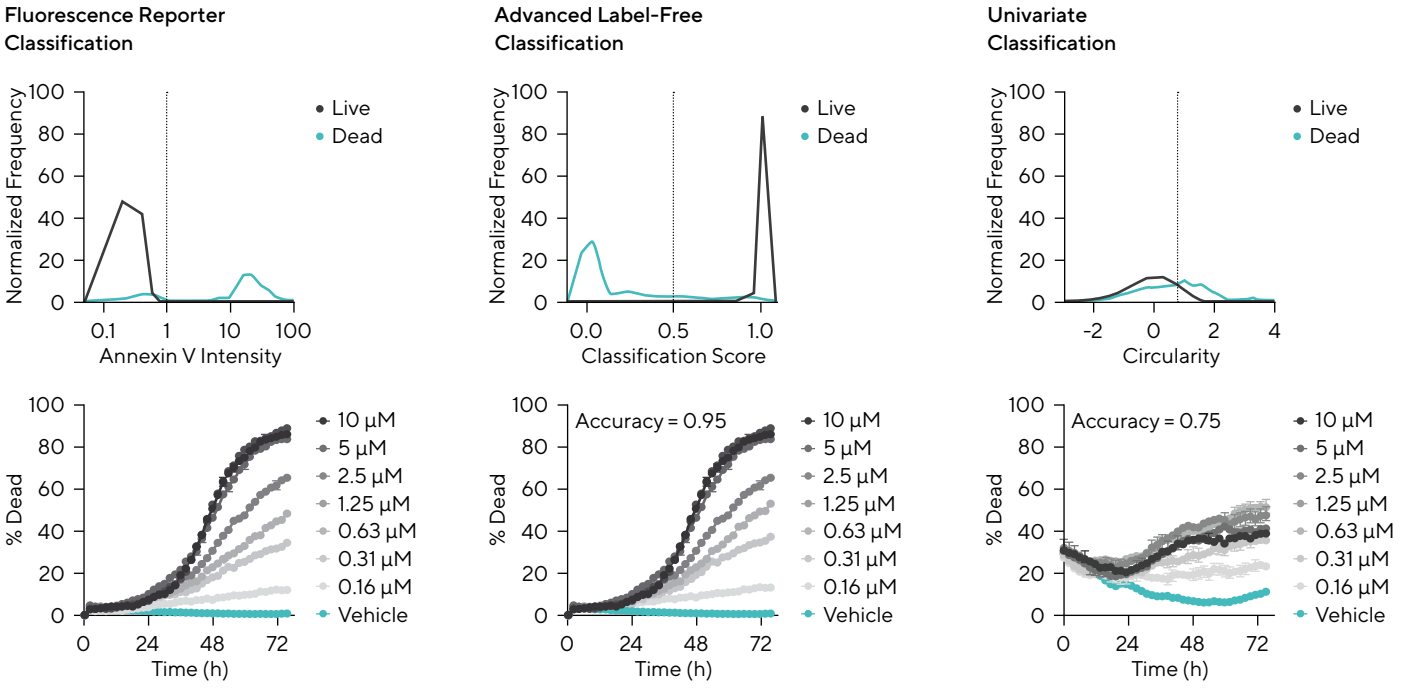
Histograms show the fluorescence intensity, advanced label-free classification score value, or a univariate circularity value for control images of live and dead cells (Figure 2, top row). Both fluorescence and Incucyte® Advanced Label-Free Classification Analysis methods show clear separation between the classes; however the use of the label-free circularity metric on its own results in overlapping populations. Thresholds were used in each case to identify live versus dead cells (indicated by the dashed line on the histogram plots), and the time courses below show the percentage of dead cells per image through time (Figure 2, bottom row).

Fluorescence and Incucyte® Advanced Label-Free Classification Analysis show similar time- and concentration-dependent increases in the percentage of dead cells. While classification based only on the circularity of the cell yields concentration-dependent effects, the time course displays a high percentage of cell death in the untreated (vehicle) cells—an observation that is not reflected upon examining the images of cells.

Incucyte® Advanced Label-Free Classification Analysis and univariate (circularity) classification methods were compared to the standard fluorescence classification method using a confusion matrix. These results confirmed that Incucyte® Advanced Label-Free Classification Analysis is more accurate (accuracy = 0.95) than the label-free univariate method (accuracy = 0.75).

Figure 2

Fluorescent, Incucyte® Advanced Label-Free, and Univariate Classification Analyses



Note. Incucyte® Advanced Label-Free Classification Analysis of live and dead cells yields similar results to the use of fluorescent Incucyte® Annexin V reagent. Univariate analysis is less accurate (relative to fluorescence classification) than multivariate analysis via Incucyte® Advanced Label-Free Classification Analysis.

Applications

This workflow can be adapted to a wide range of applications, including label-free detection of dead cells. In this application note we will demonstrate the use of Incucyte® Advanced Label-Free Classification Analysis Software Module within three biological models: 1) a label-free live | dead assay; 2) detection of mitotic cells within a cell cycle assay; and 3) label-free differentiation and morphological analysis of macrophage subpopulations.

Label-Free Live | Dead Assay

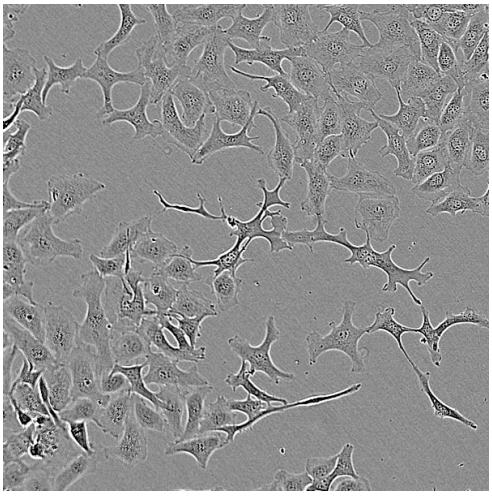
Advanced label-free classification can identify dead cells without the requirement for a fluorescent reagent. Therefore, it is an ideal solution for measurements of cytotoxicity where highly sensitive cell types are used and in cases where fluorescent channels are being dedicated to monitor other biologically relevant events. The label-free live | dead assay was validated against a panel of cancer cell lines with a wide variety of morphologies. Each cell type was treated with a concentration range of camptothecin (CMP), cisplatin (CIS), staurosporine (STP) and nocodazole (NOC) in the presence of Incucyte® Annexin V reagent for comparison. Figure 3 displays the morphology of SKOV3 ovarian cancer cells upon treatment, demonstrating that each compound results in a different morphological change.

Cell death was determined using Incucyte® Advanced Label-Free Classification Analysis Software Module, as well as fluorescence classification to identify annexin V-positive (apoptotic) cells. Phase HD images show that dead cells are visible in CMP and CIS treated conditions, while NOC treatment alters the form of the cell without cytotoxicity. STP induces rapid cell death and the apoptotic bodies are accompanied by a large amount of dead cell debris. These four compounds have different mechanisms of action and yield varied cytotoxic responses. The plate view shows the time course of percentage of dead cells calculated using the Incucyte® Advanced Label-Free Classification Analysis tool. STP induces rapid cell death even at low concentrations, while CMP induces cell death more slowly. In comparison, CIS induces only partial cytotoxicity at the highest concentration tested and NOC, which targets the cytoskeleton, appears to lack any concentration-dependent cytotoxicity.

Figure 3

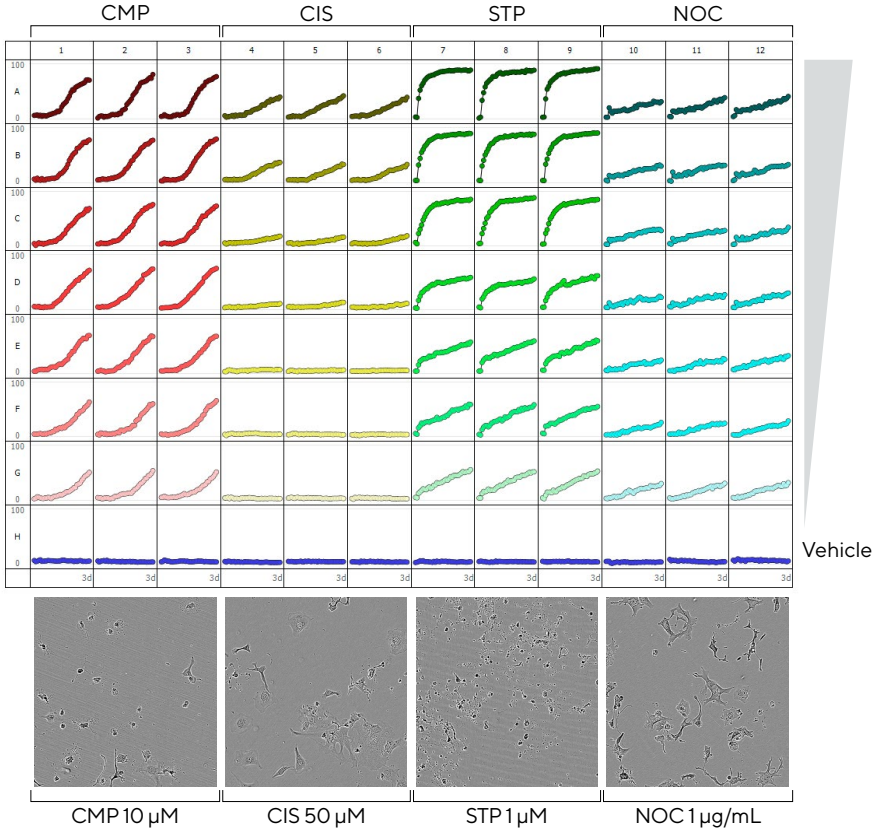
Label-Free Live | Dead Analysis of SKOV3 Cells

Vehicle SKOV3 Cell Morphology, 72 h



Note. Plate view shows the percentage of dead cells over time calculated using the Incucyte® Advanced Label-Free Classification Analysis tool. Phase HD images show cell morphology at 72 h post-treatment.

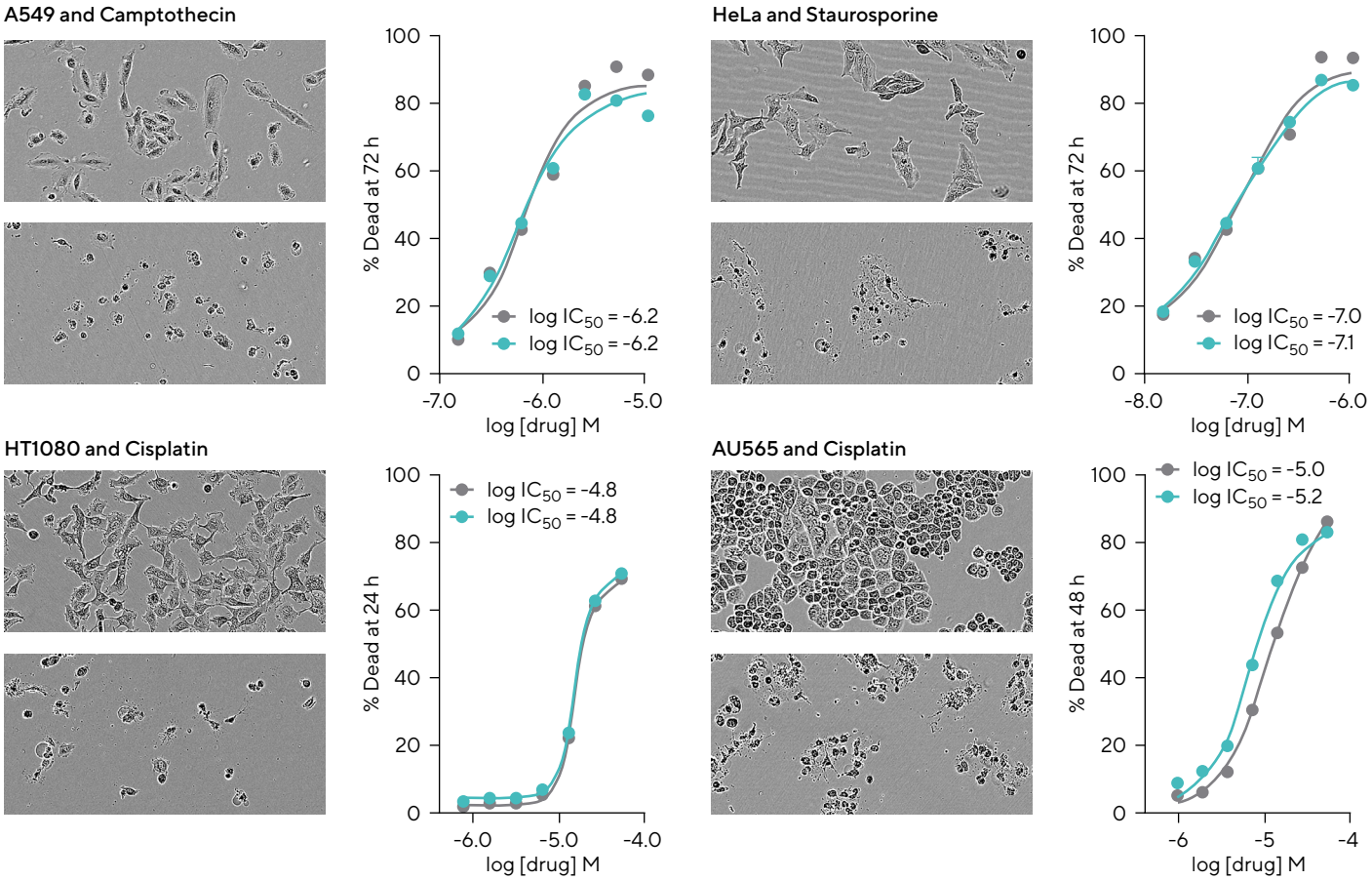
Advanced Label-Free Classification % Dead SKOV3 Cells



Validation studies revealed that Incucyte® Advanced Label-Free Classification Analysis yielded comparable results to fluorescence classification across a wide range of adherent cell types and compound treatments. Figure 4 shows that EC_{50} values for three cytotoxic compounds (CMP, STP, and CIS) calculated using Incucyte® Advanced Label-Free Classification Analysis were similar to those calculated using fluorescent cell health reagents across multiple cell types.

Figure 4

Response Curves for Incucyte Advanced Label-Free Classification Analysis and Fluorescent Reagents



Note. Diverse cell types display comparable concentration response curves using fluorescent cell health reagents (gray) and Incucyte® Advanced Label-Free Classification Analysis (teal). Pairs of images display untreated (vehicle, top) and treated cells (bottom image, highest concentration).

Label-Free Live | Dead Assay With Cell Cycle Multiplex

Label-free analysis is beneficial in circumstances where the cell type under investigation is highly sensitive, and the use of a cell health reagent is not desirable. It can also add valuable information in situations where the fluorescence channels are reporting other data such as cell cycle phase.

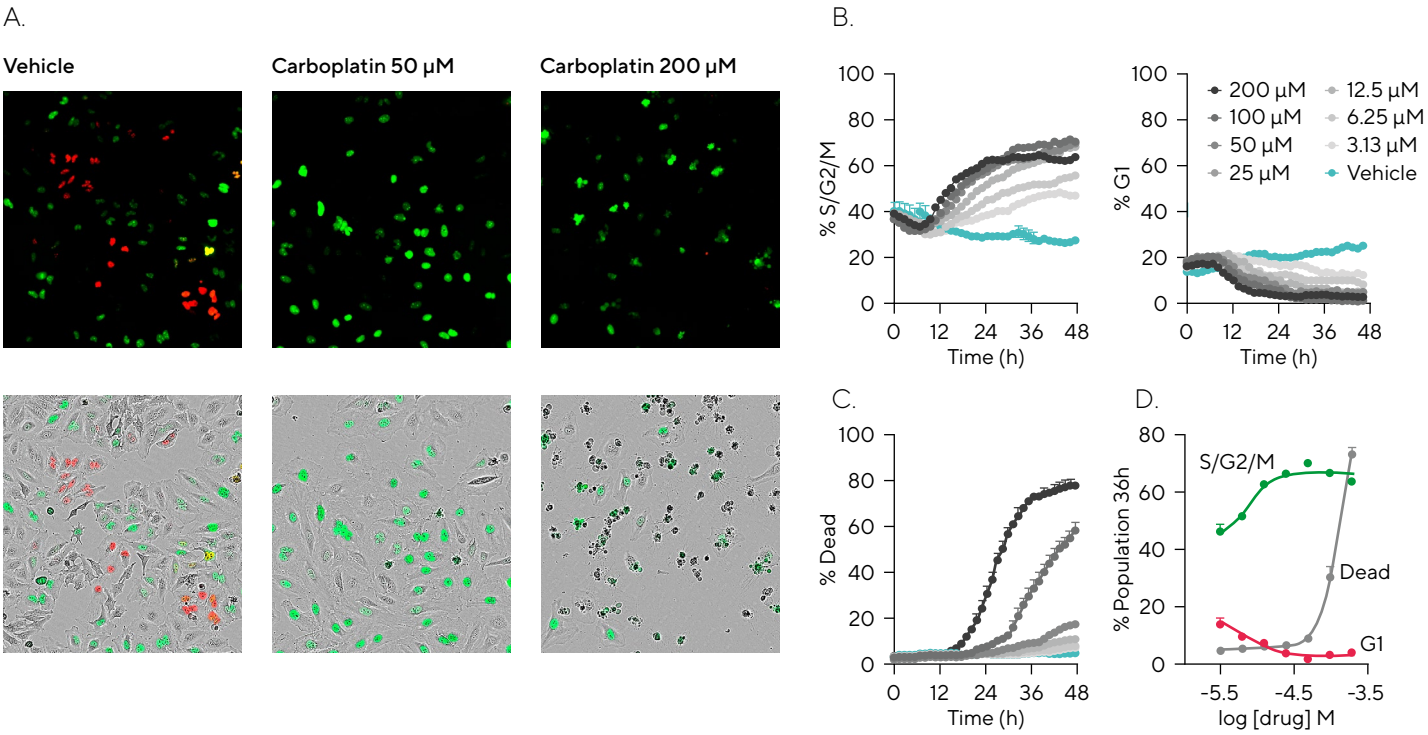
Figure 5 demonstrates the use of Incucyte® Advanced Label-Free Classification Analysis with cells expressing Incucyte® Cell Cycle Lentivirus reagent. These cells express green fluorescence in the S | G2 | M phases of the cell cycle, non-fluorescence in the transition phase M→G1, red or orange fluorescence in G1, and yellow fluorescence (red or orange + green) in the transition phase G1→S. A healthy, growing culture will display a mixture of all four populations as displayed in Figure 5A (vehicle).

HeLa cells stably expressing Incucyte® Cell Cycle Lentivirus reagent were treated with increasing concentrations of carboplatin, a DNA-binding chemotherapeutic that induces cell cycle arrest and apoptosis. Fluorescence images indicate cell cycle arrest at 50 µM and 200 µM, where a high percentage of cells are in S | G2 | M and display green fluorescence (Figure 5A, carboplatin). This observation is reflected in the time course of percent cells in S | G2 | M and G1 phases (Figure 5B).

Phase HD images reveal that in the presence of 50 µM carboplatin, cells have a normal morphology resembling that of the vehicle cells while those treated with 200 µM carboplatin have an apoptotic morphology. Incucyte® Advanced Label-Free Classification Analysis was used to identify live and dead cells, and the time course of percentage of dead cells indicates a cytotoxic effect at the two highest concentrations (Figure 5C). Overlay of the concentration response curves (Figure 5D) indicates the window between maximal cell cycle arrest and induction of apoptosis.

Figure 5

Cell Death Measurements With Incucyte® Advanced Label-Free Classification Analysis



Note. Incucyte® Advanced Label-Free Classification Analysis enables cell death measurements in cells expressing Incucyte® Cell Cycle Lentivirus reagent. A healthy, growing culture displays a mixture of all four cell populations in various phases of the cell cycle (A). After the addition of carboplatin, fluorescent images indicate cell cycle arrest, where a high percentage of cells are in S | G2 | M and display green fluorescence (B). Incucyte® Advanced Label-Free Classification Analysis indicates a cytotoxic effect at the two highest concentrations of carboplatin (C). Overlay of the concentration response curves indicates the window between maximal cell cycle arrest and apoptosis (D).

Label-Free Mitotic Cell Detection With Cell Cycle Multiplex

In addition to detection of dead cells, Incucyte® Advanced Label-Free Classification Analysis can be used to identify other morphologies of interest, such as mitotic cells. Using fluorescence classification, the Incucyte® Cell Cycle Lentivirus reagent enables users to detect four distinct populations of cells based on their stage of the cell cycle: S | G2 | M (green), M→G1 (non-fluorescent) transition, G1 (red or orange), G1→S (red or orange + green) transition. With Advanced Label-Free Classification, cells in mitosis can be identified by their unique morphology, providing quantification of a fifth population.

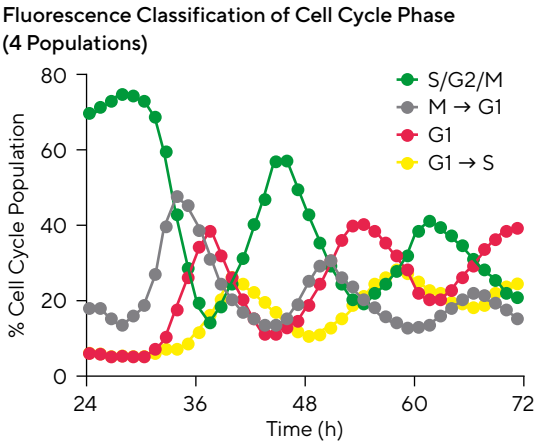
To exemplify this, HeLa cell cycle cells were synchronized using a thymidine block. Cells were treated with thymidine (2.5 mM) for 24 hours until 80% accumulated in S | G2 | M. At 24 hours, the block was removed and the cells progressed synchronously through the cell cycle and began to divide once again. The time course of four fluorescent populations in Figure 6A demonstrates that each population peaks in sequence as the cells move through the cycle.

As the schematic shows (Figure 6B), after S | G2 | M, the cells move through the non-fluorescent M→G1 transition into expressing red or orange fluorescence in G1, and red or orange + green in the G1→S transition. Approximately 9 hours after the removal of the thymidine block, images were observed that contained a high percentage of mitotic cells as indicated by their small, circular and dense morphology. Advanced training set selection enabled this mitotic cell subpopulation to be used to train a classifier to detect mitotic versus non-mitotic cells.

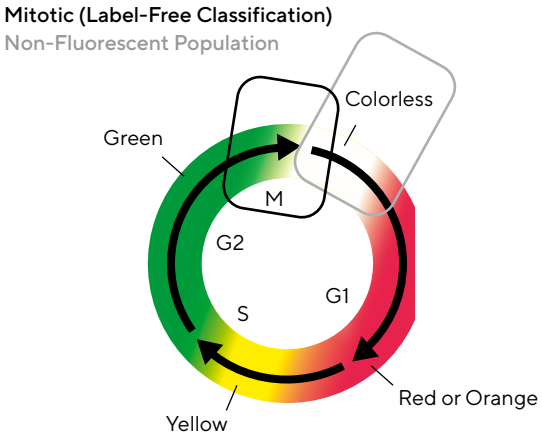
Figure 6

Identification of Five Subpopulations Within a Synchronized HeLa Cell Cycle Culture

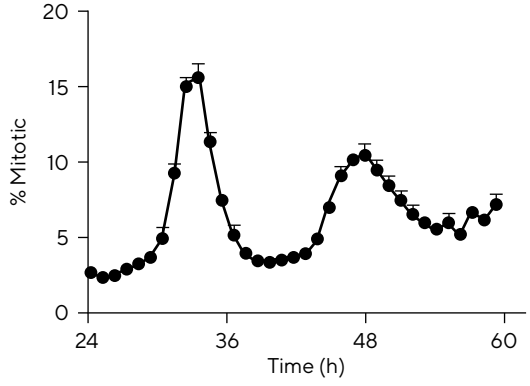
A.



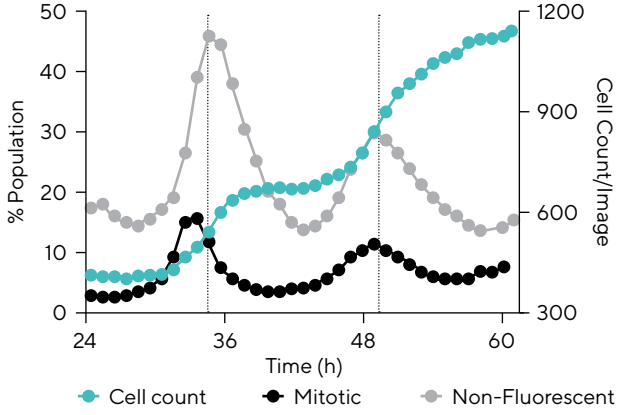
B.



C.



D.



Note. Time course of fluorescent populations (A) indicate the changing cell cycle phase (B); time course of mitotic cells (C) are indicated in black. Cell count (teal circles, D) overlays the mitotic (black circles) and non-fluorescent (gray circles) populations.

The time course of mitotic cells in Figure 6C displays the same peaking profile as the fluorescent populations. Overlay of time courses (Figure 6D) confirms that the mitotic population (black) peaks immediately prior to that of the non-fluorescent M -> G1 transition (gray), and that during this time the cell count (teal) experiences a step-wise increase.

Overall, these data demonstrate that the use of fluorescent markers with label-free morphological information enables researchers to extend the biological insight of subpopulations within live cells simultaneously.

Label-Free Differentiation Assay

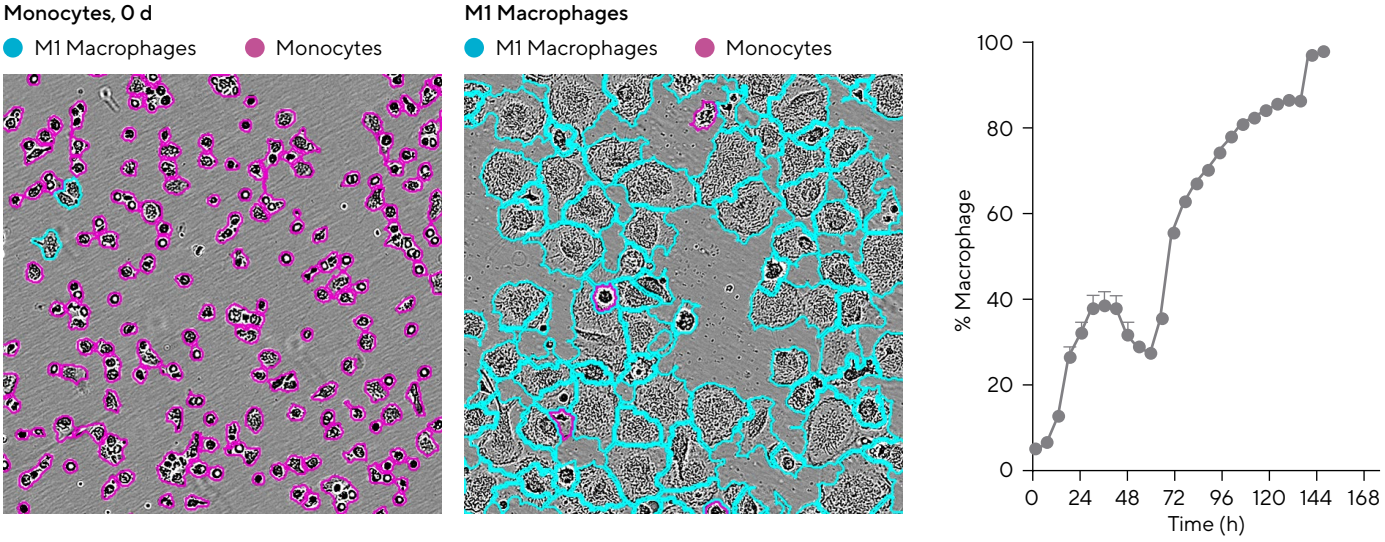
The activation and differentiation of immune cells is often accompanied by morphological changes. For example, monocytes are a key component of the innate immune system and can differentiate into a number of functional immune cells such as macrophages. Under the influence of pro- or anti-inflammatory cytokines at the site of recruitment, these macrophages can be further activated to M1 or M2 phenotypes.

Figure 7 demonstrates the morphological changes observed upon differentiation of primary human monocytes to M1 and M2 macrophages. While monocytes are small, dense cells the M1 macrophage population is comprised of large, flat amoeboid cells. Incucyte® Advanced Label-free Classification Analysis was employed to distinguish monocytes (Figure 7A, pink segmentation mask) from M1 macrophages (Figure 7A, teal segmentation mask). The time course revealed that differentiation to M1 macrophages occurred over a seven-day period; the differentiation process was non-linear.

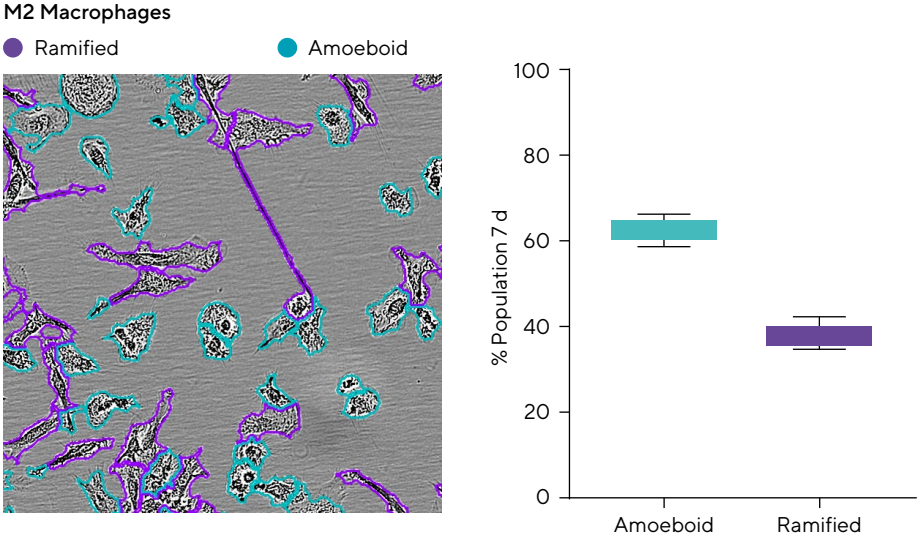
Figure 7

Quantification of Morphological Populations Within Differentiation Assays With Incucyte® Advanced Label-Free Classification Analysis

A.



B.



Note. Incucyte® Advanced Label-free Classification Analysis was employed to distinguish monocytes (A, pink segmentation mask) from M1 macrophages (A, teal segmentation mask). The M2 macrophage population displayed a mixture of ramified (long, thin) and amoeboid (round, flat) morphologies. Using Advanced Training set selection, these two subpopulations were manually identified and used to train the software. Segmentation color indicates classification results (B). The data showed that within this biological model, 38% of cells were ramified (B, purple segmentation) and 62% cells were amoeboid (B, teal segmentation).

Summary and Conclusion

Using Phase HD images of control wells, the Incucyte® Advanced Label-Free Classification Analysis can be trained following a simple workflow to yield robust and reproducible data without the need for fluorescent reporters. Advanced training set selection allows users to identify cells of interest within an image, enabling enhanced control over training set selection. This software module can be applied to a variety of biological models including a live | dead assay, which can be employed using label-free images or multiplexed with additional fluorescent readouts; identification of mitotic cells adds another population of interest to cell cycle assays. Differentiation of monocytes to macrophages can be quantified without the requirement for fluorescent reporter reagents, and mixed morphologies such as ramified versus amoeboid can be further investigated.

Incucyte® Advanced Label-free Classification is a versatile software module enabling quantification of user-defined morphological subpopulations through time.

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