Application Note

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Innovative AI Techniques for Accurate Analysis of Cell Morphology and Viability

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Abstract

Accurate segmentation of cell boundaries is the first and most critical step underlying the quantification of cell morphology. Label-free imaging enables cell behavior to be monitored in a non-perturbing environment, yet images can be challenging to analyze. Cells in culture have widely varying and fluid morphologies with certain types of cells appearing almost transparent; they grow from sparsely populated to highly confluent. In such a dynamic system, continuously and accurately detecting the boundaries between each cell presents a challenge for traditional computer vision approaches. Here we demonstrate the advantages of using a widely trained neural network for segmentation of different cellular models and using the resulting morphological descriptors to classify cells using a machine learning-based classifier.

Introduction

The use of label-free imaging for long-term cell monitoring is a powerful approach for examining live cells, providing longitudinal insight into cell behavior, function, and response to external treatment or stimulus. However, these images can be complex and display dynamic cell movement, providing a challenge for robust and accurate quantification.

We previously developed an AI-based cell segmentation tool which results in highly accurate outline of cell boundaries, allowing different types of cells to be counted over time as well as forming an accurate report of cell shape.[1] This cell segmentation was combined with a second neural network that classifies each segmented cell as alive or dead, and the resulting AI Cell Health (AI CH) Analysis module provides a fully label-free solution for quantification of cell proliferation and cytotoxicity.

Cell morphology is varied and complex, with live cells dynamically moving over time. Analysis of these morphological states is not trivial, and reporting morphology changes using single metrics such as area can be highly reductive. Machine learning based classifiers make use of many variables or metrics – known as high-dimensional data – to group cells into classes. This approach accounts for multitudes of cell features including area, roundness, texture, brightness, symmetry for every cell, and provides a simplified readout to the user.

The combined use of AI CH Analysis with Advanced Label-free Classification (ALFC) allows users not only to count live and dead cells, but also to perform further classification of cells based purely on their shape. This morphology classification is trained by the user on the morphologies specifically found in their own images and can be applied either to all cells, or selectively within either the live or dead cell subpopulations, providing deeper insights into cell behavior.

In this application note we highlight several examples in which AI CH has been used for cell segmentation and viability classification, and these cells have been further analysed using ALFC to measure morphological changes within a variety of biological models.

Materials & Methods

Cell Culture

Media formulations and materials used for cell culture and assays are described below (Tables 1 and 2). Cell lines were cultured in their respective growth medium (Table 1) and monitored using the Incucyte® Live-Cell Analysis System (Incucyte® System). A549 adenocarcinoma cell line was transfected with Incucyte® Nuclight NIR Lentivirus reagents to induce expression of a nuclear restricted fluorescence protein. A549 Nuclight NIR were maintained in medium containing 2 µg/mL puromycin, however assays were performed in the absence of puromycin.

Cell culture	Base Medium	FBS	Penicillin-Streptomycin	Puromycin	Supplements
THP-1	RPMI	10%	1%	N/A	
Growth: A549Nuclight NIR	F12K	10%	1%	2 µg/mL	
Growth: HeLa WT	F12K	10%	1%	N/A	
Assay: A549Nuclight NIR	F12K	10%	1%	N/A	
Stabilization: iPSC Microglia	Advanced DMEM F12	N/A	N/A	N/A	1X GlutaMAX, 50 µM 2-Mercaptoethanol, 1X N2, 0.1 µg/mL Doxycycline 10 µM ROCKi, 50 ng/mL M-CSF
Maturation: iPSC Microglia	Advanced DMEM F12	N/A	N/A	N/A	1X GlutaMAX, 50 µM 2-Mercaptoethanol, 1X N2, 10 ng/mL M-CSF, 100 ng/mL IL-34

Table 1. *Media formulations used for cell culture and monocyte differentiation.*

Materials	Supplier	Cat. No.	Final Concentration
ioMicroglia™	Bit.bio	Io1021	
Advanced DMEM F12	Gibco	12634010	
RPMI 1640	Gibco	21875-034	
F12K	Gibco	21127-022	
GlutaMax	Gibco	35050061	1X
2-Mercaptoethanol	Gibco	31350010	50 µM
N2	Gibco	17502001	1X
Doxycycline	Sigma	D9891	0.1 µg/mL
ROCKi (Y-27631)	Abcam	Ab144494	10 µM
FBS	Cytiva HyClone™	SH30071.03	10%
Penicillin-Streptomycin	Gibco	15140122	1%
Poly-L-Ornithine (PLO)	Merck	P4957	0.01%
Poly-L-Lysine	Sigma	P4707	0.01%
IL-34	Peprotech	200-34	100 ng/mL
M-CSF	Sartorius	CYK-0100-0031	10 – 50 ng/mL
Costar™ 96-well flat-bottom plate	Corning™	3595	
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P8139	100 nM
Camptothecin (CMP)	Sigma	C9911	10 µM
Erastin (ERA)	Merck	329600	0 – 20 µM
FIN56 (FIN)	Merck	SML1740	0 – 20 µM
RSL3 (RSL)	Merck	SML2234	0 – 20 µM
PBS	Gibco	14190-94	
Trypsin-EDTA (0.25 %)	Gibco	25200-056	

Table 2. *Materials required for cell culture and assays.*

Monocyte Differentiation

THP-1 monocytes were seeded in RPMI media at 15,000 cells per well in 96-well plates pre-coated with PLO (Table 1). For monocyte to macrophage differentiation, cells were treated with phorbol 12-myristate 13-acetate (PMA) for 4 days with a media refresh performed after 3 days. Cultures were monitored in the Incucyte® System and high-definition phase-contrast images (20X magnification) were acquired over 7 days.

iPSC Microglia Culture

Human iPSC-derived microglia were seeded at 8,000 cells per well in 96-well plates pre-coated with Poly-L-Lysine (PLL). Cell cultures were stabilized and matured with media changes every 1 to 3 days and monitored in the Incucyte® System for up to 10 days.

Senescence and Cytotoxicity Assay

A549 Nuclight NIR cells were seeded at 2,000 cells per well in 96-well plates and left overnight at 37 °C to adhere. Vehicle control (VEH) or concentration range of camptothecin (CMP) was added to the cells in 100 µL medium containing 2x final assay concentration of each compound. Plates were placed into the Incucyte® System and cell response was monitored for up to 3 days.

Compound Screen

A549 WT cells were seeded at 500 cells per well, 45 µL volume in a 384-well microplate and left overnight at 37 °C to adhere. Vehicle control (VEH) or compound treatment was added to the cells in 5 µL volume at 10x final assay concentration of each compound. Plates were placed into the Incucyte® System and cell response was monitored for up to 3 days.

Results

Analysis Overview

The datasets described in this application note have been analyzed using label-free methods; AI Cell Health (AI CH) Analysis – which segments cells and classifies them as live or dead – is followed by Advanced Label-free Classification (ALFC). No fluorescent reporters are required for this workflow.

ALFC uses multivariate analysis to classify cells based on a comprehensive list of metrics which describe cell morphology. Simply put, the user chooses images from their assay which represent morphology group A and morphology group B, and a machine learning based classifier is automatically trained based on the two different morphology patterns found in those images.

For example, during differentiation monocytes change from small, rounded cells with high contrast into slightly larger cells that still maintain a rounded morphology but with a more textured cytoplasm. Although the monocytes and macrophages cannot be reliably identified using a single metric such as area due to significant overlap, multivariate analysis using several metrics (e.g., area, roundness, texture, brightness) can ensure clear separation of the two cell populations.

The use of ALFC with AI CH has two advantages. Firstly, the AI-based segmentation accurately segments a wide range of cell morphologies, meaning that the shape is accurately reported over time. Previous segmentation algorithms were optimized towards a single morphology and often struggled to maintain accuracy while cells changed morphology, or where more than one morphological subtype was present.

Secondly, ALFC can be performed within all cells segmented by AI CH or within the live or dead subpopulations. This means that for example dead cells can be excluded from the label-free morphology classification, and different subpopulations of live cells can be identified.

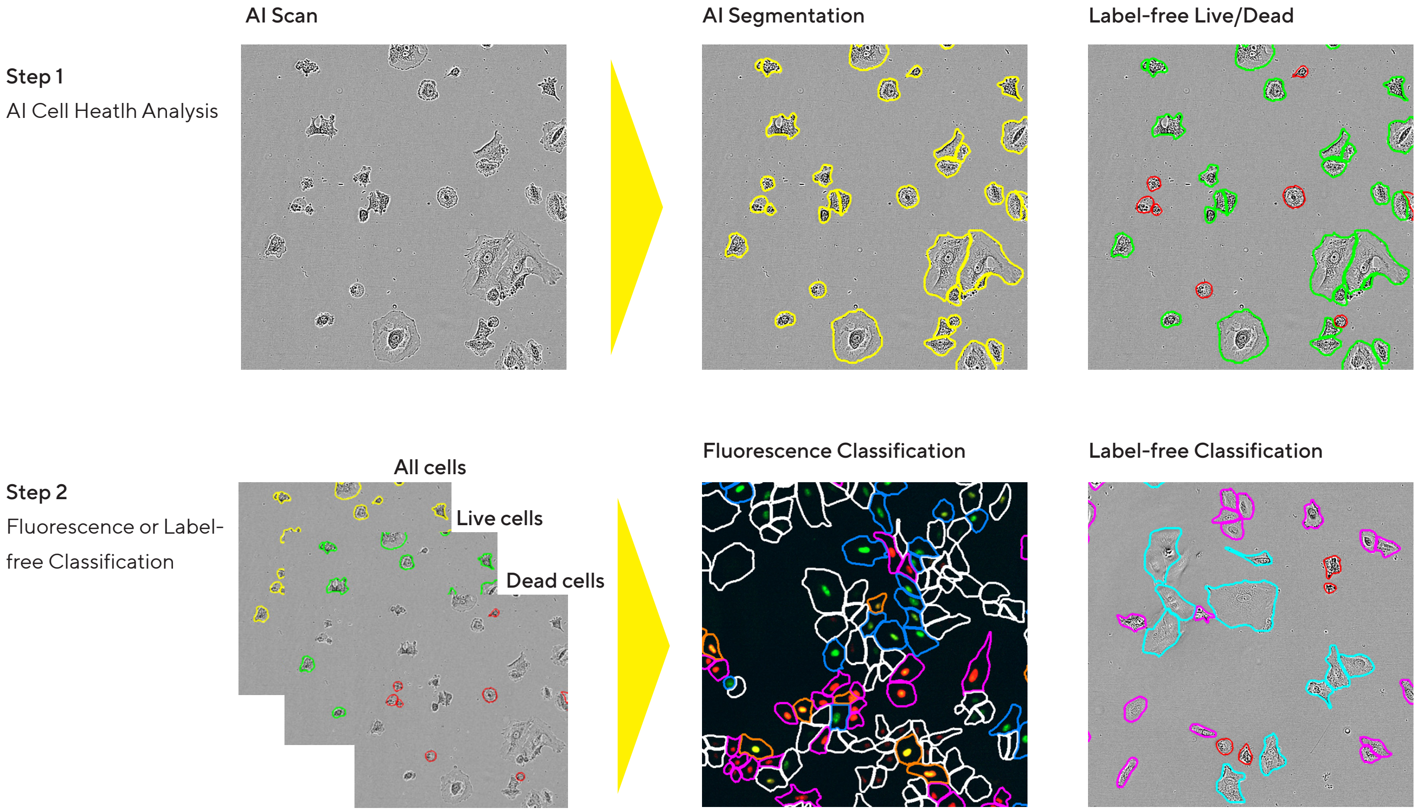

Figure 1. *Schematic showing analysis workflow. Phase contrast images are analyzed using AI CH to segment cells and classify as live or dead. Subsequent label-free classification with ALFC can separate cells into morphological subpopulations; this step can be performed on all cells or only live or dead cells.*

Improved segmentation enables more accurate morphology classification

Monocytes differentiating into macrophages undergo numerous changes, including increasing the volume of surface receptors which enable the functionality of finding and engulfing bacteria and debris. Alongside these changes in marker expression their morphology is drastically altered from rounded, free-floating suspension cells to adherent, plastic cells with the ability to rapidly move and extend the plasma membrane around target materials.

THP-1 monocytes were differentiated into macrophages by addition of the PMA. Images were acquired in the Incucyte® System over 4 days to monitor changes in cell morphology. Cells were segmented using AI CH and ALFC was then performed to quantify the differentiation process.
The AI-based segmentation method adapted well to the changing morphology with accurate outlining of all cells both as monocytes and macrophages. The label-free classifier was trained using examples of all cells at 0 hours to represent monocytes, and at 96 hours to represent macrophages. This classification reported a rapid increase in % macrophages over the first 24 hours reaching a plateau of around 80% macrophages by 48 hours (Figure 2A).
Segmentation of these cells was also performed using the Adherent Cell-by-Cell module in combination with ALFC. Using a more traditional computer vision approach, the segmentation is less robust to morphological changes and, in the case of monocytes, does not effectively delineate the boundary when several cells have formed a small cluster (Figure 2B, bottom image). This mis-segmentation not only affects the cell count readout but results in misclassification of the cluster as a macrophage. This example demonstrates the benefit of accurate segmentation in downstream analysis. Additionally, AI CH excluded a piece of debris (non-cell object, bottom left of the image) from the segmentation and subsequent classification, however Cell-by-Cell was unable to distinguish this object from true cells, leading to false segmentation and classification.

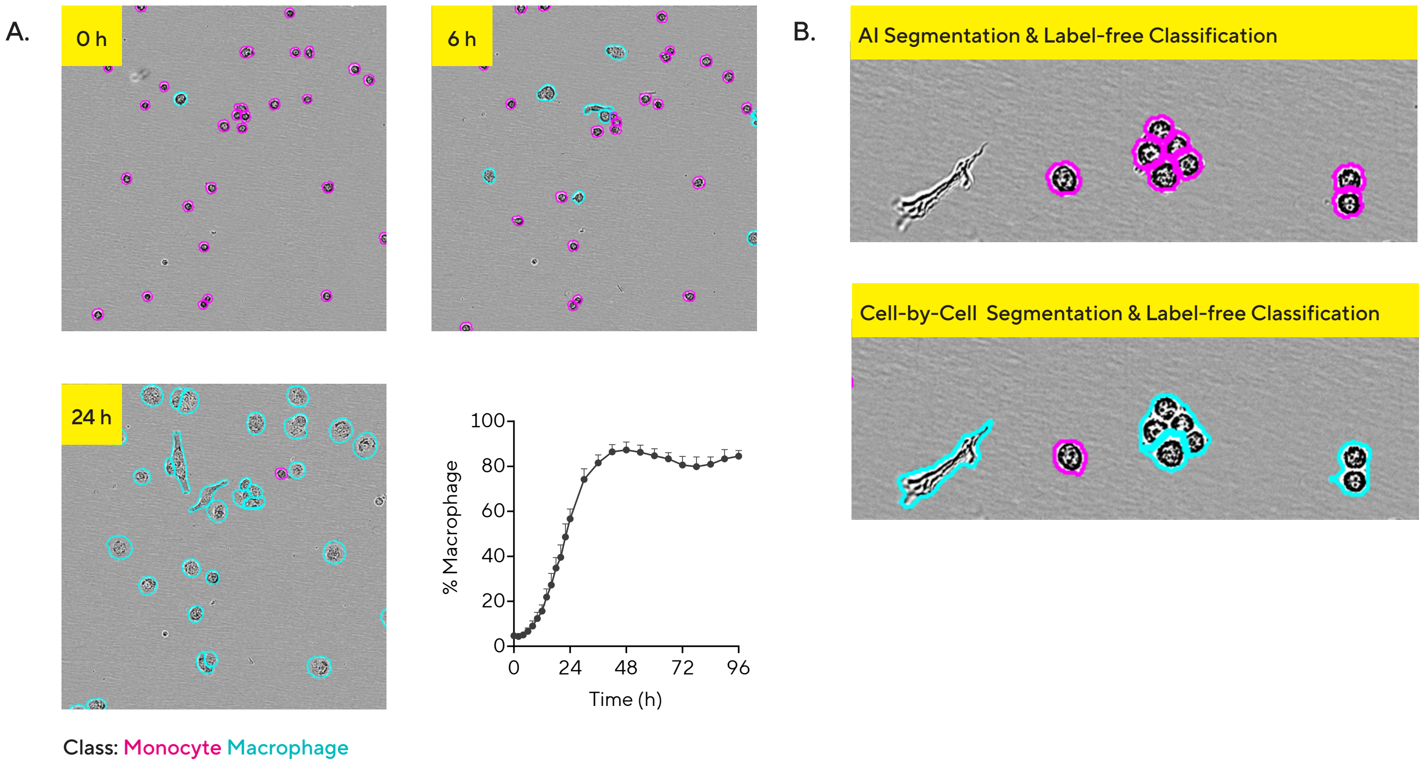

Figure 2 *Images display morphological changes as THP-1 monocytes differentiate into macrophages. Outline denotes cell segmentation and color denotes class (magenta = monocyte; teal = macrophage). Time course displays the increase in % macrophages over 96h (A). Top right image displays a cluster of monocytes segmented using AI CH and classified as monocytes using ALFC. Bottom right image displays the same cluster segmented using Adherent Cell-by-Cell segmentation and classified as macrophages using ALFC (B).*

Microglia viability and activation can be continuously monitored with label-free analysis

In vitro cell culture can result in loss of cell viability over time, particularly when using sensitive cell types that require tightly controlled conditions. Monitoring the health of these cultures is critical to the success of any assay, and the ability to exclude non-viable cells from the analytics can ensure that the resulting data truly represents the outcome of the live cell population.

Microglia are brain resident macrophages which transition between two states known as ramified – in which the cell is resting and quiescent – and amoeboid – in which the cell is functionally active, able to engulf target material and prune neurons. These two states are morphologically distinct with ramified cells presenting as elongated and highly textured whereas amoeboid cells have a star-like, adherent and motile appearance similar to that of macrophages.[2]

iPSC-derived microglia were cultured in specified media over a 6-day time frame and Phase contrast images were continuously acquired within an Incucyte® System. AI CH analysis was used to monitor cell viability during this time and ALFC was used to assess the activation state of the live cells (Figure 3A). Viability was highest in the first 36 hours of the assay, dropping rapidly thereafter during media refreshment steps (Figure 3B). By excluding these dead cells, the label-free classification results more accurately reported the proportion of live ramified vs amoeboid cells. These results revealed that in early stages of cell culture a high % of the cells were resting with around 90% ramified cells at 24 hours. However, at a later time point when the viability of the culture was lower, the live cell population was more activated with almost 60% of the live cells being classified as amoeboid at 96 hours (Figure 3C). These results demonstrate how sensitive cells can be easily stressed during routine cell culture and highlights the importance of continuous monitoring and gentle handling.

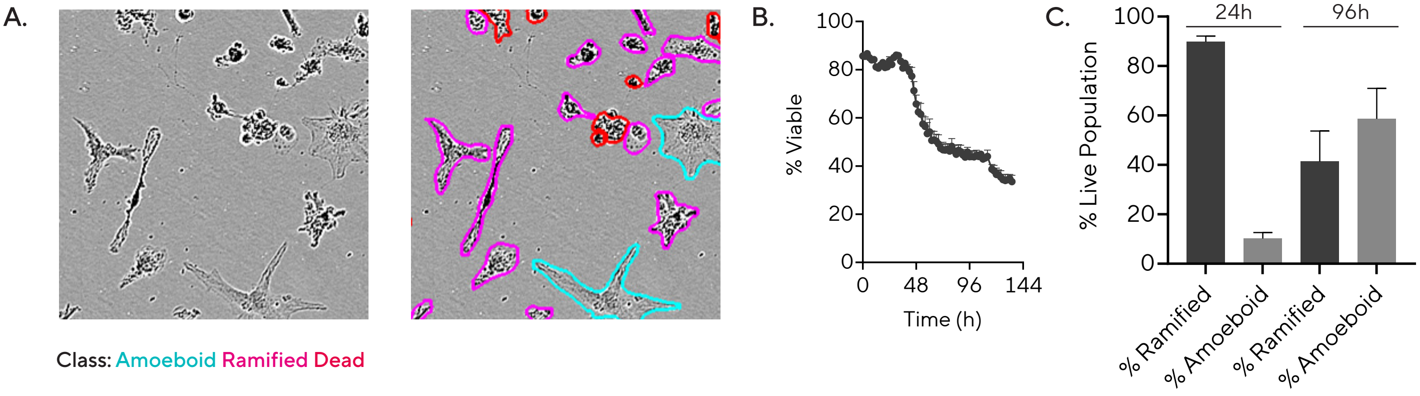

Figure 3. *Images display iPSC-derived microglia at 24h in culture. Outline denotes cell segmentation and color denotes class (magenta = live, ramified; teal = live, amoeboid; red = dead; A). Time course indicates the % live (viable) cells calculated using AI CH over 144h (B). Bar chart displays the proportion of viable ramified vs amoeboid cells at 24 and 96h (C).*

Non-toxic drug effects can be quantified

During a traditional drug screening process cells are treated with a single concentration of thousands of compounds, with the goal of comparing a single outcome for every compound – for example, death of the target cell. While this approach is high-throughput there is a risk that non-toxic or off-target effects may be missed, leading to a higher rate of false negative outcomes.

Using live-cell imaging for compound screening may reduce the number of compounds which can be tested simultaneously however the information-rich phase contrast images can yield vastly more data. For example, a compound which does not qualify as a "hit" for cytotoxicity may instead induce cell cycle arrest or senescence; in other cases, the cells move through distinct transition states before death which can yield critical information on the mechanism of action.

HeLa cells treated with a concentration range of topoisomerase inhibitor camptothecin (CMP) undergo cell senescence at low concentration ranges (10 – 100 nM) while cell death is observed at higher concentrations (1 – 10 µM).[3] AI CH analysis was used to quantify cell death; subsequent label-free classification was performed on the live cell population to identify the morphologically distinct senescent population. The images of treated cells confirm that while 10 nM CMP does not induce cell death, the proportion of senescent cells in the live population has increased. In contrast, 10 µM CMP induces death in ~70% of the population after 3 days of treatment (Figure 4A, 4B). As the concentration of CMP increases > 10 nM the % senescent cells is inversely proportional to the % dead cells (Figure 4C).

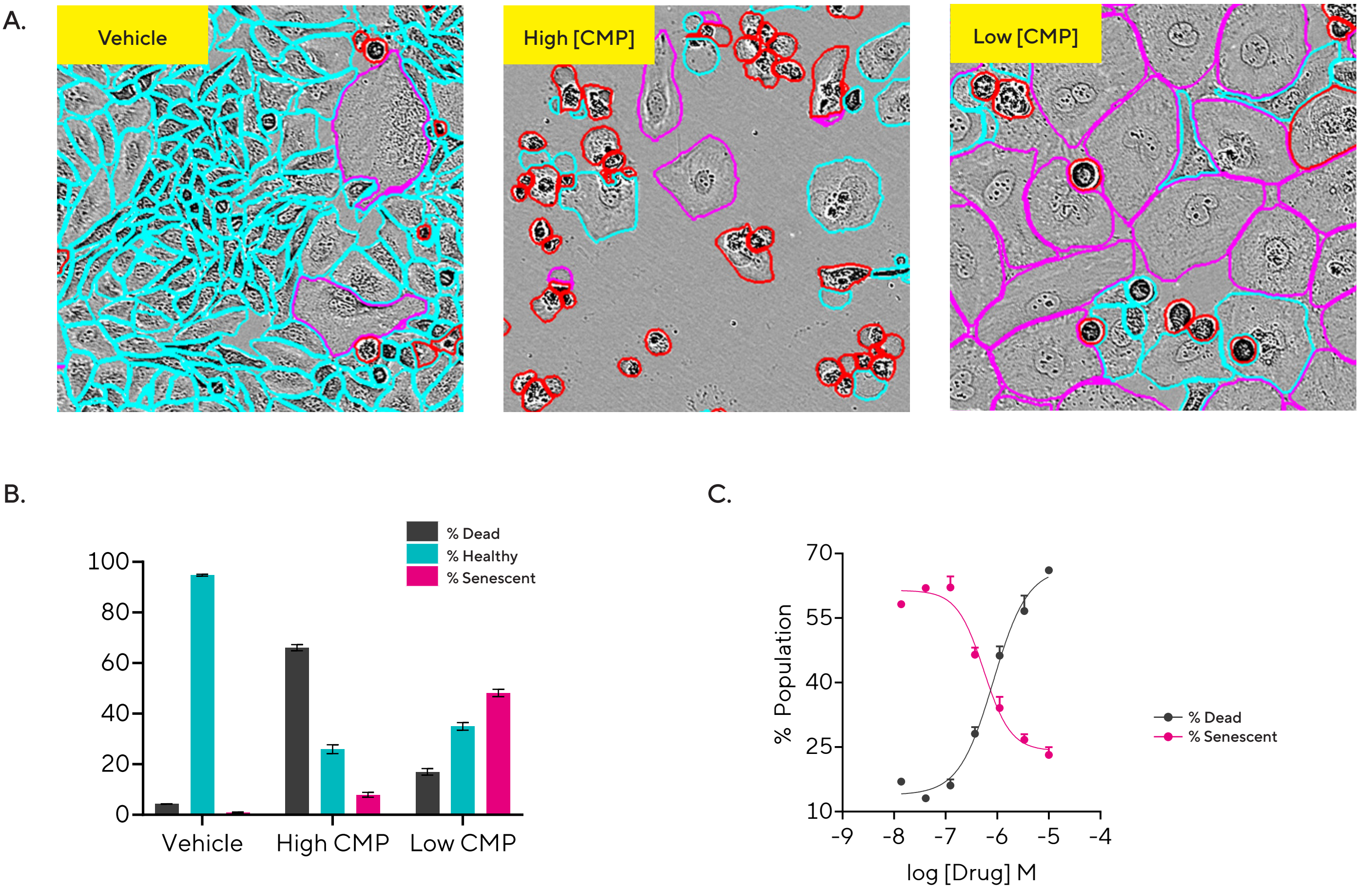

Figure 4. *Images show HeLa cells at 72h post treatment with low (10 nM) and high (10 µM) concentrations of CMP. Outline denotes cell segmentation and color denotes class (magenta = live, senescent; teal = live, healthy; red = dead; A). Bar chart displays quantification of cell viability (AI CH) and senescence (ALFC) at 72h (B). Concentration response plot indicates the decreasing proportion of % senescent cells and increasing % dead cells over the concentration range 10 nM – 10 µM (C).*

Transient morphology indicates ferroptotic cell death

Ferroptosis is an emerging cell death mechanism of interest in oncology as a potential alternative in apoptosis-resistant cells.[4] Using live-cell imaging, we determined that cells undergoing ferroptosis pass through a morphologically distinct transition state which can be identified using label-free classification (Figure 5A). GPX4 inhibitor RAS-selective 3 (RSL3) induces ferroptosis[5] which can be quantified using AI CH.[6] The time course of % dead cells (teal) displays a rapid increase in the first 12 hours which plateaus from approximately 24 hours. The % ferroptotic cells (magenta) show an even earlier peak as the cells pass through this transition state prior to cell death (Figure 5B).

The label-free classification of ferroptotic cells proved to be selective. Cells were treated with 24 compounds including inhibitors of DNA synthesis (camptothecin, CMP; etoposide, ETO; mitomycin C, MMC), receptor kinases (Dasatinib, DAS; Lapatinib, LAP; Linsitinib, LIN; Nindetanib, NIN; Palbociclib, PAL), cytoskeleton targeting compounds (colchicine, COL; Taxol, TAX; vinblastine, VIN), Akt pathway (isobavachalcone, IBV; MK-2206; PI-103; PP242; Rapamycin, RAP) as well as motor proteins (blebbistatin, BLB; Monastrol, MON) and trafficking proteins (brefeldin A; BRE) . Calculation of the area under curve (AUC) of % early ferroptosis morphology between 0 and 72h (Figure 5C) indicates that this morphology was only present where cells were treated with ferroptosis inducers Erastin (teal bar), FIN56 (magenta), and RSL3 (blue). This phenotype was also elevated in cells treated with Taxol (paclitaxel, purple), a microtubule stabilizing agent traditionally used to treat several types of cancer. Recent research has indicated that taxol-induced cell death displays several similarities to ferroptosis, including the release of intracellular iron and increasing transferrin receptor expression on the cell surface.[7]

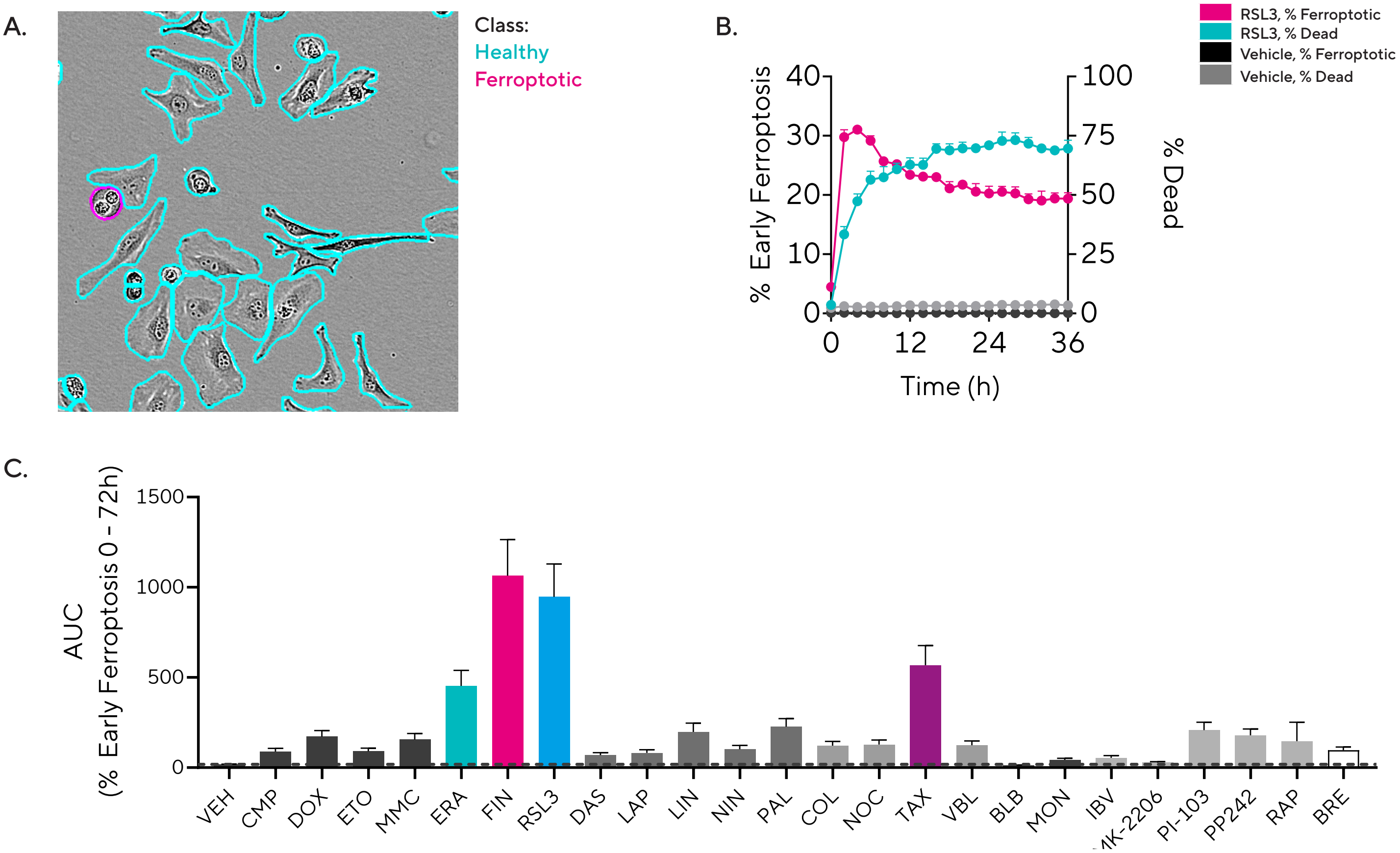

Figure 5. *Image displays cells 6h post treatment with RSL3. Outline denotes cell segmentation and color denotes class (magenta = early ferroptotic; teal = live, healthy; A). Time course displays the change in % early ferroptotic (magenta) and % dead (teal) for RSL3 treated cells and vehicle (light and dark grey respectively; B). C) Bar chart indicates area under curve (AUC) % early ferroptotic morphology from 0 – 72h.*

Summary & Outlook

The use of AI and machine learning based tools for analysis of microscopy images enables vastly more information to be gained from live-cell imaging data. Subtleties in cell behavior and temporal responses to compound treatment can be revealed by identifying morphological phenotypes including increased mitotic index, senescence, and transitional states such as that observed in early ferroptosis. Incucyte® AI Cell Health provides accurate segmentation that dynamically adapts to changes in cell shape, alongside classification of cells as live or dead. Advanced Label-free Classification enables users to classify cells based on the morphologies found in their own images by following a simple workflow. Combining these two tools yields deeper insight into cell subpopulations and derives maximal information from each label-free image.

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