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**Keywords or phrases:**

Cell Cycle, FUCCI, Cell Cycle Modulation, Differentiation, Immune Cell Killing

# Real-Time Quantification of Cell Cycle Phase in Live-Cell Models

## Introduction

Cell cycle is critical in maintaining cellular homeostasis through tightly regulated signaling pathways which control the frequency of DNA duplication and eventually cellular division. Without this regulation, cells divide in an uncontrolled manner, propagating uncorrected mutations and evading programmed cell death. Therefore, the cell cycle and its checkpoints are an attractive target for cancer therapies, which aim to arrest unchecked cell division as well as promote apoptosis<sup>1,2</sup>. Following this series of coordinated events, however, requires optimized technology and cell cycle indicator reagents. Current end-point solutions fail to study cell cycle over multiple cell divisions and do not fully integrate data acquisition and analysis. Live-cell imaging and analysis is uniquely suited to providing temporal information on the cell cycle through real-time, automated quantification and visualization of the events leading up to cell arrest, senescence or death.

## Assay Principle

The Incucyte® Cell Cycle Lentivirus reagents (Green | Red or Green | Orange) have been developed to distinguish between cells in the G1 and S | G2 | M cell cycle phase without altering cell function. As Geminin is highly expressed during S | G2 | M phase and Cdt1 during G1, fragments of these proteins are used to target fluorescent proteins for degradation during certain cell cycle phases. The Incucyte® Cell Cycle Lentivirus reagents incorporate a single cassette indicator expressing both the Geminin-TagGFP2 (green fluorescent protein) and either Cdt1-TagRFP (orange fluorescent protein) or Cdt1-mKate2 (red fluorescent protein). Therefore, cells fluoresce green during S | G2 | M and red or orange during G1; cells are colorless during the transition from M to G1 and yellow (expressing

green and red, or green and orange, simultaneously) in transition from G1 to S phase. Images of cells expressing Incucyte® Cell Cycle can be acquired and analyzed automatically in the Incucyte® Live-Cell Analysis System to identify phases of cell cycle in individual cells for real-time measurements of cell cycle dynamics. The schematic in Figure 1 shows the expression of green and red, or green and orange, fluorophores across the cell cycle, and the time course of images demonstrate the change in fluorescence as the cell divides. The indicated cell with red fluorescence is in G1 phase at 0 h. By 4 h the cell is transitioning from G1 to S, and at 7 h the cell is green indicating S | G2 | M phase. At 9 h the cell is mitotic, and by 11 h daughter cells in G1 are observed.

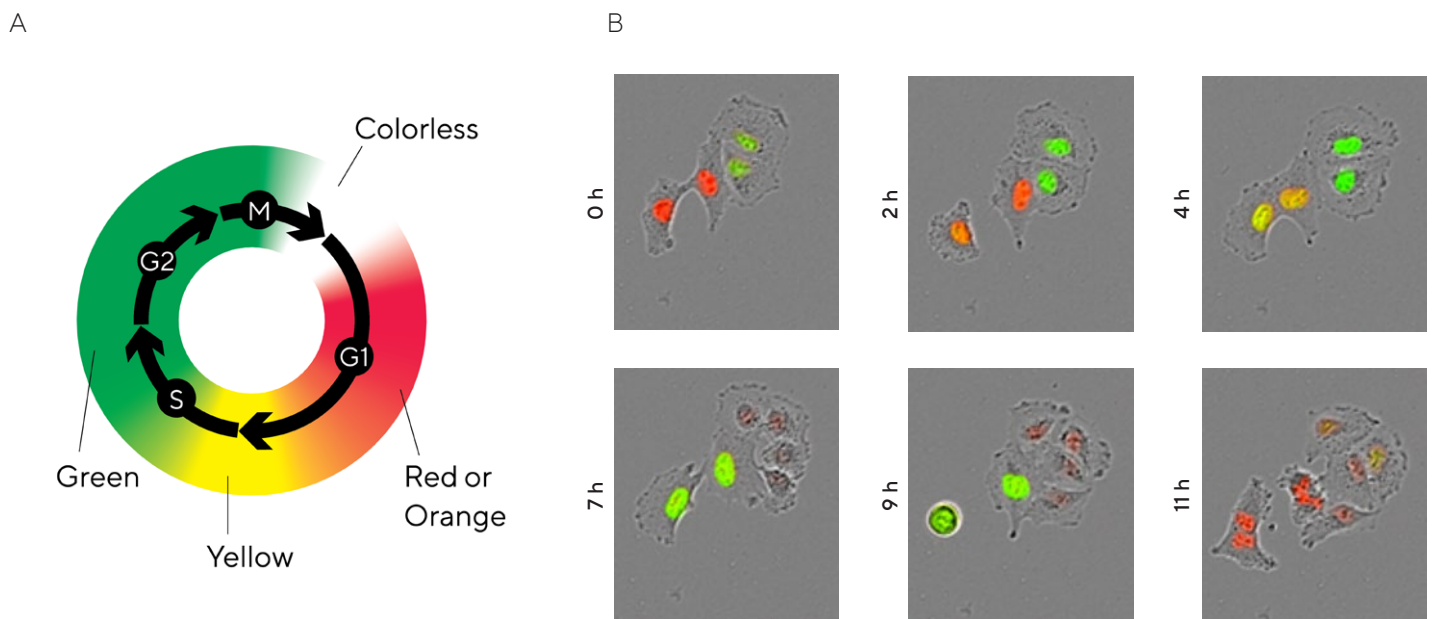


Figure 1: Incucyte® Cell Cycle Lentivirus. Schematic displays the fluorescence expressed at each stage of the cell cycle (A). Cells expressing Incucyte® Cell Cycle will exhibit green fluorescence during S | G2 | M and red or orange fluorescence during G1. Transition from M-G1 is non-fluorescent, and transition from G1-S displays yellow fluorescence, i.e., both green and red, or green and orange. Images of HeLa cells were acquired on the Incucyte® Live-Cell Analysis System (B). Sequential images show a cycling cell over time starting in G1 at 0 h with red fluorescence.

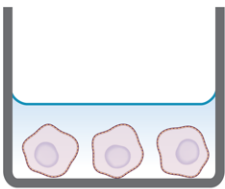
## Materials and Methods

To generate a cell line with stable Incucyte® Cell Cycle expression, the lentivirus was used to transduce the cell line of choice<sup>3</sup>. Using the Incucyte® Live-Cell Analysis System to monitor fluorescent marker expression and cell health, appropriate reagent multiplicity of infection (MOI) was chosen. When cells were observed to express the Cell Cycle reagent, puromycin selection was applied to remove non-expressing cells from the culture. By thorough

examination of cell morphology and proliferation, a number of infected and parental cell lines were compared, demonstrating that the Incucyte® Cell Cycle Lentivirus generates cell lines that have comparable morphology and growth profiles to the parental phenotype.

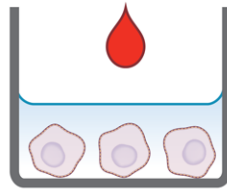
### Quick Guide

#### 1. Seed cells



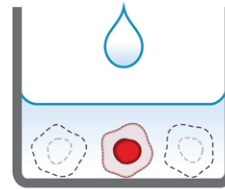
Seed cells in growth media and leave to adhere (4-24 hours). Cells should be 15-35% confluent at the time of transduction.

#### 2. Transduce



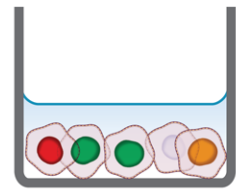
Add Incucyte® Cell Cycle Lentivirus Reagent diluted in media ± Polybrene®. After 24 hours, replace the media with fresh growth media. Monitor expression using the Incucyte® Live-Cell Analysis System.

#### 3. Apply selection



Apply Puromycin selection to derive a stable cell population or clone that expresses the genetically encoded cell cycle indicator.

#### 4. Live-cell imaging



Capture images in the presence of treatments in an Incucyte® Live-Cell Analysis System. Analyze using integrated software.

Figure 2: Quick guide to generation of a cell line stably expressing Incucyte® Cell Cycle. Using a simple protocol, cells can be treated with Incucyte® Cell Cycle Lentivirus reagent and begin expressing the fluorescent ubiquitination-based cell cycle indicator (FUCCI).

## Quantification of Cell Cycle Phase

The Incucyte® Cell-by-Cell analysis module enables individual cells in the field of view to be segmented in the phase contrast image, and metrics can be extracted per cell relating to fluorescence within the segmented boundary. Using the integrated analysis software, cell populations can be classified based on fluorescence characteristics. In combination with the Incucyte® Cell Cycle Lentivirus, this enables the percent cells in each phase to be rapidly quantified, as demonstrated in Figure 3. Cells expressing high green fluorescence (S | G2 | M) can be separated from those expressing high red fluorescence (G1), and transition phases can also be identified with high red and green (G1-S) or low red and green (M-G1). Both adherent and non-adherent cells can be quantified with this method.

Cells expressing Incucyte® Cell Cycle can also be monitored by counting the number of green and red objects (or green and orange objects, depending upon the lentivirus used) using basic fluorescence segmentation. Cells transitioning from G1-S appear yellow and can also be quantified by measuring objects with overlapping green and red fluorescence; yellow objects should be subtracted from the green object and red object counts to avoid over-estimation of these objects. This method enables evaluation of alterations in cell cycle progression in co-culture models, however information on the M-G1 phase is not available as these cells will be non-fluorescent and cannot be distinguished from a secondary non-labelled cell type.

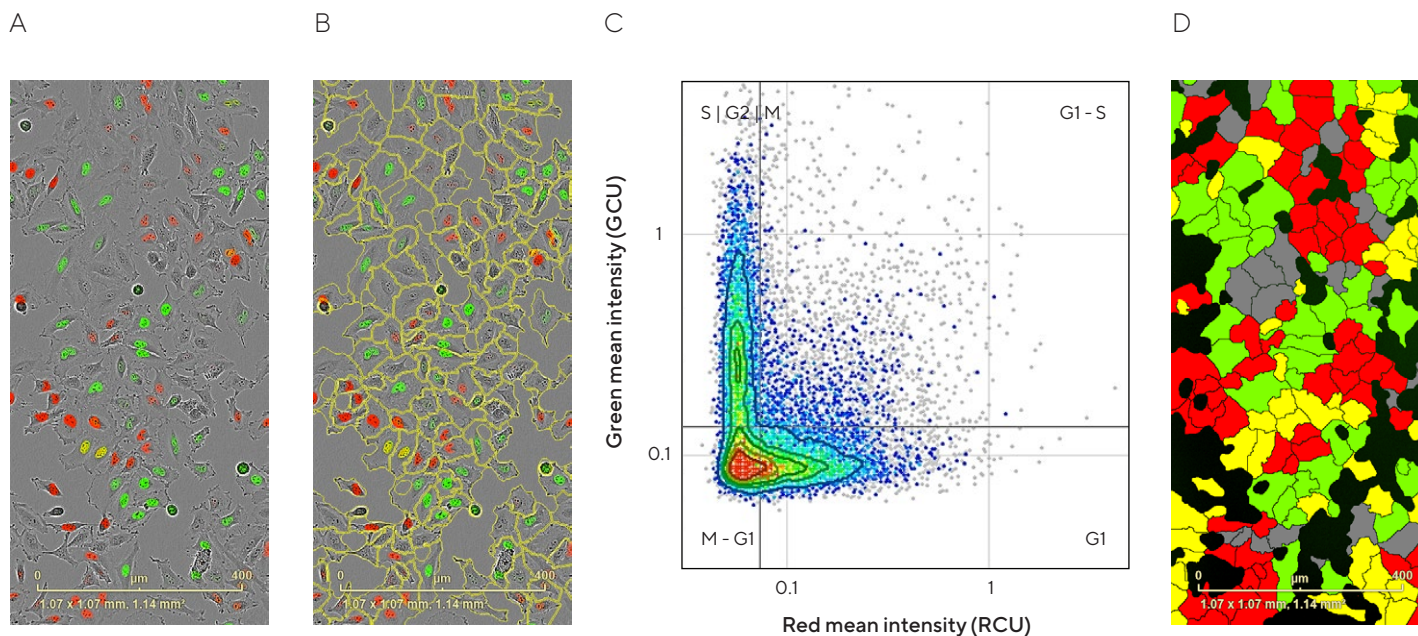


Figure 3: Cell cycle quantification. Images acquired in Incucyte® Cell-by-Cell Software Analysis Module allow individual cells to be segmented (A, B). Cells can be classified according to red and green fluorescence intensity (C). Classification mask shows cells identified as red, green, yellow or non-fluorescent (D).

## Cell Cycle Synchronization

In order to study cell cycle specific mechanisms and regulation, cells are often synchronized<sup>4</sup>. This process results in an enriched population of cells in the same phase which can be examined and quantified using Incucyte® Cell-by-Cell Analysis module. Temporary arrest is induced by serum starvation or compound treatment until all cells have reached the same phase. The block is then removed by replenishing culture media and nutrients, and synchronized cells re-enter the cell cycle at the same phase.

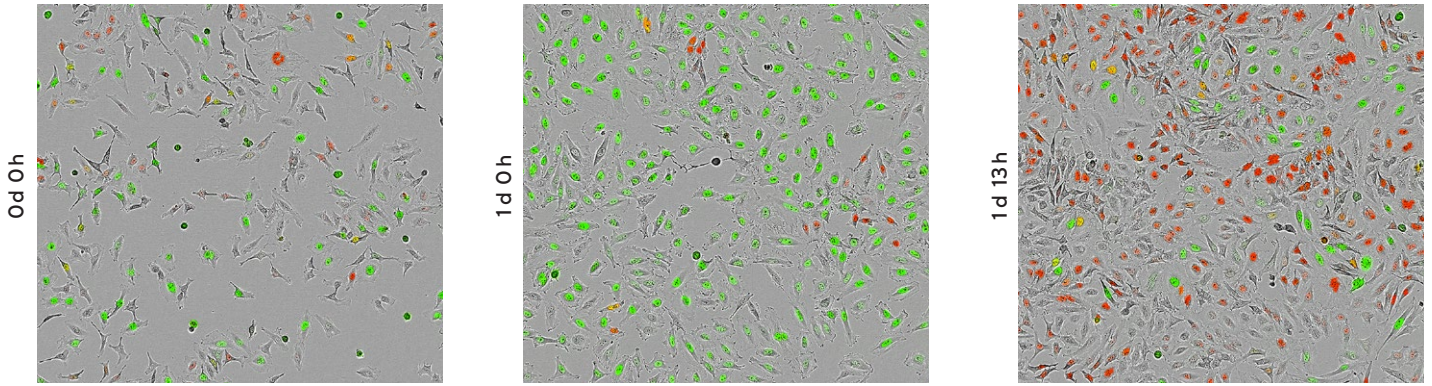
Thymidine, a DNA synthesis inhibitor, is a commonly used method of synchronizing cells by blocking progression beyond the S phase<sup>5</sup>, and Figure 4 demonstrates this process. When HeLa cells expressing Incucyte® Cell Cycle were treated with Thymidine (2.5 mM, 24 h), DNA synthesis was inhibited and cells arrested in S phase. The block was removed and cells were allowed to progress through the cell cycle. Quantification of the percent cells in each phase of the cell cycle was achieved by acquiring images in the Incucyte® Cell-by-Cell Software Analysis Module, with individual cell segmentation and subsequent classification based on red and green fluorescence intensity.

An increase in cells expressing green fluorescence was observed, with a maximum of 75% green cells reached at 24 h. Around 6 h after removal of Thymidine, the percent green cells (S|G2|M) decreased, and was followed by sequential peaks in non-fluorescent cells (M-G1 transition), red cells (G1), yellow cells (green and red, G1-S transition). Finally, another peak in green expressing cells was observed around 17 h after the previous peak in green as the cycle resumed. The temporal profiles of these peaks can yield insight into cell proliferation. For example, the time between peaks of the same color can be considered one full cell cycle. Additional cell lines synchronized with Thymidine displayed variation in peak-to-peak time, where that of MDA-MB-231 cells was 16 h, while that of SKOV3 was 24 h.

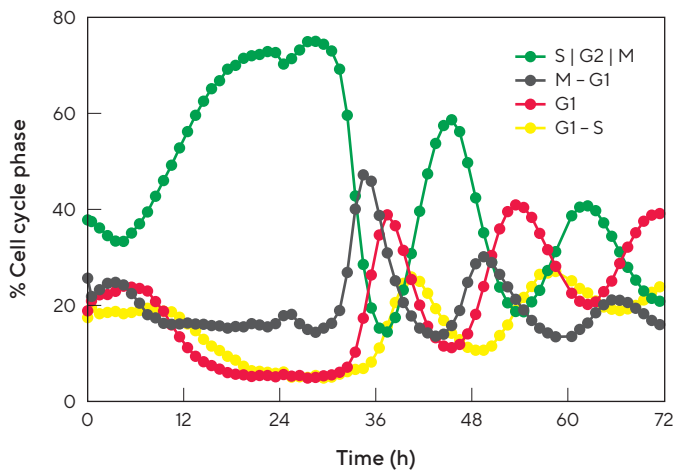
Comparison of the HeLa non-fluorescent population time course and that of label-free cell count demonstrated that the transition between M and G1 is associated with a sharp rise in cell number as cells divide (Figure 4C). This validates that mitosis is occurring during the non-fluorescent M-G1 phase.



A



B



C

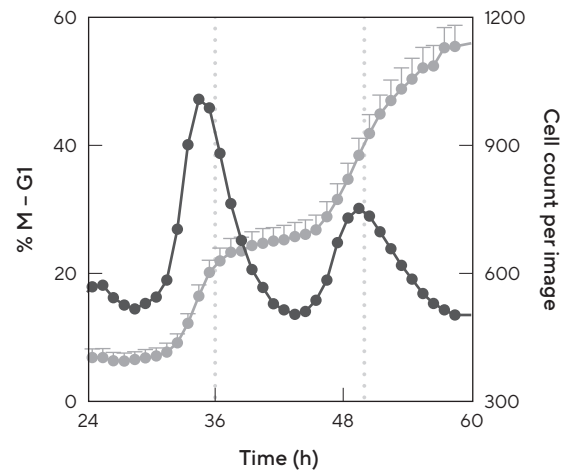


Figure 4: Synchronization of cell cycle phase. Image at 0 h shows unsynchronized cells expressing a mix of red and/or green fluorescence (A). After 1 day treatment with arresting agent, Thymidine cells have arrested in S|G2|M and are prominently green. Thymidine block was released at 1 d and 13 h later, a peak in red fluorescence is observed as synchronized cells reach G1. Quantification was achieved using Incucyte® Cell-by-Cell Analysis. Time course of cell cycle phase shows cells accumulating in S|G2|M during the first 24 h of Thymidine treatment (B). When the block is removed, consecutive peaks in M-G1, G1, and G1-S are observed. Overlay of cells in M-G1 (dark gray) with cell count (light gray) show increase in cell number during this transition period consistent with cell division (C).

## Cell Cycle Modulation

Cell cycle arrest can be induced by treatment with chemicals or via serum starvation, and is a target for novel anti-cancer therapeutic agents, such as 5-fluorouracil (5-FU)<sup>6</sup>. Using Incucyte® Live-Cell Analysis, the effect of these treatments can be visualized and quantified in 96- or 384-well throughput, to accurately track and quantify cell cycle arrest in a specific phase. Here, we highlight the ability to accurately study targeted cell cycle therapies using 5-FU and cisplatin. 5-FU depletes available nucleotides which are required for DNA synthesis, rendering cells unable to enter S phase, whereas cisplatin is a chemotherapeutic DNA intercalator which damages DNA and causes checkpoint activation and cell cycle arrest in the S|G2|M phase<sup>7</sup>.

Treatment of HT1080 fibrosarcoma cells expressing Incucyte® Cell Cycle with 5-FU (0–50  $\mu$ M) caused an increase in the percent of cells in G1 over 24 h, indicating that the cell cycle was arrested. In contrast, cisplatin treatment reduced the percent cells in G1 as cells arrested in S|G2|M. Quantification of these effects demonstrated a time- and concentration-dependent cell cycle arrest. Figure 5 shows a 96-well plate view of percent cells in G1 phase over time, which provided an overview of compound effects, as well as an indication of assay robustness and well-to-well reproducibility. EC<sub>50</sub> values were rapidly calculated within Incucyte® software to provide a measure of compound potency.

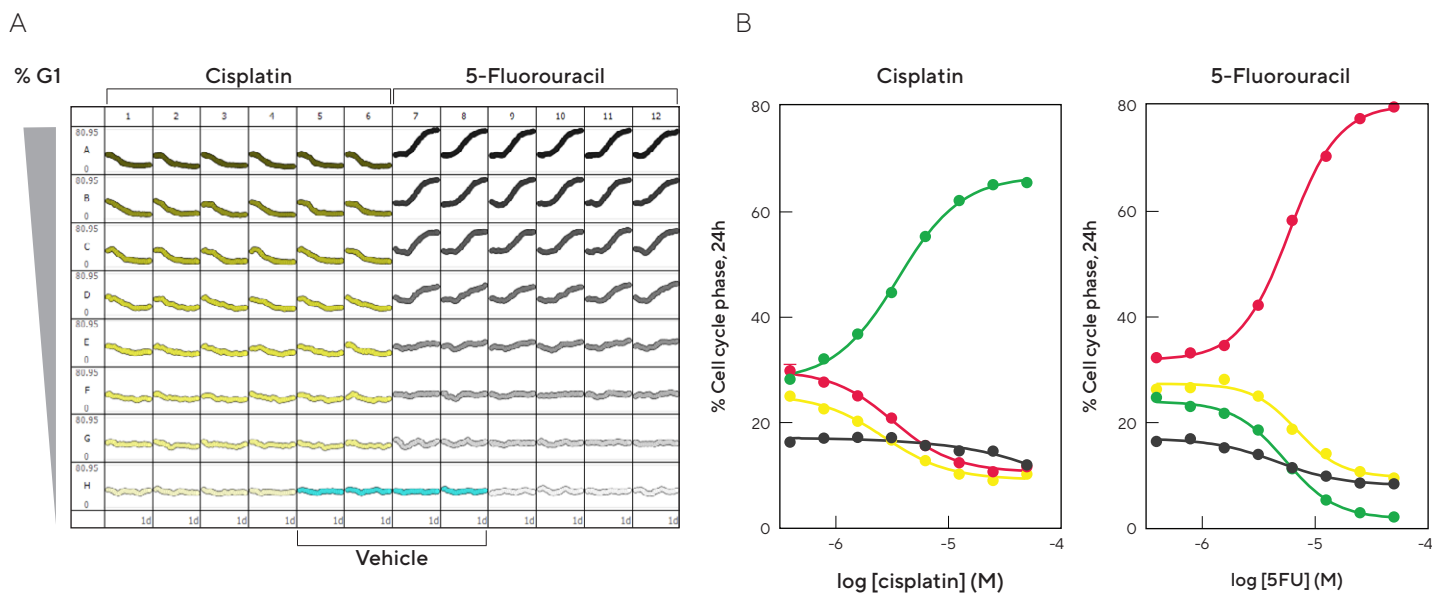


Figure 5: Concentration dependence of cell cycle modulators. HT1080 cells expressing Incucyte<sup>®</sup> Cell Cycle were treated with cisplatin (0–50  $\mu$ M) or 5-fluorouracil (5-FU, 0–50  $\mu$ M). Plate view shows the time course of percentage of cells in G1 phase in each well of a 96-well plate, over 1 day treatment with cisplatin or 5-FU. A) Concentration response curves indicate the efficacy of cisplatin and 5-FU at 1 d and percent change in each population over the concentration ranges tested (B).

## Cell Cycle Arrest Can Be Distinguished From Apoptosis

The Incucyte<sup>®</sup> SX5 Live-Cell Analysis System incorporates three fluorescence channels (green, orange, and Near IR). By multiplexing the compatible Cell Cycle Green | Orange Lentivirus with Annexin V NIR, the effect of experimental treatments on cell cycle arrest and cell viability can be determined. Figure 6 demonstrates that differential compound effects can be explored, as well as the temporal relationship between cell cycle arrest and apoptosis. Phase and fluorescence images acquired at 3 days post-treatment show the change in cell number and fluorescence induced by each compound.

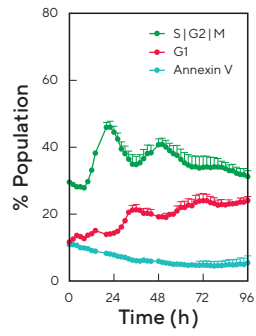
In the absence of compound treatment (vehicle), AU565 and MDA-MB-231 cells showed a mixture of green and orange fluorescence, where a small peak in green fluorescence corresponded to a small dip in orange fluorescence and vice versa. As these cells were healthy and proliferating, there was a very low number of cells which were apoptotic (Annexin V NIR positive).

Carboplatin, a non-selective DNA intercalator with a similar mechanism to cisplatin<sup>8</sup>, induced rapid arrest in S | G2 | M. In both cell types the percentage of green cells rapidly increased within 48 h, remaining stable until approximately 72 h, when the number of apoptotic cells began to increase as cells started to undergo apoptosis. Phase image shows that carboplatin has a strong morphological effect on MDA-MB-231.

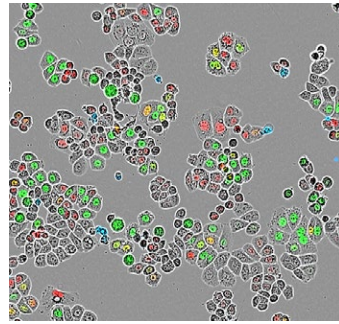
Lapatinib is a dual HER2 | EGFR inhibitor, targeting receptors expressed by the AU565 cells<sup>9</sup>. Treatment of AU565 with lapatinib caused arrest in G1, and the percentage of orange cells increased over time while other populations decreased. This effect was not observed in MDA-MB-231 cells which lack the appropriate cell surface receptors, and as the image shows, continued to proliferate in the presence of lapatinib.

Camptothecin, a cytotoxic DNA synthesis inhibitor, induced a transient increase of G1 cells in AU565 cells, which reached a peak at 48 h and then diminished. The percent S | G2 | M cells reduced over time while the apoptotic cells increased over the 96 h time course. In MDA-MB-231 cells the G1 cells plateaued 8 h after treatment, while the percentage of S | G2 | M cells dropped and the percent apoptotic increased. These data demonstrated that camptothecin has a cell cycle-dependent toxicity, and is more potent on cells in S | G2 | M than those in G1, correlating with camptothecin mechanism of action which inhibits cells in the S (DNA synthesis) phase. It is also consistent with reports that the apoptotic population of camptothecin-treated cells is derived from cells in S phase<sup>10</sup>.

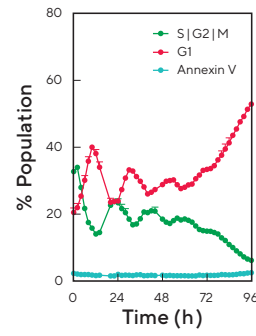
AU565



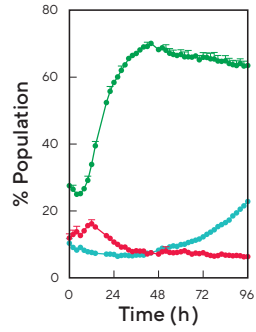
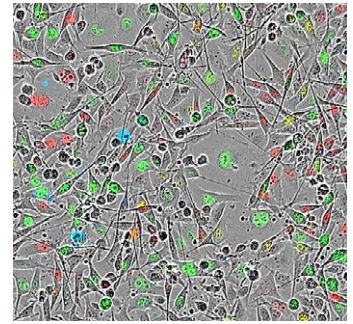
Vehicle



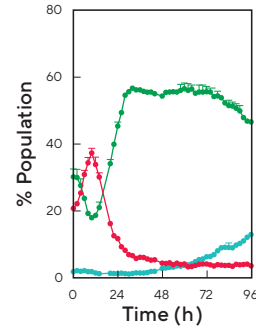
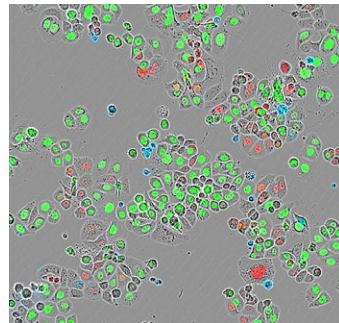
MDA-MB-231



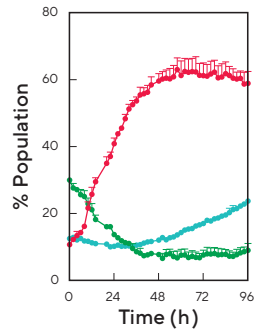
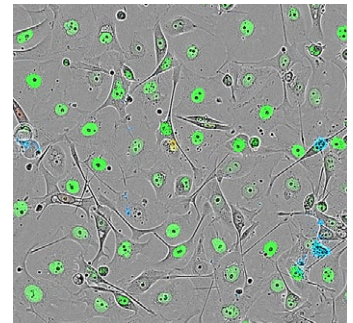
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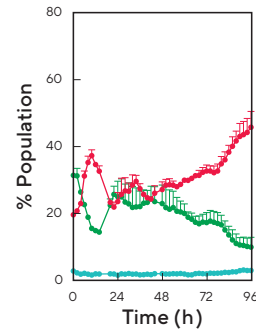
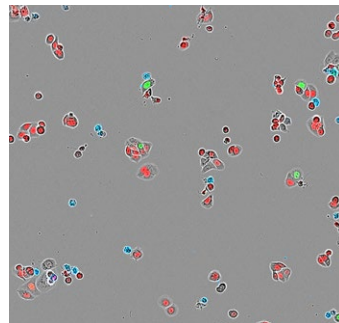
Carboplatin



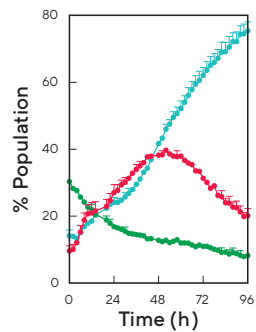
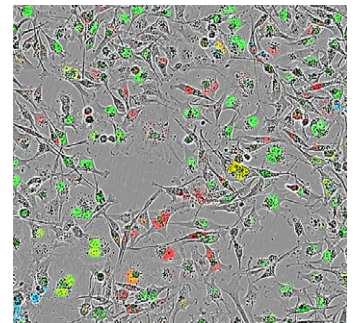
Carboplatin



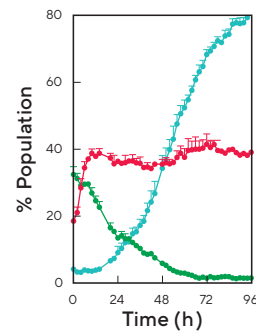
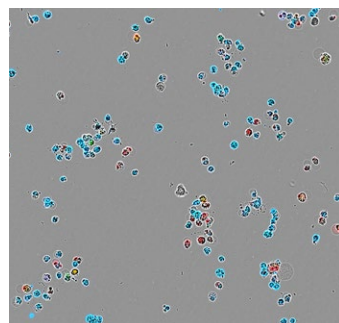
Lapatinib



Lapatinib



Camptothecin



Camptothecin

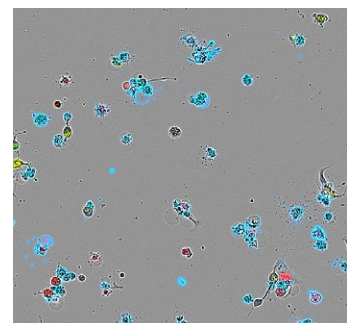


Figure 6: Combining Cell Cycle and Annexin V Reagents enables added insight into compound mechanism. AU565 and MDA-MB-231 cells expressing Incucyte® Cell Cycle Green|Orange were treated with carboplatin (100  $\mu$ M), lapatinib (0.1  $\mu$ M) and camptothecin (10  $\mu$ M) in the presence of Annexin V NIR (1:200). Scans were acquired and analyzed in Incucyte® SX5 using the Incucyte® Cell-by-Cell Software Analysis Module. Time courses show the populations of cells in S|G2|M (green) or G1 (red) overlaid with the population of Annexin V positive objects (teal). Carboplatin arrests cells in S|G2|M. Lapatinib targets specific cell receptors and is, therefore, effective on AU565 but not MDA-MB-231. Camptothecin induces apoptosis in both cell lines. Phase and fluorescence (green, orange NIR) blended images show AU565 or MDA-MB-231 cells at 3 d post treatment.



## Effect of Differentiation on Cell Cycle and Function

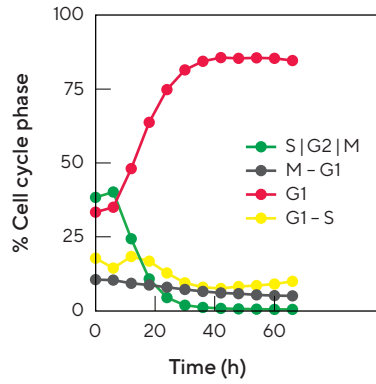
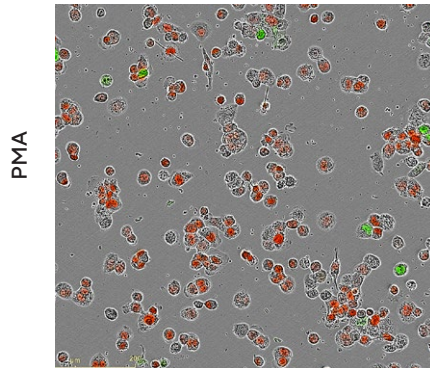
Incucyte® Cell Cycle Lentivirus reagents enable quantification of cell cycle phase by determination of percentage of cells in each phase of the cell cycle. Non-fluorescent metrics can also be derived from the Phase HD channel relating to cell count, area, and eccentricity. Combining these data with the reagent provides additional insight into morphological changes and cell behavior.

The THP-1 cell line is commonly used as a model of human monocyte function and for differentiation into macrophages<sup>11,12</sup>. In order to determine the relationship between cell differentiation and cell cycle, THP-1 cells expressing Incucyte® Cell Cycle were stimulated using two common treatments: PMA (100 nM) or LPS with IFN $\gamma$  (both 100 ng/mL). Studies of the cell cycle phase (Figure 7) indicated that both stimulants caused cells to arrest in G1, with an increase in cells expressing red fluorescence. Morphological differences between cells activated with PMA vs. LPS|IFN $\gamma$  were noted, with cells treated with PMA changing from non-adherent to adherent morphology, with an increase in cell area and eccentricity, whereas LPS|IFN $\gamma$ -

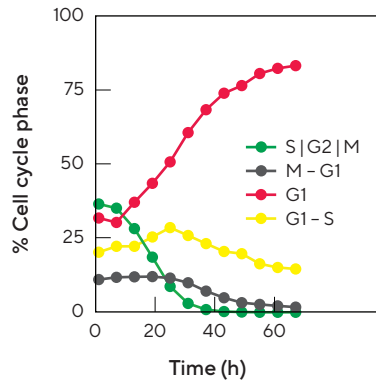
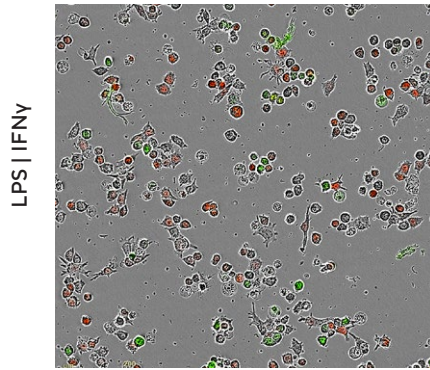
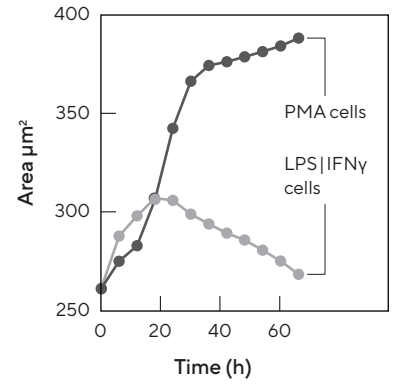
treated cells displayed a mixed phenotype containing small, rounded, star-like shapes. Although the cell cycle was arrested, cell functionality was retained and stimulated cells (PMA and LPS|IFN $\gamma$ ) demonstrated the ability to phagocytose bioparticles. To assess phagocytosis, the total phase channel confluence of zymosan bioparticles was compared in the presence and absence of PMA or LPS|IFN $\gamma$  treated cells. In the absence of cells, bioparticles settled to the bottom of the well within 12 h and the confluence remained constant after this time. In the presence of functionally active cells, bioparticles were engulfed and the confluence decreased over time. Cells differentiated with PMA were highly phagocytic, achieving 30% clearance of bioparticles, while those treated with LPS|IFN $\gamma$  achieved approximately 15% clearance of bioparticles. LPS|IFN $\gamma$  stimulation is known to increase the phagocytic capacity of THP-1 cells<sup>12</sup>, however PMA treatment produces a macrophage-like phenotype which has a higher phagocytic potential<sup>11</sup>.



### A. Differentiation



### B. Morphology



### C. Function

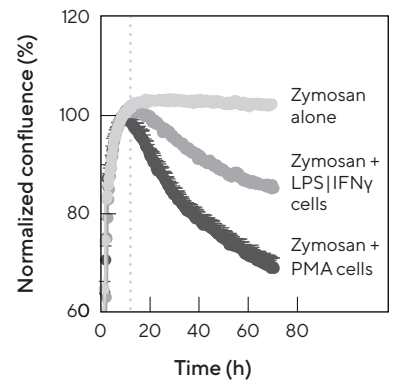


Figure 7: Differentiation of THP-1 arrests cell cycle, alters morphology, and results in phagocytic cells. THP-1 cells expressing Incucyte® Cell Cycle Lentivirus were differentiated by treatment with PMA (100 nM) or LPS|IFN $\gamma$  (100 ng/mL each) for 62 h (A). Blended phase and fluorescence images show cells with altered morphology and a high proportion of red cells. Time courses of cell cycle phase indicate that both treatments induce arrest in G1, with PMA acting more rapidly than LPS|IFN $\gamma$ . Average cell area shows a rapid increase in the size of PMA treated cells while LPS|IFN $\gamma$ -treated cells become slightly enlarged before returning to approximately 260  $\mu\text{m}^2$ . To assess functional capacity, stimulated cells were washed with media to remove non-adherent and dead cells, and Zymosan bioparticles (1.5  $\mu\text{g}$  per well) were added (B). Total confluence was measured using basic phase segmentation, and once the bioparticles had settled (confluence of bioparticles alone reaching a maximum value at 12 h), confluence was normalized to 100%.

## Immune Cell Killing and Cell Cycle Arrest

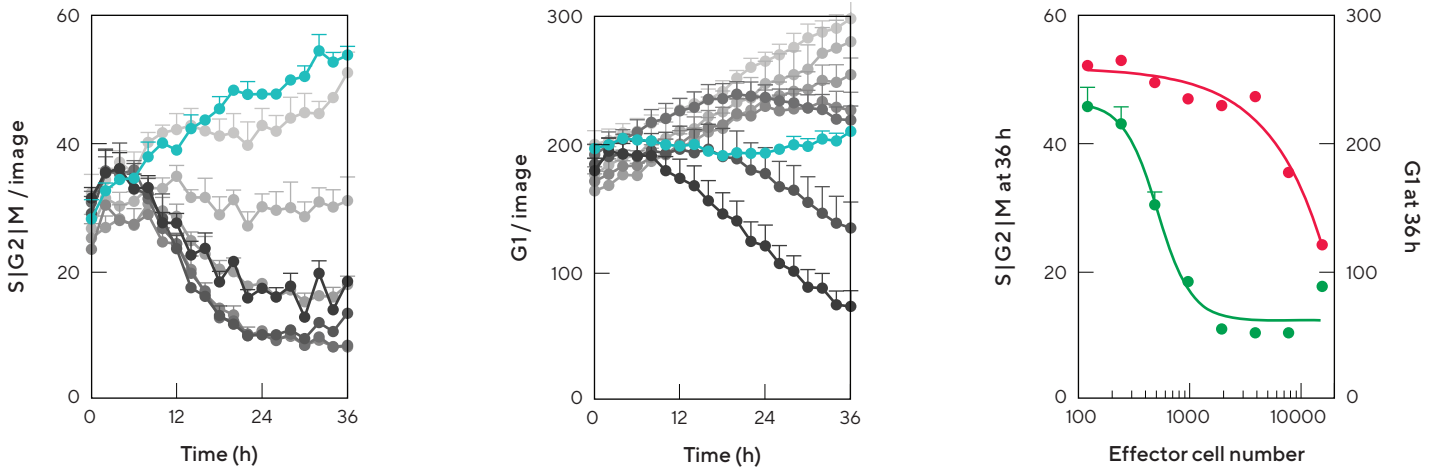
While simplistic 2D monocultures can be quantified using Incucyte® Cell Cycle Assay, more complex co-culture models can be evaluated for deeper insight into cellular interactions and functional changes. For example, the interplay between tumor and immune cells is critical in understanding the ability of immune cells to induce cell death or suppress growth of tumor cells.

To evaluate if tumor cell death by activated immune cells is cell cycle dependent, peripheral blood mononuclear cells (PBMCs) were co-cultured with target tumor cells expressing Incucyte® Cell Cycle. Using basic fluorescence masking and analysis of green and red objects, tumor cell cycle status was continuously monitored throughout the assay as activated immune cells induced apoptotic death (Figure 8).

T47D cells expressing Incucyte® Cell Cycle sensor were exposed to increasing densities of activated, freshly isolated PBMCs. The time courses of green and red objects indicate that the cells in S|G2|M were preferentially targeted as the green object count drops rapidly, while the cells in G1 were less affected by the PBMCs. Red object count only decreased at the two highest PBMC densities, at a slower rate than the green objects. At lower PBMC densities the red object count continues to increase, suggesting that cells are arresting.

MDA-MB-231 cells exhibited different behavior in the presence of PBMCs to T47D cells. The green object count plateaued but did not decrease, and red object count was entirely unaffected by the presence of immune cells. Together, these data suggest a cytostatic mechanism consistent with reports that T cells can suppress tumor cell growth via IFN $\gamma$ -mediated cell cycle arrest<sup>13</sup>.

T47D



MDA-MB-231

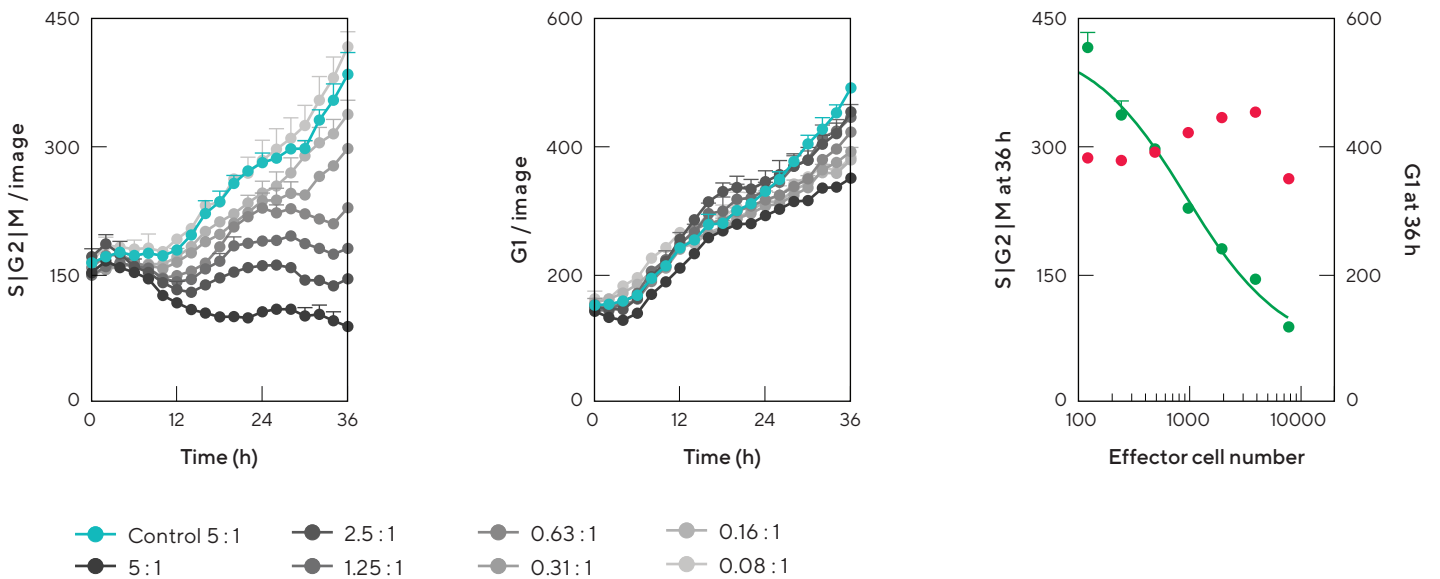


Figure 8: Incucyte® Cell Cycle Lentivirus reagent elucidates mechanism of T cell interactions with tumor cells. T47D and MDA-MB-231 breast cancer cells expressing Incucyte® Cell Cycle were incubated with increasing ratio of pre-activated PBMCs (anti-CD3, 72 h). Phase and fluorescence images were acquired over 3 days and green and red fluorescent objects were counted using basic fluorescence segmentation. S|G2|M cells were quantified by subtracting overlap (yellow) object count from green object count. G1 cells were similarly quantified. Time courses for T47D cells (top row) show a density-dependent decrease in S|G2|M and G1 cells, with cells in S|G2|M (green objects) being affected more rapidly and at lower PBMC density than cells in G1 (red objects). This is highlighted by the data showing cell populations at 36 h, which show a reduction of cells in S|G2|M at low PBMC densities, while loss of cells in G1 is only observed at the two highest PBMC densities. Time courses for MDA-MB-231 show that only cells in S|G2|M are affected by the presence of activated PBMCs while the number of cells in G1 is unaffected. This is confirmed by data showing populations at 36 h.

## Summary and Outlook

Cell cycle is a dynamic process, and following this transition is critical in many therapeutic areas, such as oncology and immuno-oncology. Using Incucyte® Cell Cycle Lentivirus reagents with real-time image acquisition and analysis, a quantitative picture of this changing landscape can be achieved. Combining the cell cycle data with cell morphology, function and cell health readouts enable

multiple aspects of a treatment or culture conditions to be considered in a single well. More advanced cell models, such as co-cultures with immune cells, can also benefit from the information yielded by the Incucyte® Cell Cycle Lentivirus reagents, under physiologically relevant conditions, to deepen our understanding of temporal patterns of cell cycle progression.

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