SARTORIUS

Application Note

February 25, 2022

Keywords or phrases:
Proliferation, Incucyte®, Live-Cell Analysis, Cell Growth, Confluence

Kinetic Quantification of Cell Proliferation Using Live-Cell Analysis

Nicola Bevan,[1] Gillian Lovell,[1] Jasmine Trigg,[1] Clare Szybut,[1] John Rauch,[2] Libby Oupicka,[2] Kalpana Barnes[1]

[1] Sartorius UK Ltd., Units 2 & 3 The Quadrant, Newark Close, Royston Hertfordshire SG8 5HL UK

[2] Sartorius Corporation, 300 West Morgan Road, Ann Arbor, MI 48108 USA

Correspondence
Email: AskAScientist@sartorius.com

Introduction

Cell proliferation assays are a cornerstone of cancer therapeutic, developmental biology, and drug safety research. Analysis of the sustained signaling pathways that underlie the progression of tumors, for example, accounts for > 12,000 manuscripts in PubMed, the majority of which use cell proliferation analysis to evaluate tumor cell growth. However, there are a number of technical challenges associated with this seemingly simple activity: cell growth takes time, which makes temporal monitoring and non-perturbing measurements important for high-quality results. Live-cell analysis is ideally suited to this, providing valuable information without interfering with cell growth to ensure reliable, reproducible, and comparable data. It enables the real-time quantification of living cells' behavior using time-lapse imaging, integrated image analysis, and on-the-fly data visualization for cell-based experiments over days, weeks, or even months. In this way, the need for multiple assays with different end points is eliminated, allowing the continuous collection of data without the risk of missing a key biological event. The noninvasive nature of this technology makes it complementary to methods such as flow cytometry and multimode microplate readers and can be used in combination using label-free or specialized labeling approaches to expand into more complex assays.

Find out more: www.sartorius.com/incucyte

In this application note, we illustrate how proliferation assays using the Incucyte® Live-Cell Analysis System in conjunction with fit-for-purpose software tools and non-perturbing reagents enable kinetic quantification of cell proliferation. This can be performed at microplate scale, for both non-adherent and adherent cell cultures, in mono- and co-culture. Additional assay strategies will be introduced to generate measurements of cell type-specific growth rates in co-cultures, including use of Incucyte® Nuclight Reagents to fluorescently label nuclei and determine true counts of viable cells.

General Assay Principles of Incucyte® Proliferation Assays

A variety of strategies for kinetic measurement of proliferation are possible using the Incucyte® Live-Cell Analysis System. Selection of label-free or fluorescent assays depends on the specific scientific question being asked and cell models studied. Continuous live-cell assays for both adherent and non-adherent cells are possible as cells stay stationary inside a standard tissue culture incubator while the Incucyte® optics move. Incucyte® Live-Cell Imaging and Analysis enables noninvasive, label-free measurement of cell growth based on area (confluence) or cell number (count) metrics, both of which are generated via segmentation (masking) of high-quality phase images. To resolve the challenge of quantifying low-contrast cells that can be difficult to identify in phase contrast images, Incucyte® Nuclight Reagents for live-cell analysis can be used to fluorescently label nuclei. Fluorescent Incucyte® images can then be acquired over time and analyzed to generate nuclear counts and derive doubling times in either mono- or co-cultures. Additionally, Incucyte® Proliferation Assays can be multiplexed with Incucyte® fluorescent reagents for cell health assessments. These will not be covered in this application note, but more information can be found on the Sartorius webpage here: Apoptosis | Sartorius. Cell boundaries can be identified using the Incucyte® Morphological Analysis, enabling cell counting, and simultaneous assessment of cell death or viability achieved by measuring the fluorescence intensity originating from within the individual cell boundary.

We do recommend some optimization steps to ensure high-quality results:

- Key to tracking proliferation is ensuring your cells are healthy at the beginning of the assay and present in the well at an appropriate density. This can be achieved by pre-imaging your cells before the assay begins to visually assess if they demonstrate the expected morphology and are evenly distributed within the well.
- For growth assays, a lower starting confluency is recommended, but if assessing both inhibition and growth this may need to be higher. As part of pre-optimization, we recommend testing several plating densities so the most appropriate can be chosen on the day. If conducting co-culture experiments, the expected growth patterns of the individual cells may need to be taken into consideration.
- For non-adherent cells, a biologically inert plate coating is recommended (e.g., Poly-L-Ornithine [PLO]) to ensure cells are lightly adhered to the plate to remain within the field of view for the experiment.

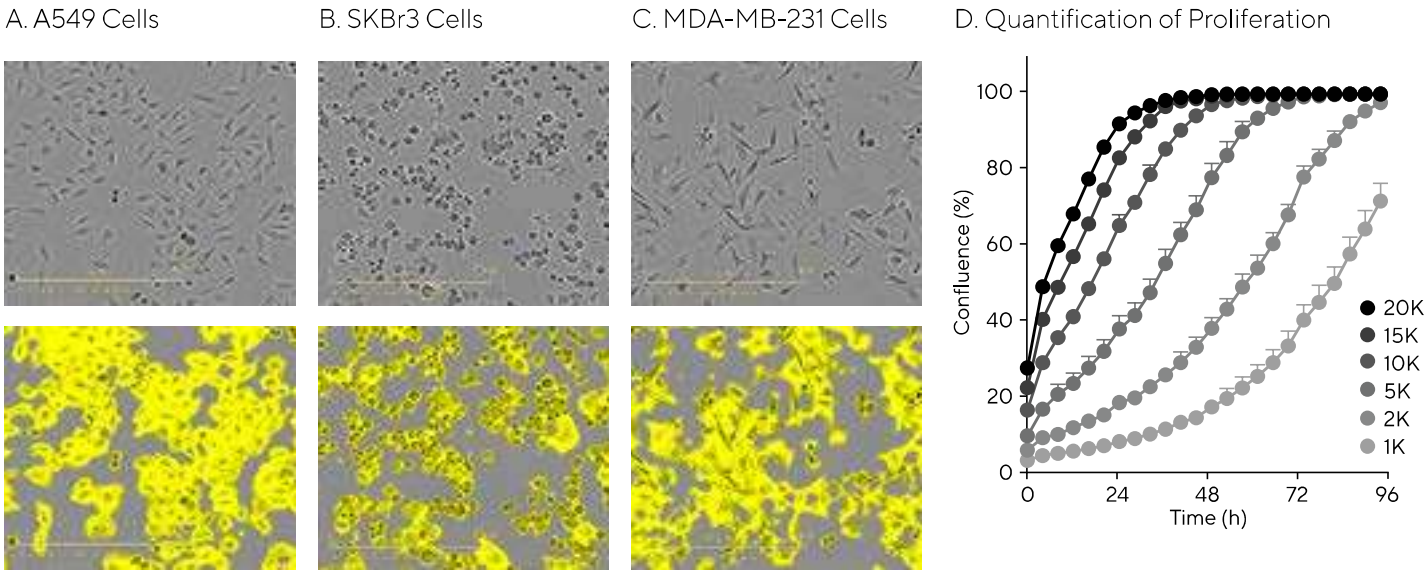
Label-Free Analysis of Cell Proliferation

Label-free analysis is a non-perturbing and noninvasive method, enabling the study of undisturbed cell populations. This makes it well suited to applications where label addition may lead to changes in cellular behavior over time, particularly for primary tissues. Eliminating the need for any manipulation of cells ensures that any observed activity at the cellular level is a result of the test conditions, not the label or labeling process.

Depending on the level of rigor required, confluence assays can be a high value and valid approach, routinely used as a surrogate for cell number.[1] They are simple to implement and can distinguish concentration-dependent effects on proliferation. Cells in monoculture are seeded into 96- or 384-well plates and images collected over time.

Image analysis is performed using the Incucyte® Live-Cell Analysis System with Base Analysis Software to achieve a confluence mask, which identifies cell coverage in the well (Figure 1A–C). To demonstrate the type of data that can be quantified and graphed within the integrated software, MDA-MB-231 cells were seeded at various starting densities (1–20 K/well) and imaged using the Incucyte® over 4 days (Figure 1D). The graph displays the proliferation over time for this cell line. This method has been demonstrated to successfully measure proliferation of multiple different adherent and non-adherent cell types, for studying cell type-specific effects of pathways including growth, inhibition, and differentiation.[2] The ubiquitous functionality of this approach makes it ideally suited to simple proliferation assays as well as a quality control tool.

Figure 1: *Kinetic, Label-Free Analysis of Cell Proliferation*

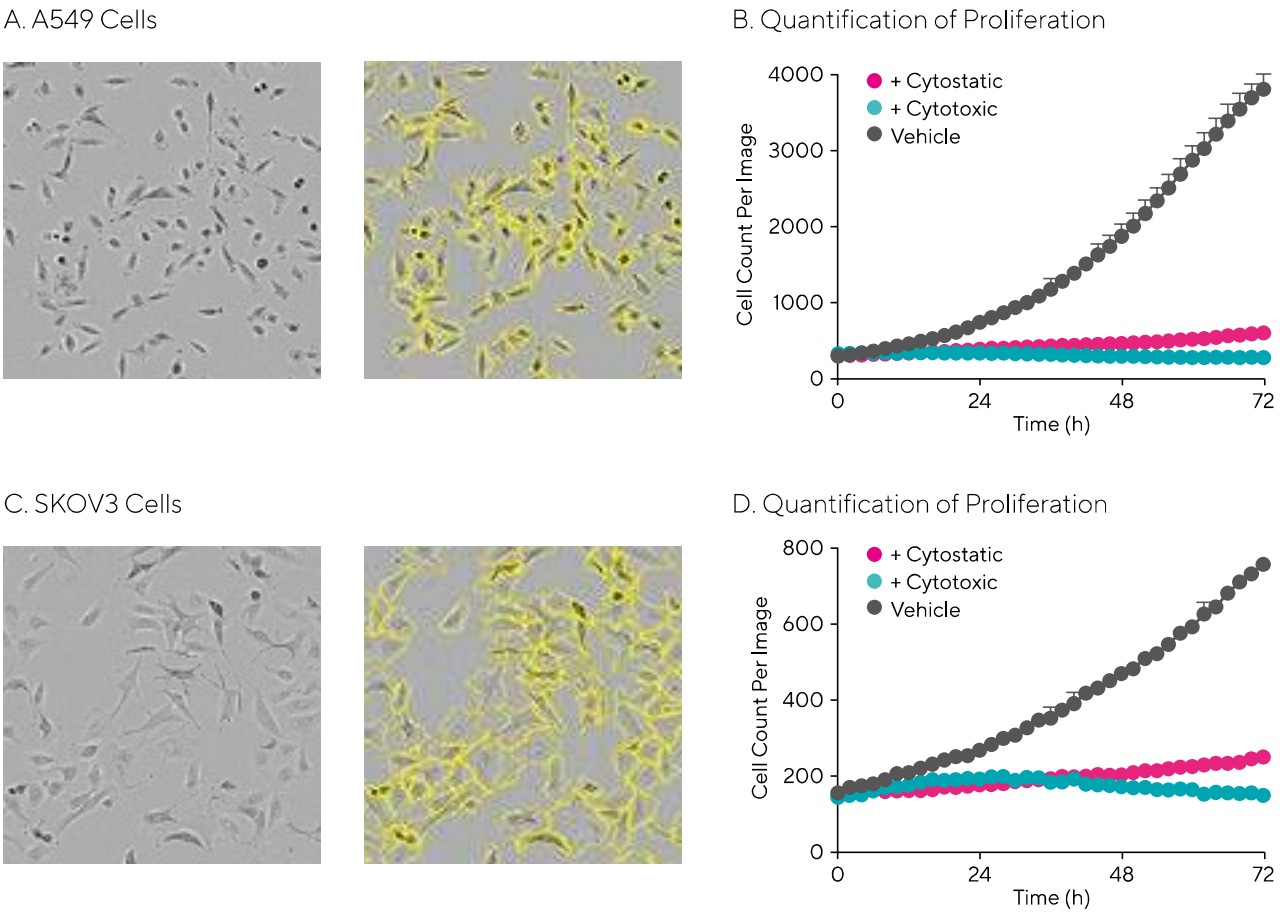


Note. A549 (A), SKBr3 (B), or MDA-MB-231 (C) cells were seeded in a 96-well plate, and images were collected and analyzed using the Incucyte® Live-Cell Analysis System with Base Analysis Software. Shown are the original images (top row) and the generated confluence mask in yellow (bottom row). The graph (D) shows quantification of various starting densities of MDA-MB-231 cells proliferating over time. Data shown as mean ± SEM for 4 wells.

If a true cell count is required, label-free cell counting can be accomplished with the Incucyte® Cell-by-Cell Analysis Software Module, which utilizes proprietary image acquisition strategies and algorithms to identify individual cells in high definition (HD) phase-contrast images. As before, cells are seeded in 96- or 384-well plates and images collected using the Cell-by-Cell Software Module for either adherent or non-adherent cell types. Image analysis generates a mask to identify individual cells (Figure 2A and C), which can then be counted.

The graphs show the inhibitory effect on proliferation of a cytotoxic reagent camptothecin (10 µM) or a cytostatic reagent cycloheximide (1 µM), which was added to the well post-plating (Figure 2B and D). Both reagents result in a similar inhibition of cell proliferation. Parameters of individual cells can also be quantified using the Cell-by-Cell Software Module such as area, eccentricity and, if a label is introduced, fluorescence within the cell boundary.

Figure 2: *Kinetic, Label-Free Cell Counting*



Note. A549 (A) or SKOV3 (C) cells were seeded in a 96-well plate, and images collected and analyzed using the Incucyte® Live-Cell Analysis System with Cell-by-Cell Analysis. Shown are the original images (left column) and the generated segmentation mask in yellow (right column). The graphs (B and D) show quantification of proliferation in the presence of vehicle, a cytotoxic reagent camptothecin (10 µM), or a cytostatic reagent cycloheximide (1 µM) over time. Data shown as mean ± SEM for 4 wells.

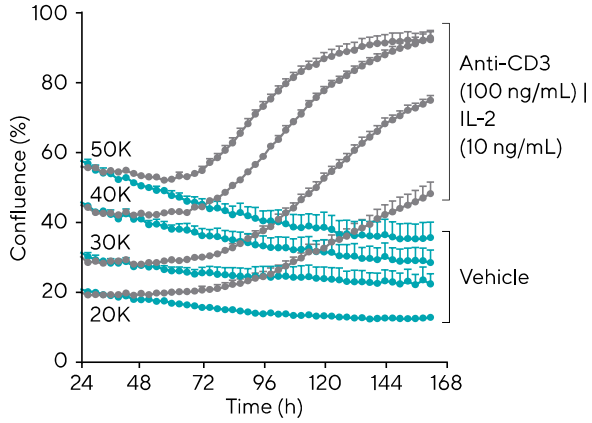
To further interrogate your cells without labeling and maximize your label-free analysis with advanced machine learning, the Incucyte® Advanced Label-Free Classification Analysis Software Module can be added to the Incucyte® Cell-by-Cell Analysis Software Module, expanding the automated identification and quantification of morphological changes. Using HD phase-contrast images, you can specify label-free cells of interest based on their morphology—such as apoptotic cells—and quantify them in real-time. See Morphological Analysis | Sartorius.

Continuous Live-Cell Proliferation for Non-Adherent Cells

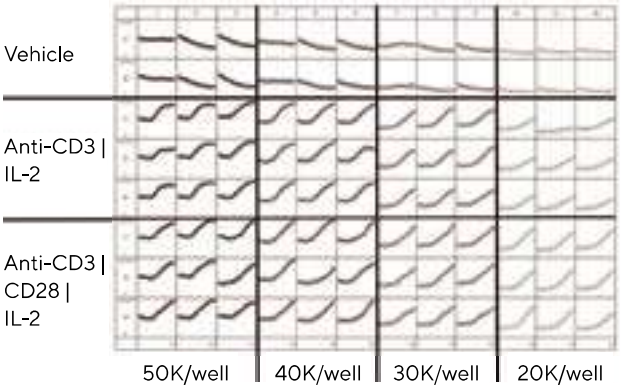
Confluence and Cell-by-Cell Analysis is also suitable for analysis of non-adherent cell types. To investigate the effect of seeding cell density on primary T cell proliferation, isolated human peripheral blood mononuclear cells (PBMCs) were seeded at various cell densities (20–50 K/well) on PLO-coated flat bottom 96-well plates. Cells were grown in the absence or presence of T cell activators anti-CD3 (100 ng/mL), IL-2 (10 ng/mL) and HD phase-contrast images were captured on an Incucyte® Live-Cell Analysis System. Images were analyzed for phase confluence (%) as a measure of cell proliferation. The kinetic graph (Figure 3A) demonstrates little or no proliferation under basal conditions (teal lines) but rapid proliferation in the presence of activators (grey lines), which is seeding cell density-dependent. The confluence in unstimulated PBMCs can be seen to drop over time due to the possible presence of phagocytes. The plate view (Figure 3B) displays confluence data over time using various activation cocktails.

Figure 3: *T Cell Proliferation Is Seeding Density-Dependent*

A. T Cell Proliferation



B. Automated 96-Well Continuous Analysis



Note. T cells demonstrate little or no proliferation under basal conditions but proliferate (A) when activated (e.g., by IL-2, anti-CD3, anti-CD28). Plate graph of change in confluence time-course reveals seeding density-dependent differences under various activation regimes (B).

Use of Fluorescence Labeling to Track Proliferation

Not all biology questions can be addressed using label-free techniques, so another common technique is to label cellular features using fluorescent tags.

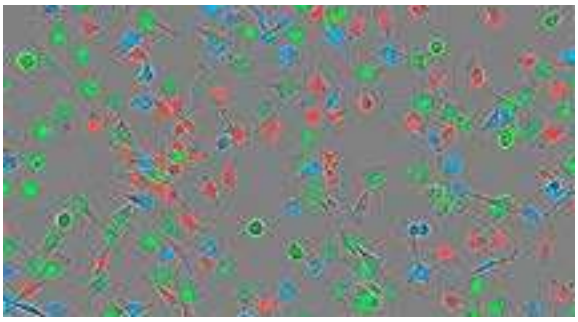
- Cells can be labeled with fluorescent dyes, for example Incucyte® Nuclight or Cytolight Rapid Dyes. These offer a simple, short-term approach, but signals can diminish over time as cell division occurs.
- Alternatively, stable expression of fluorescent proteins such as Incucyte® Nuclight or Cytolight Lentivirus provides a more robust, long-term approach but requires cell manipulation such as transduction.
- Finally, fluorescently tagged antibodies—for example Incucyte® Fabfluor Reagents—can be used for selective labeling in a mixed population of cell types based on surface protein expression.

Combining fluorescent labeling with live-cell imaging can aid in the analysis of cellular responses by providing visual monitoring to count individual cells and monitor growth and is a common technique for tumorigenic studies.[3,4] Nuclear counts can be performed using Incucyte® Nuclight Reagents. Incucyte® Nuclight Rapid Dye is a cell-permeable DNA stain that specifically stains nuclei in cells, using a mix-and-read protocol. Alternatively, Incucyte® Nuclight Lentiviruses are compatible with convenient transduction protocols and provide homogenous expression of a nuclear-restricted fluorescent protein in your choice of primary, immortalized, dividing, or non-dividing cells, without altering cell function and with minimal toxicity. Loss of viability is indicated by a loss in fluorescence when fluorescent protein passes out of the nucleus as nuclear membrane integrity is lost. These reagents are ideal for generating stable cell populations or clones using puromycin or bleomycin selection.

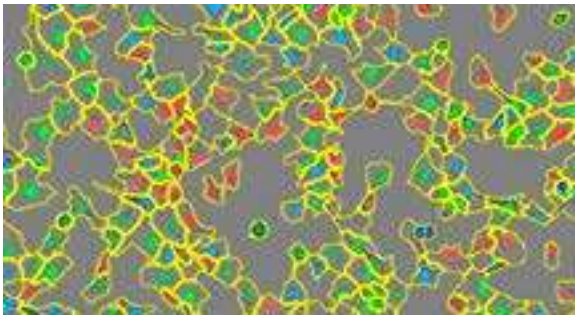
When using co-culture models, identification of each cell type allows the determination of specific effects. The data (Figure 4) shows a tri-culture of HT-1080 cells expressing different fluorescent markers and the ability to track all three populations using the Incucyte® SX5 Live-Cell Analysis System in combination with the Incucyte® Cell-by-Cell Software Module. A similar approach can be used to create more advanced co-culture models; for example, immune cell killing assays using labeled target cells with non-labeled effector cells to track the proliferation of the target cells over time.

Figure 4: *HT-1080 Nuclight Cell Tri-Culture*

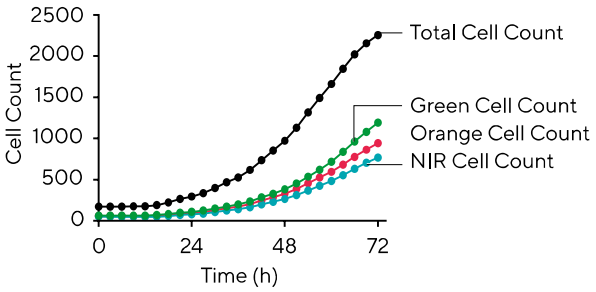
A. Cell Image (48 h)



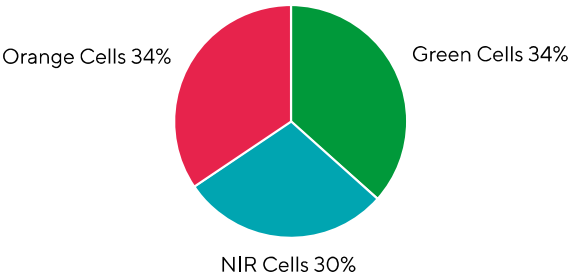
B. Segmented Image



C. Proliferation



D. Population (60 h)



Note. HT-1080 fibrosarcoma cells stably expressing Incucyte® Nuclight Green, Orange, or NIR Lentivirus were monitored for 72 hours. Representative images taken at 48 hours, with and without the label-free Incucyte® Cell-by-Cell Analysis mask (A, B), automatically identify the entire population of cells and quantify percentages of green-, orange-, or NIR-expressing cells (C, D).

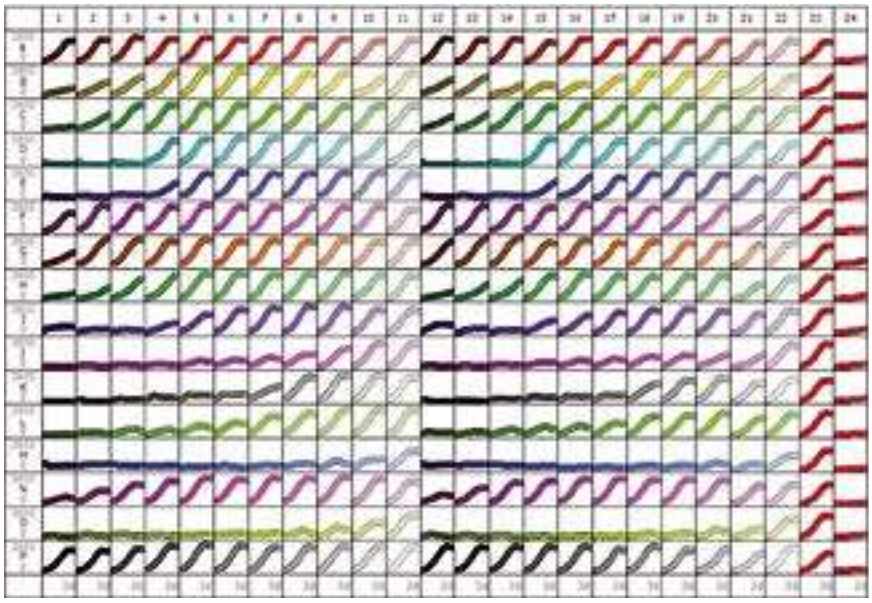
High Throughput Compound Testing Using Incucyte® Nuclight Green HT-1080 Cells

High throughput compound testing is essential for efficiently advancing promising compounds through the drug discovery pipeline. To examine the ability of the Incucyte® Live-Cell Analysis System to meet this need, cell proliferation was measured over time in a higher throughput format. To assess many pharmacological agents simultaneously, 16 literature-standard compounds (Table 1) were applied to Incucyte® Nuclight Green HT-1080 cells in a 384-well plate (Figure 5). An 11-point concentration response curve was constructed for each compound (Figure 6). Of the 16 compounds tested, the rank order of potency for inhibition of cell proliferation was: doxorubicin = staurosporine = camptothecin > mitomycin C > cycloheximide = RITA > PD-98059 > FAK inhibitor 14 = cisplatin > 10-DEBC = chrysin = compound 401. The compounds TAME, PAC-1, KU-0063794 and FPA-124 had little or no effect on cell proliferation under the conditions of the experiment.

Table 1: *Drugs Identified in Literature as Relevant to Cell Proliferation*

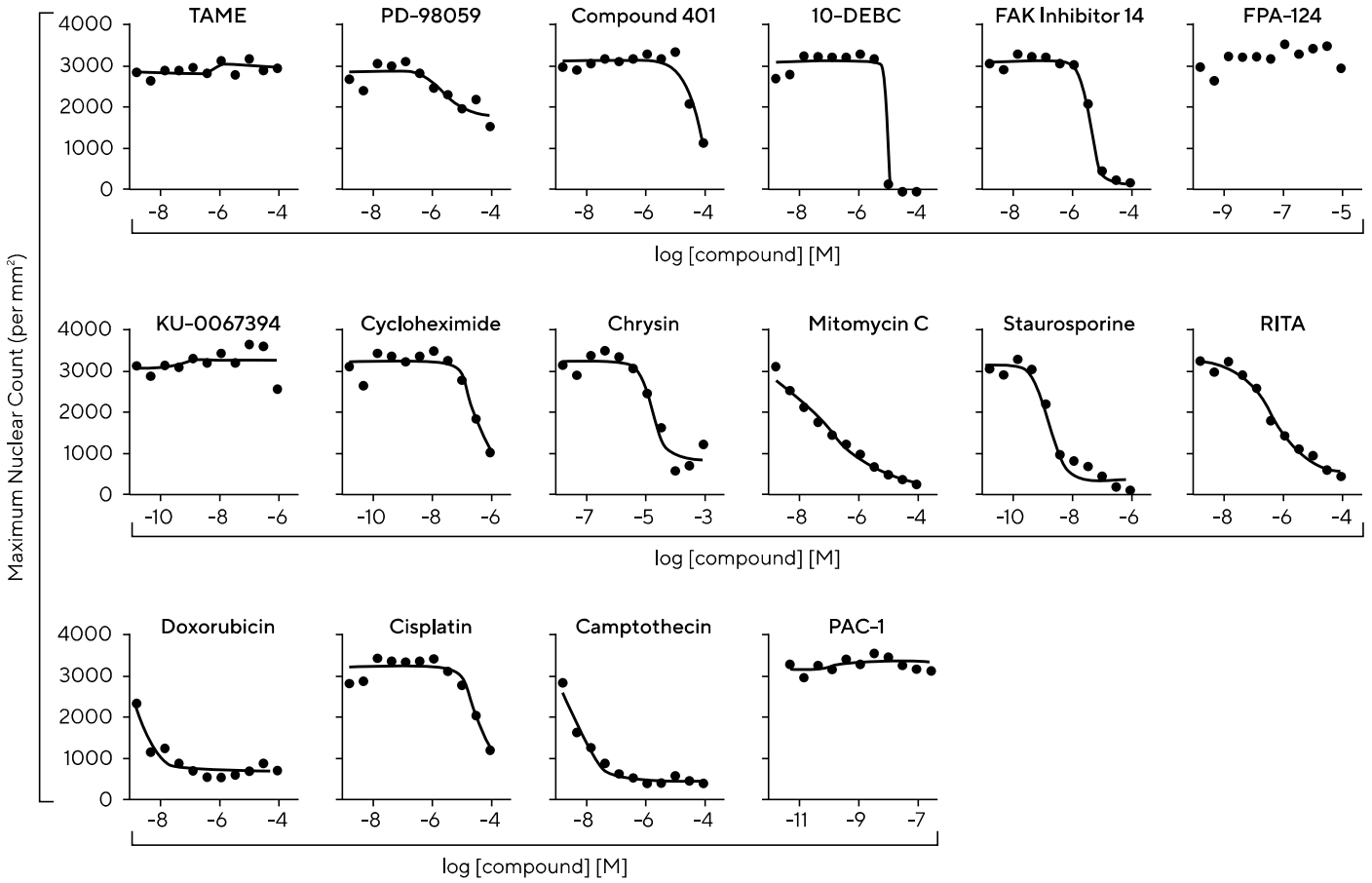
Drug	Description
Doxorubicin	Chemotherapy drug, intercalates DNA[5]
Camptothecin	Alkaloid inhibits topoisomerase, causing DNA damage[5]
Staurosporine	Potent alkaloid inhibitor of protein kinase[6]
Mitomycin C	Chemotherapy drug, alkylates DNA[7]
Cycloheximide	Protein synthesis inhibitor[8]
RITA	(Reactivation of p53 and induction of tumor cell apoptosis) a small molecule, binds p53[9]
PD-98059	MAPK1 | 2 inhibitor[10]
Cisplatin	Chemotherapy drug acts through crosslinking DNA[11]
FAK inhibitor 14	Selective inhibitor of focal adhension kinase[12]
10-DEBC	Selective inhibitor of Akt[13]
Chrysin	A flavonoid observed to inhibit growth in cancer cells[14]
TAME	Tert-Amyl methyl ether; a gasoline additive with suspected toxic effects upon inhalation[15]
PAC-1	(Procaspase activating compound-1), a small-molecule activator of procaspase-3 to caspase-3[16]
KU-0063794	Specific inhibitor of mTORC1 | 2[17]
FPA-124	Akt inhibitor[18]
Compound 401	Inhibitor of DNA-dependent kinase and mTOR[19]

Figure 5: *384-Well Microplate View of Incucyte® Nuclight Green HT-1080 Cell Proliferation With 16 Different Compounds*



Note. 11-point concentration-response curves in duplicate (different colors, high to low concentrations left to right). Columns 15 and 16 are vehicle (0.5% DMSO) and CHX (3 µM) controls, respectively. Note the potent concentration-dependent inhibition of cell proliferation for certain compounds (e.g., Row J, Row M, Row O), and weaker effects | inactivity of others (e.g., Row A, Row P). Abscissa: Time (0–72 hours), ordinate: Fluorescent Object Count per well (0–3800).

Figure 6: *Concentration Response Curves*



Note. Concentration-response curves for a selected set of literature standard inhibitors of cell proliferation in Incucyte® Nuclight Green HT-1080 cells. Abscissa: -log M [compound], ordinate: Maximum Nuclear Count (per mm²). Each point represents the average data from two wells from a single 384-well compound plate. The lines of best fit are a 4-parameter logistic equation and can be calculated using Incucyte® Integrated Analysis Software.

Discussion and Summary

The experiments described in this application note demonstrate:

1. Label-free solutions for assessing either cell confluence or cell count providing a validated assay for tracking cell proliferation for both adherent and non-adherent cell types.
2. The introduction of fluorescent labels enables kinetic proliferation assays based on direct, true cell (nuclear) count to be performed in both mono- and co-culture models.
3. Proliferation assays can be run in microplates (96-well and 384-well) with high precision and reproducibility. In 384-well plates, a mix and read assay is exemplified whereby full concentration-response curves of 16 standard antiproliferative agents were compared. In a single Incucyte® Live-Cell Analysis System, 6 x 384-well plates can be monitored providing > 2000 wells of parallel data acquisition.

Additionally, all data and time points can be verified by inspecting individual images and/or time-lapse movies. Cell morphology observations provide additional validation and insight into mechanistic differences between treatments or conditions.

We conclude that our label-free image analysis solutions and non-perturbing fluorescent reagents in combination with easy to use software tools provides a powerful solution for kinetic cell proliferation measurements and pharmacology assays.

References

1. Miettinen T, Kang J, Yang L, Manalis S. Mammalian cell growth dynamics in mitosis. *eLife*. 2019; 8: e44700. Accessed February 25, 2022.
2. Szemes M, Melegh Z, Bellamy J, et al. A Wnt-BMP4 signaling axis induces MSX and NOTCH proteins and promotes growth suppression and differentiation in neuroblastoma. *Cells*. 2020;9(3):783. doi: 10.3390/cells9030783.
3. Velazquez E, Brindley T, Shrestha G, et al. Novel monoclonal antibodies against thymidine kinase 1 and their potential use for the immunotargeting of lung, breast and colon cancer cells. *Cancer Cell Int*. 2020;20:127. doi: 10.1186/s12935-020-01198-8.
4. Härkönen K, Oikari S, Kyykallio H, et al. CD44s assembles hyaluronan coat on filopodia and extracellular vesicles and induces tumorigenicity of MKN74 gastric carcinoma cells. *Cells*. 2019;8(3):276. doi: 10.3390/cells8030276.
5. Sappal, DS, et al. Biological characterization of MLN944: A potent DNA binding agent. *Molec. Cancer Ther.* 2004, 3(1):47
6. Seynaeve, CM. et al. Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. *Mol. Pharmacol.* 1994, 45(6):1207-1214
7. Palom, Y. et al. Bioreductive metabolism of mitomycin C in EMT6 mouse mammary tumor cells: cytotoxic and non-cytotoxic pathways, leading to different types of DNA adducts. The effect of dicumaro. *Biochem. Pharamcol.* 2001, 61(12):1517-1529.
8. Chang, TC et al. Effects of transcription and translation inhbitors on a human gastric carcinoma line. Potential role of Bcl-X(S) in apoptosis triggered by these inhibitors. *Biochem. Pharmacol.* 1997, 53(7)96-977.
9. Roh, JL et al. The p53-reactivating small-molecule RITA enhances cisplatin-induced cytotoxicity and apoptosis in head and neck cancer. *Cancer Let.* 2012, 325(1):35-41
10. Aravena, C et al. Potential Role of Sodium-Proton Exchangers in the Low Concentration Arsenic Trioxide- Increased Intracellular pH and Cell Proliferation. *PLoS One* 2012, 7(12): e51451
11. Hu, W. The anticancer drug cisplatin can cross-link the interdomain zinc site on human albumin. *Chem. Commun.* 2011, 47(21):6006-6008.
12. Cabrita, MA. Focal adhesion kinase inhibitors are potent anti-angiogenic agents. *Molec. Oncology* 2001, 5(6):517-526.
13. Janjetovic, K. Metformin reduces cisplatin-mediated apoptotic death of cancer cells through AMPK-independent activation of Akt. *Europ. J. Pharmacol.* 2011, 651(1-3):41-50.
14. Shao, J et al. AMP-activated protein kinase (AMPK) activation is involved in chrysin-induced growth inhibition and apoptosis in cultured A549 lung cancer cells. *Biochem. Biophys. Res. Comm.* 2012, 423(3):448-453.
15. Ahmed, FE. Toxicology and human health effects following exposure to oxygenated or reformulated gasoline. *Tox. Letters* 2001, 123(2-3):89-113.
16. Boldingh Debernard, KA. Cell death induced by novel procaspase-3 activators can be reduced by growth factors. *Biochem. Biophys. Res. Comm.* 2011, 413(2):364-369.
17. Garcia-Martinez, JM. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem. J. 2009, 421(1):29-42.
18. Strittmatter, F et al. Activation of protein kinase B/Akt by alpha1-adrenoceptors in the human prostate. *Life Sciences* 2012, 90(11-12):446-453.
19. Ballou, LM et al. Inhibition of Mammalian Target of Rapamycin Signaling by 2-(Morpholin-1-yl)pyrimido[2,1-α] isoquinolin-4-one. *J. Biol. Chem.* 2007, 282(33):24463-70.

North America
Sartorius Corporation
300 West Morgan Road
Ann Arbor, Michigan 48108
USA
Phone +1 734 769 1600

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202

Kinetic-Proliferation-Assay-Application-Note-en-L-Sartorius Status: 03 | 2022