APPLICATION NOTE

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Real-time Multiparametric Pharmacological Screening with Live Cell Imaging

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Abstract

A comprehensive understanding of animal cell proliferation underpins a wide range of cellular models, from simple monocellular systems used in basic life sciences to more complex co-culture systems employed in more translational and commercial settings. Many pathologies are indeed characterized by different degrees of deranged cell proliferation, and live cell imaging represents the ideal tool to study and understand this behavior for both discovery and monitoring purposes. Different qualitative and quantitative metrics can be used as proxies of cellular growth, allowing researchers to better understand the impact that experimental conditions might have on the proliferation of cells in different systems. In this application note, we show how the CELLCYTE X™ platform can be used to conveniently obtain a comprehensive, robust, multiparametric and real-time overview of cell growth in multi-well plates. We have demonstrated that the Enhanced Contour-based cell confluence percentage in combination with fluorescence signal intensity and nuclear or cellular counting metrics can be used to characterize the pharmacology of drugs in different human and murine cell types over multiple days.

Introduction

Cell proliferation, an increase in the number of cells in a population, is a crucial metric in both basic and translational research, particularly in drug discovery for immune-oncology applications. As mitosis directly results from biomass accumulation and successful cell cycle completion, abnormal proliferation patterns represent a primary readout for the breakdown of natural molecular mechanisms regulating the balance between cell cycle arrest and cell proliferation, as observed in tumorigenesis and cancer progression.

Changes in cell proliferation are commonly assessed with end-point assays, such as quantifying DNA synthesis via the incorporation of nucleoside analogues BrdU and EdU, or by analyzing the expression of proliferation reporter proteins, such as Ki-67 and the Proliferating Cell Nuclear Antigen (PCNA). However, in the past two decades, live cell imaging has proven to be a far superior technique because it relies on the acquisition and analysis of images of growing cells within an incubator over multiple days or weeks, providing real-time data of cell populations in controlled, consistent environmental conditions.

This application note demonstrates how the CELLCYTE X, a high-throughput live cell imaging system, monitors cell proliferation in real time using multiple parameters. The following sections detail how the CELLCYTE X platform can be used to quantitatively and qualitatively assess the pharmacology of different drugs just by using label-free confluence measurements in murine embryonic fibroblast (MEF) cells (**Figures 3 and 4**) or via multiparametric analysis of cells expressing a fluorophore (**Figures 5 and 6**).

Materials and methods

Cell culture and treatments

RPE-1, MEF and MDA-MB-231 cells were cultured in complete Dulbecco's Modified Eagle Medium/F-12 media (DMEM/F-12 media supplemented with 10% fetal bovine serum [FBS], 1% penicillin/streptomycin, 1% nonessential amino acids and 1 mM sodium pyruvate) at 37°C with 5% CO_2. One day before initiating the experiments, cells in the exponential growth phase were detached with TrypLE solution and seeded in a 96-well plate in a volume of 150 µL. For untreated conditions, cells were seeded in 200 µL. The cells were then allowed to settle at room temperature for 20 minutes to ensure a homogeneous distribution of cells across the well. The following day, compound solutions were added on top of each well in a volume of 50 µL per well. All drug stock solutions were prepared in Dimethyl sulfoxide (DMSO) and diluted as required in complete growth media. Vehicle controls were achieved using a volume of DMSO corresponding to the highest concentration of drug, either 0.2% or 0.1%. Before imaging, the multi-well plates were placed into the CELLCYTE X for 30 minutes to equilibrate their temperature at 37°C in order to minimize condensation. Imaging parameters, including imaging channels, fluorescence exposure time and gain, scan intervals and total duration of the run, were set up and established for each experiment using the CELLCYTE Studio and Analysis software.

Results and discussion

Multiparametric analysis of cell proliferation

As exemplified in **Figure 1**, growing Retinal Pigment Ephitelial-1 (RPE-1) cells were imaged in a high contrast form of brightfield called Enhanced Contour (EC) as well as in the green (green FL) and red (red FL) fluorescent channels to monitor the expression of a nuclear and cytoplasmic fluorophore, respectively. In the second step, the CELLCYTE X software was used to analyze these images in parallel in order to extrapolate quantitative measurements of cell proliferation in the form of EC confluence percentage, green FL cell count and red FL nuclei count.

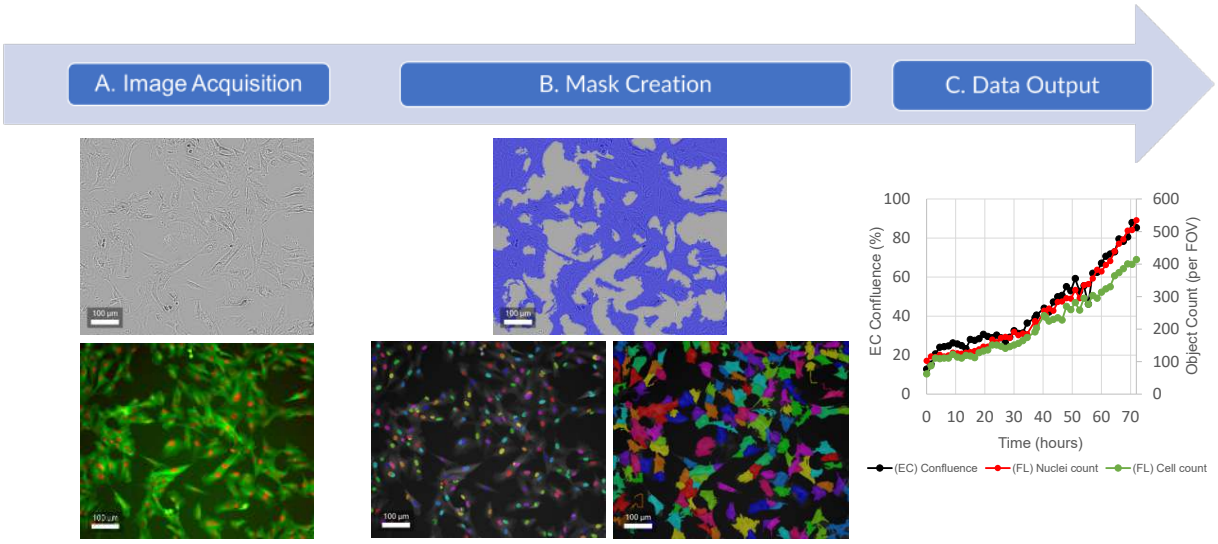

Figure 1. *Real-time analysis of cell proliferation in RPE-1 cells expressing YFP-tagged α-tubulin and mRFP-tagged histone H2B.* ***A****) Representative images of RPE-1 cells acquired with the CELLCYTE X in Enhanced Contour (top) and overlain green and red (bottom) fluorescent (FL) channels.* ***B****) Corresponding fitted masks for EC confluence (top), nuclei (left) and cell (right) counting metrics were created and analyzed using the CELLCYTE Studio and Analysis software.* ***C****) Kinetic analysis of EC confluence, nuclei and cell count metrics of RPE-1 cells over a 72-hour period. Each data point is extrapolated from one field of view (FOV) acquired every 1.5 hours. Fluorescent images were acquired with 100 ms of exposure to blue light and 0 dB of gain for the green FL channel, and 150 ms of exposure to amber light and 5 dB of gain for the red FL.*

The ability to measure multiple fluorescent signals in parallel makes the CELLCYTE X the ideal platform for real-time analysis of co-culture systems (**Figure 2**). In this study, the proliferation of a breast cancer cell line expressing either a green or red fluorophore was analysed by considering the relative area occupied by each fluorescent signal using the fluorescence confluence area percentage metric (**Figure 2B**). The fluorescent object count per each field of view (FOV), as seen in **Figure 2C**, clearly complements this finding, indicating a superior proliferation for GFP-expressing cells. Lastly, the fluorescence intensity (a.u.) metrics (**Figure 2D and 2E**) give a more comprehensive view of the individual cellular proliferation behavior over time. Indeed, the overall red fluorescent intensity indicates that despite the lower proliferation of mCherry expressing cells, the fluorescent signal intensity is equivalent to the GFP signal (**Figure 2D**). This is explained by the individual fluorescent metrics, which can be analyzed both as the population average over time as shown in **Figure 2E** or at the individual cell level across all the images as shown in **Figure 2F**, confirming that mCherry-expressing cells are fewer in number but emit a more intense fluorescent signal compared to GFP-expressing cells.

These findings can be reconciled considering that the exposure time used for the red channel is 350 ms against 100 ms for the green channel, reflecting less efficiency from mCherry compared to GFP. Furthermore, this study suggests that cells expressing GFP proliferate faster than those expressing mCherry, possibly as a result of the genetic instability the latter engineered cell line exhibits.

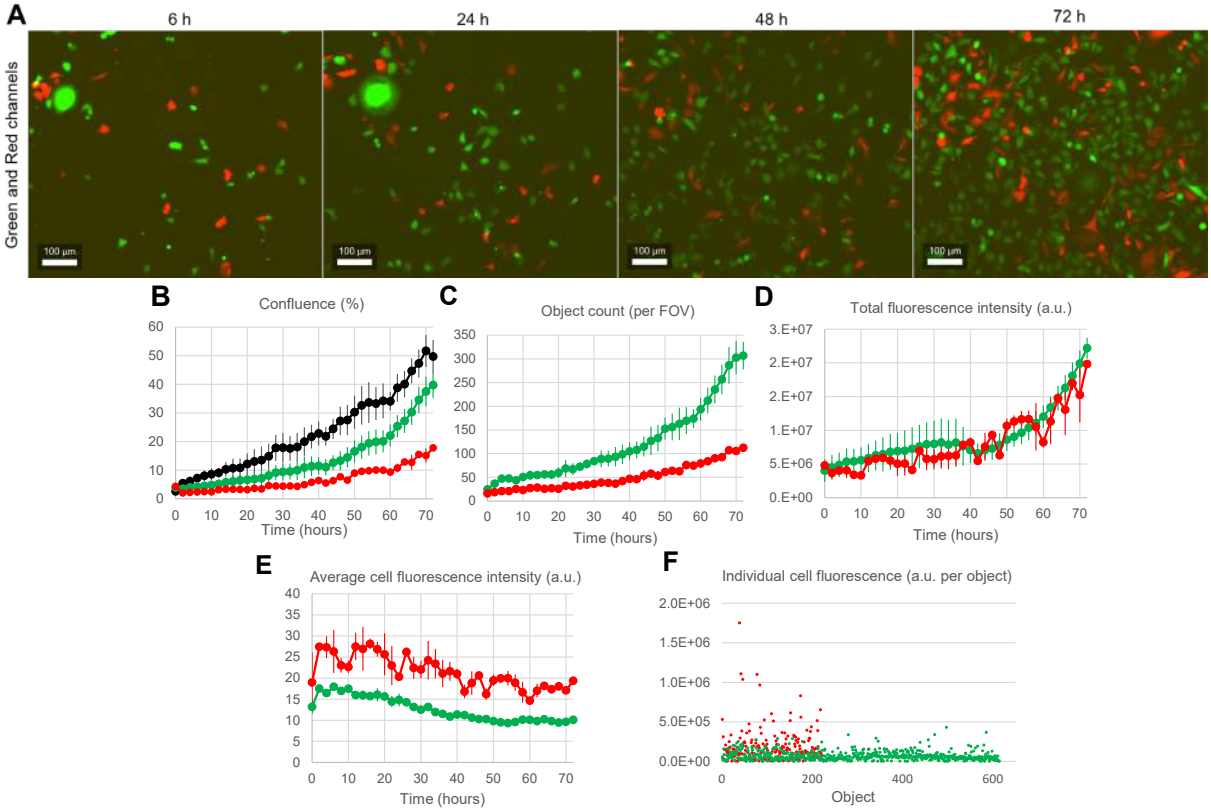

Figure 2. *Representative images and co-culture study analysis of MDA-MB-231 cells expressing either GFP or mCherry. (**A**) The proliferation of a heterogeneous mixture of MDA-MB-231 cells stably expressing either GFP or mCherry was monitored by acquiring the indicated timepoint images in the green and red fluorescent channels (top row) using 100 ms of exposure 0 dB of gain and 350 ms of exposure and 5 dB of gain, respectively. (**B**) Quantitative analysis of the cell confluence in EC without consideration of fluorophores expression (black line) or for each individual fluorescent channel. The same mask was used to compute the fluorescent object count metric (**C**) and the fluorescent intensity metrics (**D**, **E**, **F**). Cell population average fluorescence intensity calculated for each field of view in the respective channel (**E**), and population fluorescence intensity plot at the individual cell level at 72 hours (**F**). All data are shown as representative averages ± SEM of 2 FOVs. In (**F**), all cells detected in 2 FOVs were included.*

With the CELLCYTE X Studio Software, users can also tailor the fluorescent mask sensitivity to include only cells expressing a certain level of fluorophore, which is useful when culturing a heterogeneous population of cells showing different degrees of expression. By leveraging these metrics, it is possible to account for variable fluorophore expression in high-throughput formats, including genetic screenings of CRISPR/Cas9 gRNA libraries or FACS-sorted cells.

Label-free dose-response analysis of drugs inhibiting cell proliferation

Dose-response assays are critical tools for evaluating the effects of various compounds in the drug discovery pipeline, especially when evaluating the impact of drugs on the proliferation of different types of cells over multiple days. The CELLCYTE X Enhanced Contour imaging modality is a form of high contrast brightfield microscopy that can be used to obtain accurate label-free cell confluence measurements.

To validate the applicability of the cell confluence metric to a high-throughput screening platform, we monitored the proliferation profiles of MEF cells treated in a 96-well plate with Cycloheximide, a commonly used cytotoxic protein synthesis inhibitor drug (Lawana, 2014), and Camptothecin, a DNA Topoisomerase I inhibitor historically used for its anti-tumoral activity (DrugBank Online, 2021).

To evaluate the translatability in terms of sensitivity and granularity of the EC confluence metric, a 1:3 dilutional dose-response curve was derived for the two drugs, covering 2-log concentrations range from 0.123 µM to 10 µM.

Robust compound concentration-dependent dose responses were obtained for both the Camptothecin and Cycloheximide treatments (**Figure 3**). MEF treated with Camptothecin, particularly at the lower doses, showed an increase in confluence for the first 6 hours after applying the compound. Afterwards, the cytotoxic effects were soon apparent. There was a steady decrease in cell confluence at the lowest concentration of 0.123 µM and a kinetically equivalent exponential decrease in confluence for concentrations of 1.11 µM up to 10 µM (**Figure 3A**).

On the other hand, Cycloheximide-treated cells showed a wider range of cytotoxicity kinetics. While a minor increase in confluence was also observed during the first 3 hours, it was followed by a slower and steadier decline with the fastest kinetics seen with the highest concentration (**Figure 3B**).

The different potencies of these compounds are reflected in their half-maximal inhibitory concentrations (IC50), which were calculated with the cell confluence data at 48 hours. MEF cells were clearly more sensitive to Camptothecin (IC50 = 0.15 µM, CI95 0.12 – 0.17 µM) than to Cycloheximide (IC50 = 1.15 µM, CI95 1.03 – 1.48) (**Figure 3C**).

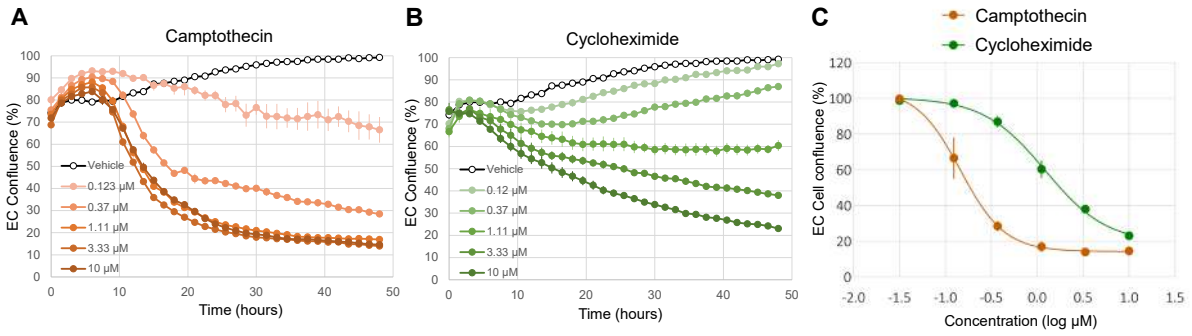

Figure 3. *Label-free analysis of concentration-dependent growth inhibition (**A**, **B**) and pharmacological potency analysis (**C**) of Camptothecin and Cycloheximide in 7,500 MEF cells. (**A**, **B**) EC-based cell confluences were extrapolated every 1.5 hours. 2 FOV were acquired per well with a total of 4 wells per condition in a 96-well plate. (**C**) Label-free drug potency analysis of Camptothecin and Cycloheximide at 48 hours. Data are shown as averages ± Standard Error of the Means (SEM) of 4 wells and potency curves were created with GraphPad Prism 9.2.0 for Windows using a sigmoidal 4PL interpolation.*

This quantitative data, indicating a wider pharmacological window for Cycloheximide, is also reflected in the qualitative morphology changes that can be appreciated with the high-resolution EC images generated with the CELLCYTE X (**Figure 4**). While neither drug at 1.11 µM seemed to affect the typical fibroblast morphology within 6 hours, there was a clear difference by 48 hours. With Camptothecin, extensive cell death can be seen in the form of rounded up high-contrast cells.

The mask used for the quantification of EC confluence was tailored not to recognize the extensive debris (**Figure 4, middle right panel**) in order to more accurately reflect the drug cytotoxicity. On the other hand, the protein synthesis inhibition instated with Cycloheximide was seen not only in the form of a moderate proliferation inhibition as quantified by EC confluence, but also morphologically in the extensive accumulation of high contrast perinuclear structures (**Figure 4, bottom right panel**), features that can be appreciated thanks to the high resolution EC images.

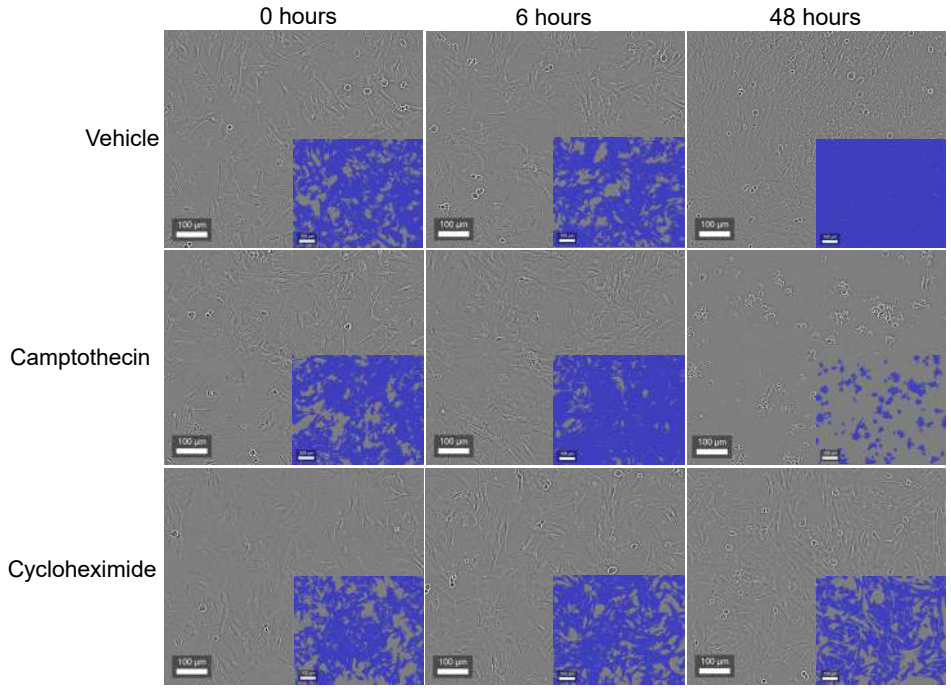

Figure 4. *Representative EC images of 7,500 MEF cells treated with 1.11 µM Camptothecin or Cycloheximide. The relative insets shown in blue showcase the fitted masks used to recognize and compute the area occupied by the cells at the indicated timepoints.*

Multiplexing compound screening using Enhanced Contour confluence and fluorescent channel metrics

Screening libraries of prospective anti-proliferative drugs is often done by analysis in high-throughput setups with a single metric reflecting different measurements of cellular health at pre-determined endpoints. By using the CELLCYTE X's Enhanced Contour and fluorescence imaging modalities, the impact of drug treatments on cellular proliferation can be evaluated in real time with multiple metrics.

To validate the capacity of the CELLCYTE X to quantify cell proliferation sensibly and accurately, we treated a rapidly-growing breast cancer cell line with a wide concentration range of cytotoxic drugs and imaged it in real time using the instrument's EC and green fluorescent channels.

MDA-MB-231 cells constitutively expressing the GFP protein were treated with a 1:2 dilutional dose-response curve from 156 nM to 20 µM of Staurosporine and Camptothecin. Staurosporine is a potent protein kinase inhibitor that induces cell death via Caspase-3 mediated apoptosis (Eidet, 2014).

This drug has also been documented to cause a unique cell morphological change to a characteristic stellated shape in a spectrum of cell lines, from tumor to mesenchymal to lymphocytes, a phenomenon likely ascribed to the inhibition of actin microfilaments polymerization (Deshmukh, 2000 and Yang, 1997).

As shown in **Figures 5A** and **5D**, both Staurosporine and Camptothecin impacted the EC confluency metrics with a peak after 6 to 12 hours, followed by a slow but steady decrease over the next 3 days, particularly at the highest doses. Fluorescent images were also acquired and analyzed with the CELLCYTE Studio Software, obtaining both the green intensity (**Figures 5B and 5E**) and green object count metrics (**Figure 5C and 5F**). Indeed, these two parameters gave a complementary view of the effect of these drugs. The green intensity of Staurosporine-treated cells (**Figure 5B**), which reflect the degree of overall GFP expression, remained constant for the first 6 to 12 hours and decreased steadily, while the control-treated cells displayed a 6-fold increase. For Camptothecin, cells showed a more nuanced dose-dependent constant kinetics over the 3 data (**Figure 5E**). The third metric of interest was the green object count which, contrary to the intensity, showed more granular dose-dependent behavior with Staurosporine (**Figure 5C**) than with Camptothecin (**Figure 5F**).

These 3 metrics provide a distinct but complementary set of quantitative measurements that can be reconciled by examining the images that were acquired and analyzed.

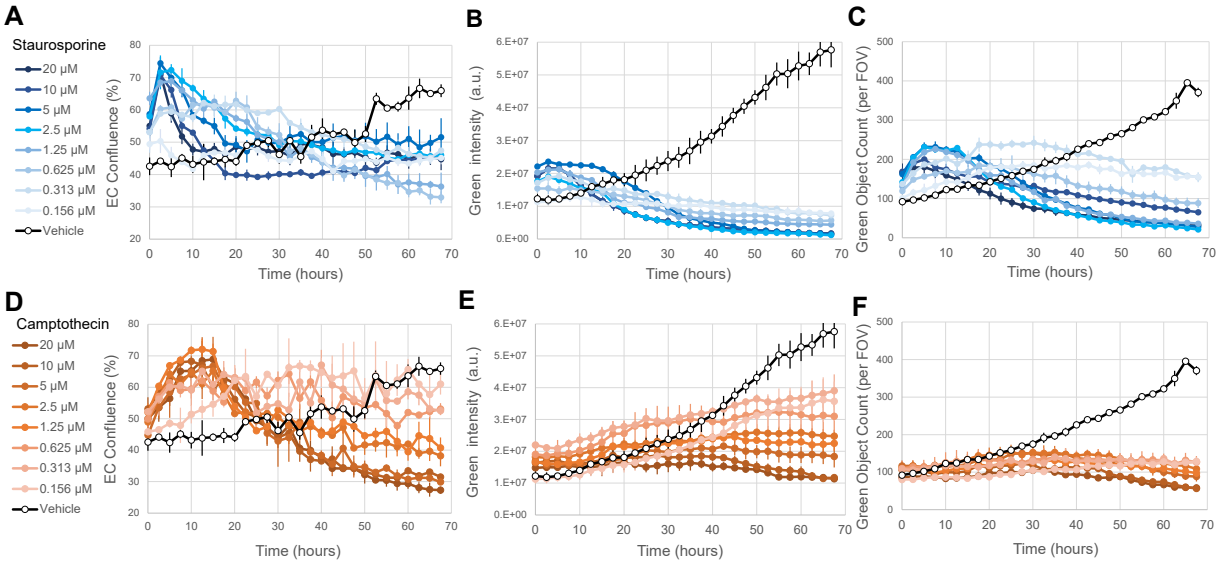

Figure 5. *Proliferation curves of GFP-expressing MDA-MB-231 cells treated with a wide concentration range of Staurosporine (**A**, **B**, and **C**) and Camptothecin (**D**, **E**, and **F**). Confluence percentage (**A**, **D**), green intensity (**B**, **E**) and green object count metrics (**C**, **F**) were calculated from EC and green FL images acquired in parallel every 2.5 hours over a period of 72 hours. Each data point represents the average of 2 wells with error bars indicating the SEM. Green FL images were acquired with an exposure time of 50 ms and 0 dB of gain.*

Cells treated with Staurosporine acquired a stellate morphology that manifested prominently after 10 hours (**Figure 6**). The EC-confluence mask (**Figure 6**) was therefore overestimating the increase in overall occupied area. Over time, cell growth was indeed inhibited and despite the stellate morphology, extensive cell death could still be observed with the EC confluence metric, which depicted a steady decline after the first 10 hours. The values recorded for the highest concentrations were affected by extensive cellular debris.

In this scenario, a complementary metric to consider is the fluorescent signal. When examining the green fluorescence, the accumulation of GFP in apoptotic bodies in Staurosporine-treated cells explained the increase in green objects count seen quantitatively in **Figure 5C** and morphologically in **Figure 6**. Over the following days, with the complete loss of membrane integrity and necrosis, the fluorescent object numbers quickly decreased in a more dose-dependent fashion, with minimal signal seen after 72 hours in Staurosporine-treated cells (**Figure 6**).

On the other hand, the rapid increase in EC confluence seen in cells treated with Camptothecin is explained by the increase in number of oversized anaplastic cells that grow in biomass but do not undergo mitosis (**Figure 6**), a phenomenon that has been described in literature (Tharanga, 2018). This cell cycle blockage reflects the static green cell count metric shown in **Figure 5F and Figure 6**, while biomass expansion is more accurately and dose-dependently reflected in terms of GFP expression, as seen by the green intensity metric signal in **Figure 5E**.

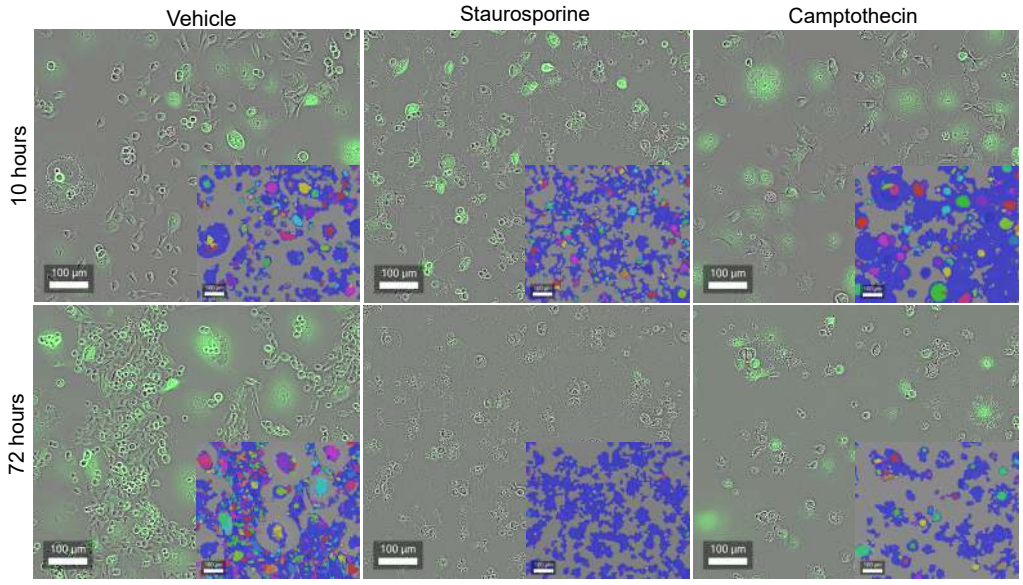

Figure 6. *Representative overlain EC and green FL images and relative fitted masks of MDA-MB-231-GFP cells treated with 20 µM Staurosporine and Camptothecin for either 10 or 72 hours. Small insets show in blue the corresponding fitted EC-based cell confluence masks, while the tailored green FL mask is overlain with each independent object indicated by a different color.*

Conclusions

The CELLCYTE X live cell imager offers automated, continuous kinetic cell growth analysis for pharmacological screening. The experiments described in this application note demonstrate that:

- EC confluence percentage, nuclei count and cell count are used to monitor cell proliferation
- Multiple fluorescent channels enable flexible cell proliferation analysis in co-culture systems
- Label-free, high-throughput setting is well suited for sensitive, kinetic and morphological dose-response pharmacological analysis of growth inhibiting drugs
- Comprehensive pharmacological appraisals is achieved via qualitative morphological analysis alongside quantitative measurements of EC confluence percentage, fluorescence intensity signal and fluorescent object count.

References

1. Lawana V, Korrapati MC, Mehendale HM. Cycloheximide. In Wexler P, ed. *Encyclopedia of Toxicology*. 3rd ed. Academic Press; 2014: 1103–1105. DOI:10.1016/B978-0-12-386454-3.00298-0.
2. DrugBank Online. Camptothecin. Accessed 12 October 2021. https://go.drugbank.com/drugs/DB04690.
3. Eidet JR, Pasovic L, Maria R, et al. Objective assessment of changes in nuclear morphology and cell distribution following induction of apoptosis. *Diagnostic Pathology*. 2014; 9(1): 92. DOI:10.1186/1746-1596-9-92.
4. Deshmukh M, Johnson EM. Staurosporine-induced neuronal death: multiple mechanisms and methodological implications. *Cell Death & Differentiation*. 2000; 7(3): 250–261. DOI:10.1038/sj.cdd.4400641.
5. Yang R, Fu W, Wang S, et al. Mechanism of the morphological changes induced by staurosporine in rat osteoblasts. *Calcified Tissue International*. 1997; 61: 68–73. DOI:10.1007/s002239900297.
6. Tharanga Jayasooriya R, Gayani Dilshara M, Neelaka Molagoda I, et al. Camptothecin induces G2/M phase arrest through the ATM-Chk2-Cdc25C axis as a result of autophagy-induced cytoprotection: Implications of reactive oxygen species. *Oncotarget*. 2018; 9(31): 21744–21757. DOI:10.18632/oncotarget.24934.

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