High content imaging analysis for basic and applied research

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Introduction

High content imaging analysis systems that are integrated with fluorescence microscopes were initially designed for the drug discovery industry and have been used in that setting for over 20 years. However, those systems have limitations which have prevented smaller labs – including basic research labs – from gaining access to them. Issues with how image analysis software handles very large datasets, large footprints and prohibitively high costs have all proved problematic.

In this eBook, we take a look at the CELENA® X High Content Imaging System from Logos Biosystems to help you understand how its integrated image analysis software, benchtop capability, affordable cost and versatility can fulfil the high content imaging needs of any small laboratory.

The automated fluorescence microscope

The CELENA® X High Content Imaging System is a technological solution, designed for the benchtop, that integrates an automated fluorescence microscope with sophisticated quantitative image analysis software capable of processing large datasets at an affordable cost. It is capable of high content, multi-color fluorescence imaging via a user-friendly design and intuitive data analysis software. Its interface allows a user to run multi-well or multi-spectral experiments with only a few clicks. The microscope provides for a multitude of fluorescence cell imaging possibilities by supporting all objective magnification from 1.25x to 100x, both brightfield and phase contrast illumination, and with LED filter cubes.

This study utilized the CELENA®X to effectively quantify and monitor the apoptosis of McCoy cells under treatment with a

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topoisomerase inhibitor. This application note shows how the CELENA® X can be employed in the high throughput screening of phagocytosis of E. coli cells in real time.

Versatility and compatibility

The CELENA® X is a versatile system that is flexible enough to meet both the demands of the drug discovery industry and the basic research needs of the smaller laboratory. The system is compatible with a wide range of cell culture vessels such as multi-well plates, dishes, flasks and slides to cover a wide variety of assay types. Depending upon the application, either image-based or laser-based autofocus methods can be used.

The CELENA® X can be used for image confluency in McCoy cells seeded in 96-well plates over 48 hours with brightfield image-based autofocusing, demonstrating how the system can be modified and applied as a high throughput method for various cell-based assays. With laser-based autofocusing and multiple filter cubes, this method utilized the CELENA® X to image the dose-dependent effects of anti-cancer drugs throughout the cell cycle in HeLa cells seeded in 96-well plates, demonstrating how the system can be used in a multivariate drug screening process.

Image analysis software

An analysis of high content images with large datasets can cause problems with most types of analytical software. Each new assay requires the creation of new modules, which can be challenging for lab staff and often involves IT staff. The CELENA® X provides an easy-to-use modular-design analysis software based on a powerful CellProfiler engine. Tens of thousands of images can be analyzed automatically to obtain quantitative information with a complex software setting or optimization.

The following series of wide-ranging application notes demonstrate how the system's easy-to-use modular-design analysis software can be harnessed in various assays:

- Learn how the CELENA® X can be used for high throughput monitoring of intracellular calcium flux in bovine aortic endothelial cells under the influence of adenosine triphosphate (ATP), demonstrating how six of the system's built-in modules were used to quantify membrane potentials of calcium mobilization.

- Understand how the system can be applied to a quantitative assay of cytotoxicity in HeLa cells, in which two analysis pipelines make use of four different built-in modules to identify individual cells, assess the surface area they occupy, quantify their size and shape, and measure signal intensity, all in a 96-well format.

- Discover how the CELENA® X can be used for the highly reproducible assessment of the effect of plasmid concentration on transfection efficiency in HeLa cells in a 96-well format. This case study shows how three different analyzer modules are used to study green fluorescent protein (GFP) expression, including the FilterObjects module which sets a fluorescence intensity threshold to eliminate false positives.

- Learn how straightforward capturing and analyzing adherent cell images in a 96-well plate format can be, to determine cell counts in a high content capacity. This case study uses two IdentifyPrimaryObjects modules in sequence.

- Find out how the CELENA® X can analyze a wound healing assay, based on image segmentation, to determine bovine aortic endothelial cell migration *in vitro* in a 6-well format. Five of the system's analyzer modules are used in this application, including the EnhancedEdges and Smooth modules.

- Appreciate how the system can be harnessed for automated, non-destructive, highly reproducible monitoring and quantification of cytotoxicity in live HeLa cells, based on inhibition of cell confluency. Five analyzer modules are again used in this case study, including the OverlayOutlines module for visual confirmation of cell segmentation precision.

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Application Note

Apoptosis, McCoy cells, Caspase-3/7, Camptothecin (CPT), high content imaging, high content analysis

Fluorescence-based apoptosis assay using CELENA® X

Key features

- High throughput screening of apoptosis using the CELENA® X high content imaging system.
- Optimized analysis pipeline to evaluate apoptosis.

INTRODUCTION

Apoptosis is a form of programmed cell death (PCD). It is a highly controlled cellular process that acts to eliminate unwanted and/or damaged cells during a cell's early growth and development. There are two pathways, intrinsic and extrinsic, that induce apoptosis. Despite being distinct pathways both the intrinsic and the extrinsic pathway require activation of caspase 3 and caspase 7. As a result, monitoring caspase 3/7 activity is used as a proxy for monitoring apoptosis. Here, we demonstrate the ability of the CELENA® X High Content Imaging System to monitor and quantify apoptosis using the CellEvent™ Caspase-3/7 Green Detection Kit.

APPLICATION

Cell preparation

McCoy cells were counted using the LUNA-II™ Automated Cell Counter, seeded at a density of 1 x 10^3 cells/100 µL/well in a 96-well plate and grown overnight. Nuclei were counterstained with Hoechst 33342 (Life Technologies, H3570). Cells were washed twice using the cell culture media to remove excess dye before imaging.

Imaging and analysis

Apoptosis was evaluated by measuring the increase in Caspase-3/7 activity over time after inducing using CellEvent™ Caspase-3/7 Green Detection Reagent (catalog number C10423; Life Technologies). The CellEvent reagent only becomes fluorescent when bound to DNA after cleavage by Caspase-3/7 which making it an ideal tool for monitoring apoptosis. Imaging and analysis were performed using the CELENA® X High Content Imaging System, CELENA® X Cell Analyzer software, and the CX Stage Top Incubator Pro [Tokai] (95 % humidity, 5 % CO_2, and 37 °C). Time lapse image acquisition was performed at T0 (time point) and then at 15-minute intervals for 2 hours. The cell culture media were exchanged with the fresh media containing 2 µM CellEvent™ Caspase-3/7 Green Detection Reagent and incubated for 30 minutes. Camptothecin was then added to cells with the final concentration of 0 µM and 2.5 µM to induce apoptosis. Live-cell imaging started immediately after adding Camptothecin. Images were captured using an Olympus 10X LWD high NA objective, a DAPI filter cube (Ex375/28, Em460/50) and an EGFP filter cube (Ex470/30, Em530/50).

Quantifying apoptotic events was done by measuring the area occupied by green fluorescence (Caspase-3/7 activity) occupied and the area occupied by blue fluorescence (Nuclei). Objects containing green and blue fluorescence were identified using the 'IdentifyPrimaryObjects' module. The area of fluorescence was measured using the 'MeasureImageAreaOccupied' module (Table 1). To visualize the data, the 'MaskImage' module was used to eliminate areas of low signal The 'GrayToColor' module was used to convert the grayscale images to color images (Figure 1).

Order	Module	Use
1	*IdentifyPrimaryObjects*	Detects blue fluorescing Hoechst 33342
2	*IdentifyPrimaryObjects*	Detects green fluorescing CellEvent™ Green Detection Reagent
3	*MaskImage*	Eliminates cells with light levels below background
4	*MeasureImageAreaOccupied*	Measures area occupied by fluorescing CellEvent™ Green Detection reagent and Hoechst 33342
5	*GrayToColor*	Produces color images from gray scale

Table 1. *Description of the pipeline used to assess apoptosis with CELENA® X Cell Analyzer*

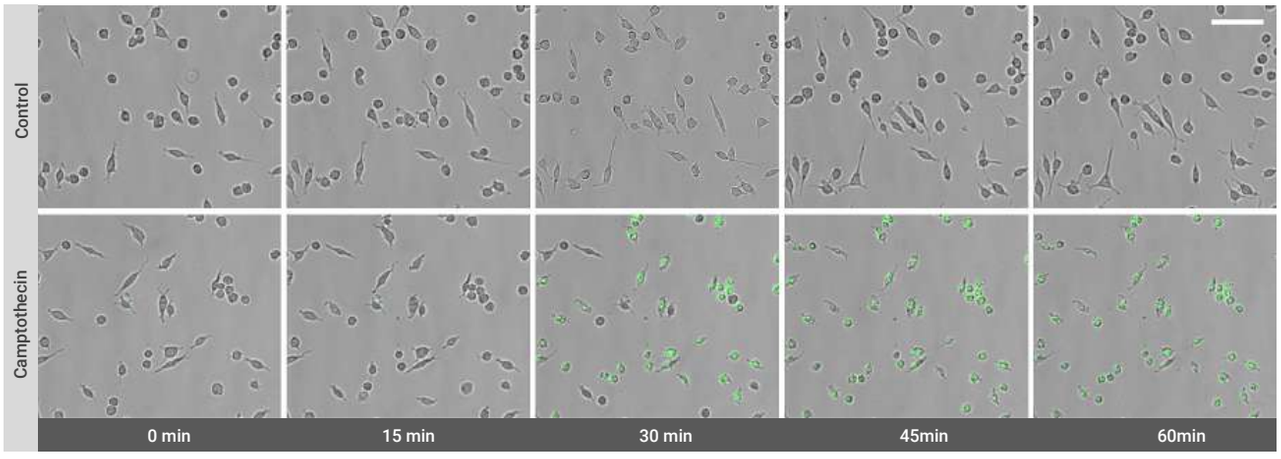

Figure 1. *Time-lapse images acquired using CELENA® X system showing green fluorescence increasing over time (scale bar: 100 µm).*

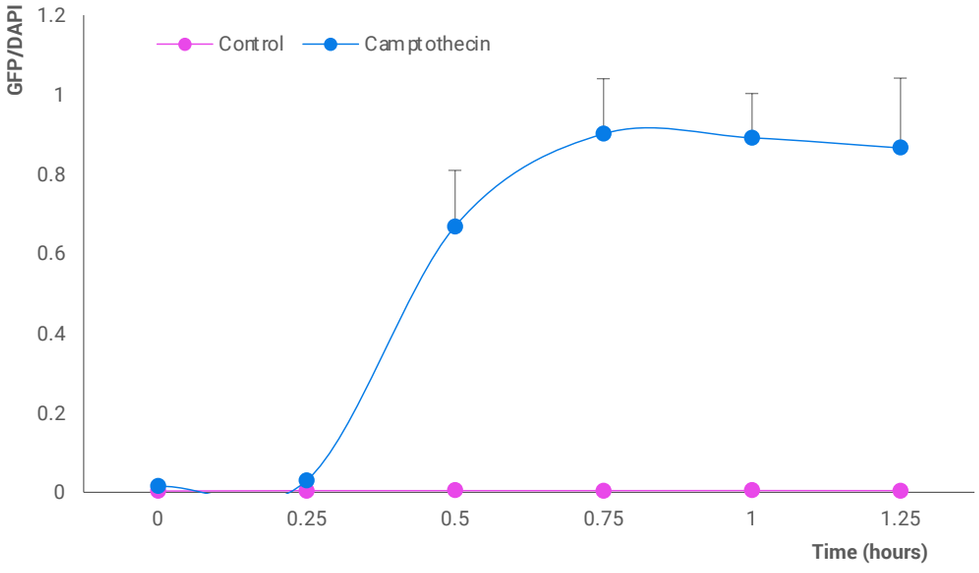

Figure 2. *Quantification of apoptosis. The area occupied by green fluorescence (showing caspase activity) was divided by DAPI (showing Nuclei). Camptothecin treatment increases caspase activity over time.*

CONCLUSION

In this study, we analyzed apoptotic events after Camptothecin treatment using the CELENA® X High Content Imaging System. Images were automatically taken and analyzed to identify objects, measure area, and produce images showing Caspase-3/7 activity. As shown in Figure 1 and 2, caspase activity was increased, and cells underwent apoptosis over time. Establishing the analysis pipeline was simple and straightforward. Overall, the CELENA® X High Content Imaging System, CELENA® X Cell Analyzer software, and CX Stage Top incubator proved to be an effective and efficient system for quantifying and monitoring apoptosis activity.

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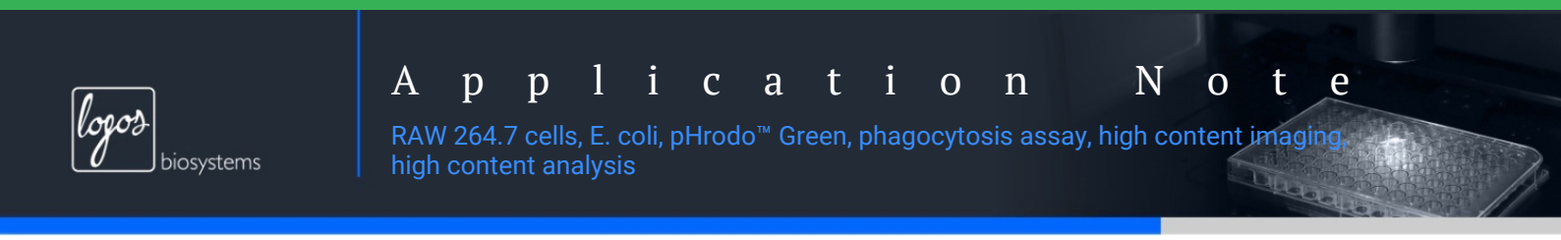

Application Note

RAW 264.7 cells, E. coli, pHrodo™ Green, phagocytosis assay, high content imaging, high content analysis

Live cell fluorescence-based phagocytosis assay using CELENA® X High Content Imaging System

KEYWORDS

RAW 264.7 cells, *E. coli,* pHrodo™ Green, phagocytosis assay, high content imaging, high content analysis

Key features

- High throughput screening (HTS) of phagocytosis with the CELENA® X High Content Imaging System
- The optimized analysis pipeline used to evaluate phagocytosis of pHrodo™ Green labeled *E. coli* over time

INTRODUCTION

Phagocytosis, simply put, is a process by which certain live cells, called phagocytes internalize foreign matter. This defensive reaction against infection is key in the study of immunology and plays an important role in immune responses, tissue homeostasis, and continuous clearance of apoptotic cells. Generally, phagocytotic activity is assayed using flow cytometry. However, this process only provides quantitative data and does not provide the means to monitor phagocytosis in real time. Here, we demonstrate the ability of the CELENA® X High Content Imaging System to monitor and quantify phagocytosis using pH-sensitive fluorescent particles, pHrodo™ Green.

APPLICATION

Cell preparation

RAW 264.7 cells were counted using the LUNA-II™ Automated Cell Counter and seeded at a density of 1 x 10^4 cells/50µL/well in a half area 96-well plate overnight. Nuclei were counterstained with Hoechst 33342 (Life Technologies, H3570) for laser autofocusing. Cells were washed twice using the cell culture media to remove excess dye before imaging.

Imaging and analysis

Phagocytic activity was assayed by measuring the increase in fluorescence of RAW 264.7 cells over time after incubation with pHrodo™ conjugated E. coli (green). The use of pHrodo™ Green reduces signal variability and improved timing in sensitive experiments. In addition, fluorescence of the pHrodo™ Green dye dramatically increases as pH decreases from neutral to acidic making it an excellent tool for the study of phagocytosis. Imaging and analysis were performed using the CELENA® X High Content Imaging System, CELENA® X Cell Analyzer software, and the CX Stage Top Incubator Pro [Tokai] (95% humidity, 5% CO_2, and 37℃). Time lapse image acquisition was performed at T0 (time point) and then at 30-minute intervals for 20 hours. Prior to starting live-cell imaging, the cell culture media was replaced with the fresh media containing 10 µg, 3 µg, and 0 µg of pHrodo™ Green conjugated *E. coli* (catalog number P35366; Life Technologies). Live-cell imaging started immediately after replacing the media. Images were captured using an Olympus 10X LWD high NA objective and an EGFP filter cube (Ex470/30, Em530/50).

To quantify phagocytic activity over time, we measured the area and level of green fluorescence occupied by bright green fluorescence. Objects containing green fluorescence were identified using the 'IdentifyPrimaryObjects' module. The area of fluorescence was measured using the 'MeasureImageAreaOccupied' module (Table 1).

To visualize the data, the 'MaskImage' module was used to eliminate areas of low signal. The 'GrayToColor' module was used to convert the grayscale images to color images (Figure 1).

Table 1. *Description of the pipeline used to assess phagocytosis with CELENA® X Cell Analyzer*

Order	Module	Use
1	*IdentifyPrimaryObjects*	Detects green fluorescing pHrodo™ bioparticles
2	*MeasureImageAreaOccupied*	Measures area occupied by fluorescing pHrodo
3	*MaskImage*	Eliminates cells with light levels below background
4	*GrayToColor*	Produces color images from gray scale

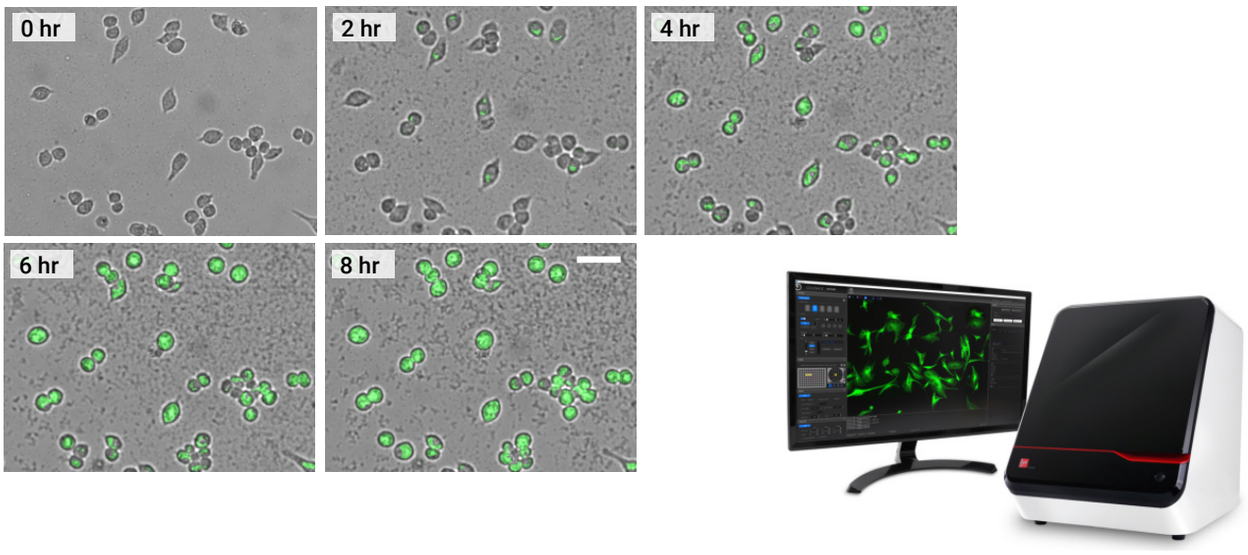

Figure 1. *Time-lapse images acquired using CELENA® X system. The area occupied by bright green fluorescence increased over time (scale bar: 50 µm).*

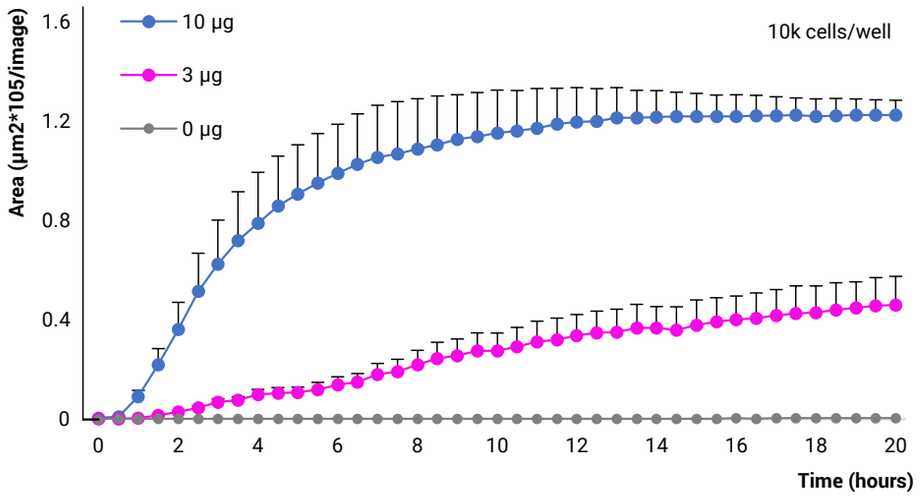

Figure 2. *Quantification of phagocytosis. The area occupied by E. coli bioparticles with high GFP signal was measured. Phagocytosis was dependent on the quantity of E. coli bioparticles.*

CONCLUSION

In this study, we analyzed phagocytic activity against pHrodo conjugated *E. coli* bioparticles using the CELENA® X High Content Imaging System. Images were automatically taken and analyzed to identify objects, measure area, and apply colors to images for visualizing data. Phagocytic activity increased with a higher quantity of *E. coli* bioparticles as shown in Figure 1 and 2. Establishing the analysis pipeline was simple and straightforward. Overall, the CELENA± X High Content Imaging System, CELENA® X Cell Analyzer software, and CX Stage Top incubator proved to be an effective and efficient system for quantifying and monitoring phagocytotic activity.

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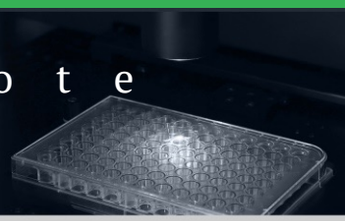
Application Note

McCoy, confluency, cell-based assay, automated scanning, and high throughput analysis



Monitoring confluency of adherent cells in multi-well plates using the CELENA® X High Content Imaging System

INTRODUCTION

Confluency is best described as the percentage of surface area covered by adherent cells in a culture medium. In cell culture biology, confluency assessment is important for visually determining the timing to proceed with cell-based experiments such as cell passage, harvest, transfection, or drug treatment. This is important because properly timing experiments is critical for maintaining cell quality and integrity. Generally, researchers directly view and judge cell confluency with the use of a standard microscope. However, using the CELENA® X High Content Imaging System and its laser auto-focusing modules, images can be rapidly and conveniently captured. The integrated CELENA® X Cell Analyzer software provides accurate data for quantitative analysis of cell confluency. Here, we describe how to take brightfield cell images and analyze confluency using the CELENA® X High Content Imaging System.

APPLICATION

For the confluency experiment, we used the McCoy cell line. McCoy cells were counted with the LUNA II™ Automated Cell Counter, and seed to 2 x 10^4 cells/ml in 96well plate. Cells were prepared in four replicates and placed in the CELENA® X Stage Top Incubator Pro with 95% humidity and 5% CO_2 at 37℃. Cells were photographed automatically at 20-minute intervals for 48 hours with the CELENA® X High Content Imaging System using 10X LWD high NA objective and image-based autofocusing. Acquired images were analyzed using CELENA® X Cell Analyzer software.

The method of analyzing cell confluency using CELENA® X Cell Analyzer was to identify overall cells based on the brightfield image of the cells; then measure the area occupied by these objects. A pipeline was created in CELENA® X Cell Analyzer to automatically batch process and analyze images (Table 1). To do this, the *Enhance Edges* module was used to create binary images that distinguished the foreground (the cells) from the background. The *Smooth* module was used to reduce intensity irregularities by homogenizing cells, smoothing edges, and removing debris from the background. The resulting segmented area was identified as cells using the *Identify Primary Objects* module and then measured using *Measure Image Area Occupied* module to quantify the surface area occupied by the cells within the field. The *Overlay Outlines* module was used to overlay the original brightfield images with the outlines of the segmentation precision.

Figure 1 shows the segmentation of brightfield images for 48 hours. Image segmentation was obtained using the method described in Table 1 in CELENA® X Cell Analyzer. Cell images at 0, 16, 32, 48 hours were shown, and confluency was measured to be 17.1%, 47.0%, 62.4%, and 66.8%, respectively. The experiment was repeated with a total of 4 wells, and as time passed, it was confirmed that confluency increased.

Table 1. *Daescription of the pipelines used to measure confluency with CELENA® X Cell Analyzer.*

Order	Module	Use
1	*EnhanceEdges*	To enhance the edges of cells in BF image
2	*Smooth*	To smooths or blurs images to remove small aritfacts
3	*IdentifyPrimaryObjects*	To find cells
4	*MeasureImageAreaOccupied*	To measure area occupied by cells
6	*OverlayOutlines*	To place outlines around identified cells

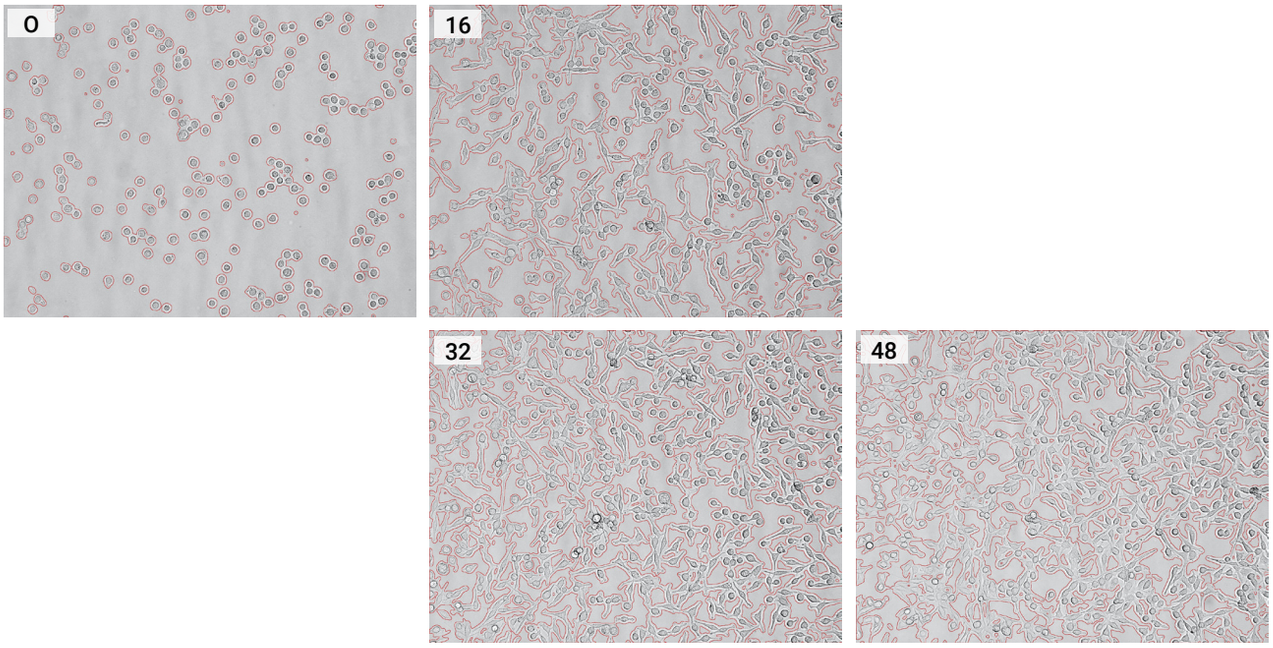


Figure 1. *Image analysis of McCoy cells showing cell growth over time. Red borders illustrate the separation between background and the areas covered by intact cells.*

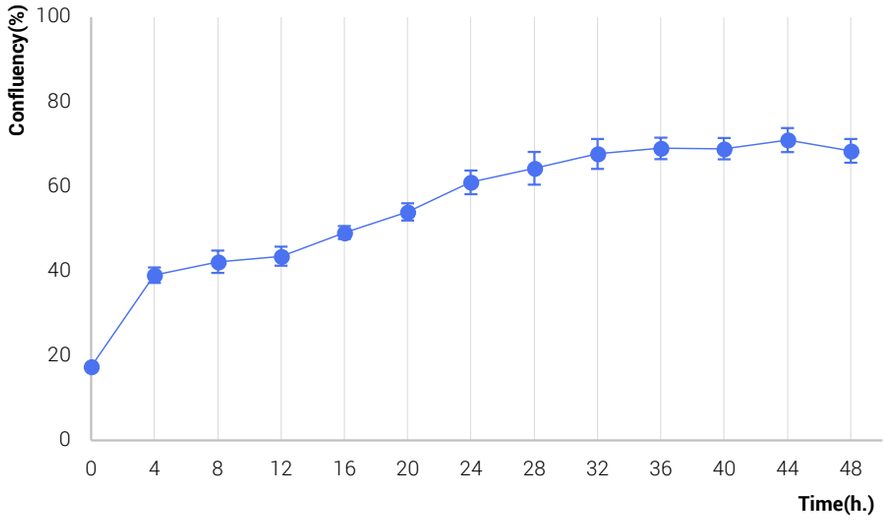


Figure 2. *Curve of confluency change over time. The pictures were taken at 20-minute intervals for a total of 48 hours and were indicated by dots on the graph at 4 –hour intervals. N=4.*

CONCLUSION

In this study, we used the CELENA® X High Content Imaging System to measure time-varying cell confluency. Live cell imaging combined with analysis of the cell surface area occupying each well is a reproducible and quantifiable approach for measuring confluency accurately and conveniently. CELENA® X Cell Analyzer software makes it possible to create pipelines, so that confluency can be simply and objectively quantified rather than subjectively judged. Since the CELENA® X High Content Imaging System and the CELENA® X Cell Analyzer software can be modified and applied to various experimental conditions and multi-well plates, it can be used as a high-throughput method for various cell-based assays.

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Application Note

Cell cycle, HeLa cells, paclitaxel, thymidine, DNA synthesis, high content imaging, high content analysis

A high content anti-cancer drug screening using automated cell cycle assay by the CELENA® X High Content Imaging System

Key features

- Assessment of the effect of paclitaxel and thymidine treatment on cell cycle using the CELENA® X High Content Imaging System.
- The optimized analysis pipeline to evaluate the effect of drugs on the cell cycle.

INTRODUCTION

A key first step in the drug discovery process for anti-cancer treatment is understanding the relationship between a dysregulated cell cycle and the formation of abnormal, cancerous cells. Since many anti-cancer drugs are designed to specifically target DNA synthesis and/or mitosis, there are a multitude of cell cycle assays that are used to monitor and quantify the impact of these treatments have on the cell cycle process.

Here, we demonstrate how the CELENA® X High Content Imaging System and the CELENA® X Cell Analyzer software can be used for monitoring and screening the dose-dependent effects of the anti-cancer drugs thymidine and paclitaxel through the cell cycle.

APPLICATION

Cell Cycle Assay

We utilized the CLICK-IT™ EdU Cell Proliferation Kit (Life Technologies, C10337) and a Phospho-Histone H3 (Ser10) Rabbit Polyclonal Antibody (pHH3; Life Technologies, PA5-17869) to visualize the effects of thymidine (Sigma-Aldrich, T1895) and paclitaxel (Sigma-Aldrich, T7402) treatments on HeLa Cells. EdU is a thymidine analog and marker for DNA replication. pHH3 is a marker for chromosomal condensation that occurs in mitosis. For this procedure, HeLa cells were grown, seeded in 96-well plates at a density of 1.2 X 10^4 cells/well (LUNA-II™ Automated Cell Counter), and incubated overnight. The cells were subsequently treated with either thymidine or paclitaxel for 16 hours, as indicated in Figure. 1. Afterward, half the media was removed, replaced with a pre-warmed 2X EdU solution to bring a final concentration of 10 µM, further incubated for 3 hours, and then fixed with 4 % PFA. After fixation, the cells were treated with the Click-IT™ reaction cocktail for 30 minutes at room temperature and added with pHH3 antibody at 1:150 dilution followed by an AF 680-labeled secondary antibody (Life Technologies, A10043). The cell nuclei were counterstained with Hoechst 33342 (Life Technologies, H3570). The cells were washed with 1X PBS between each step.

Imaging and analysis

For imaging, we used the CELENA® X High Content Imaging System with an Olympus 10X LWD high NA objective, a DAPI filter cube (Ex375/28, Em460/50), an EGFP filter cube (Ex470/30, Em530/50), and a Cy5 Long Pass filter cube (EX620/60, Em665lp). Desired wells were selected and scanned using laser autofocus.

Cell cycle assessment was quantified by filtering objects without green and far-red signals from all nuclei. Objects containing blue fluorescence were identified using the 'IdentifyPrimaryObjects' module. The median intensity of blue, green, and far-red signals from all nuclei was measured using the 'MeasureObjectIntensity' module. The number of nuclei with green or far-red signals were counted using the 'FilterObjects' module (Table 1).

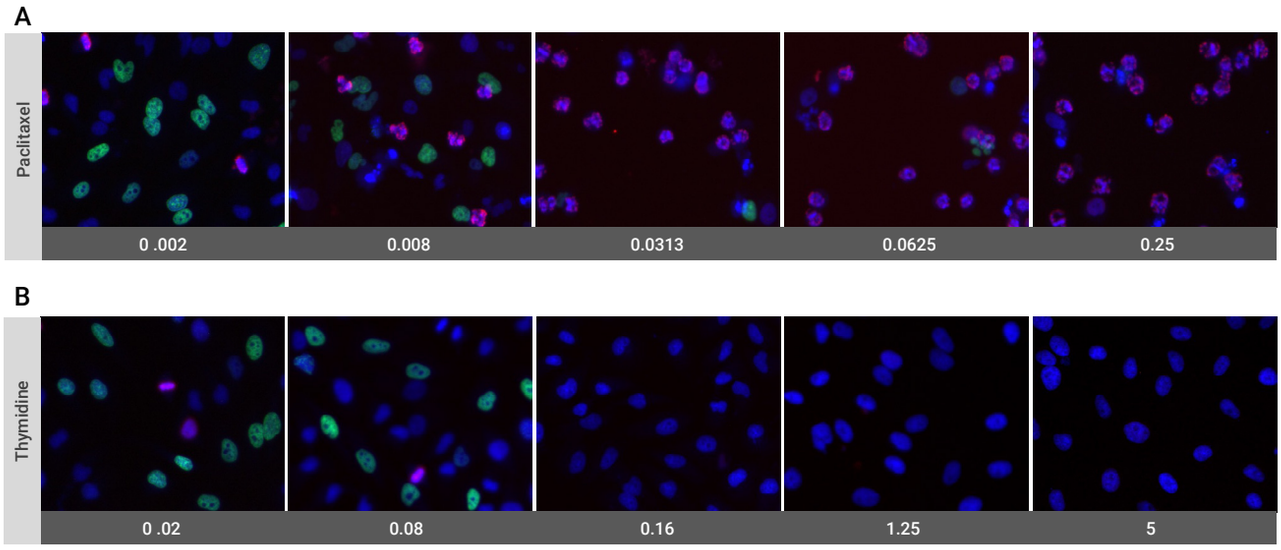
To visualize the data, the 'GrayToColor' module was used to produce color composite images from grayscale images (Figure 1A, B). GraphPad Prism® software (GraphPad Software, Inc.) was used to plot sigmoidal dose-response curves and calculate EC50 values.

Order	Module	Use
1	*IdentifyPrimaryObjects*	Detects nuclei stained with Hoechest 33342
2	*MeasureObjectIntensity*	Measure median intensity of blue, green and farred signal from all nuclei
3	*FilterObjects*	Filter nuclei without green signal
4	*FilterObjects*	Filter nuclei without farred signal
5	*GrayToColor*	Produces color images from gray scale

Table 1. *Description of the pipeline used to assess cell cycle with CELENA® X Cell Analyzer*

Results

Both thymidine and paclitaxel act to arrest the cell cycle, but at different phases. Paclitaxel arrests the cell cycle in the M phase (mitosis) by preventing the dissociation of microtubules and halting progression from metaphase to anaphase. As expected, paclitaxel treatment decreased the EdU-positive cells, but increased the pHH3-positive cells (Figure 1A, C), indicating that paclitaxel induced the M phase arrest. By comparison, thymidine blocks DNA replication and a cell's entry into the S phase (DNA synthesis) by interrupting deoxynucleotide metabolism through competitive inhibition. Thymidine treatment decreased both EdU-positive cells and pHH3-positive cells (Figure 1B, D), showing the G1/S phase arrest.



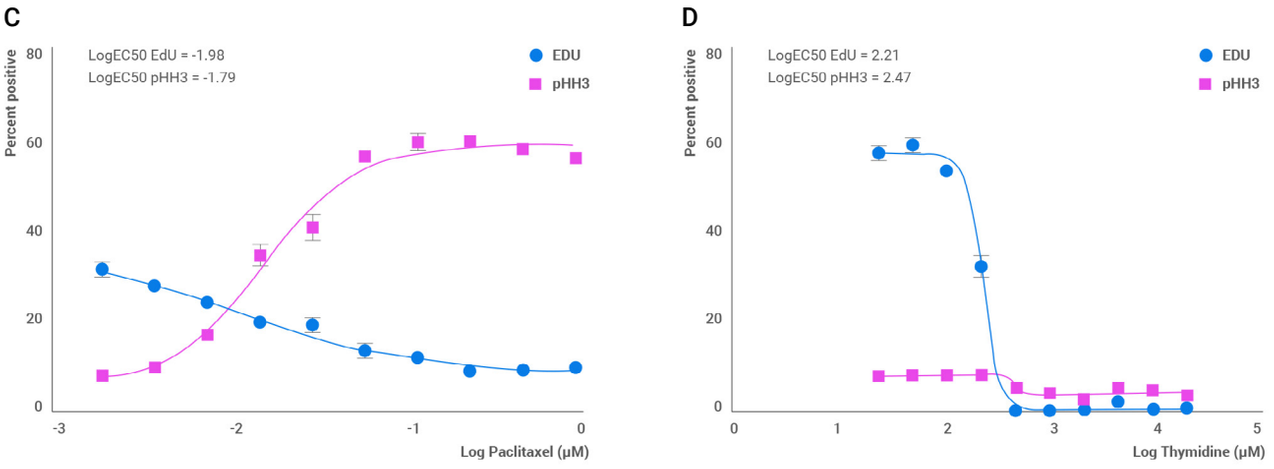
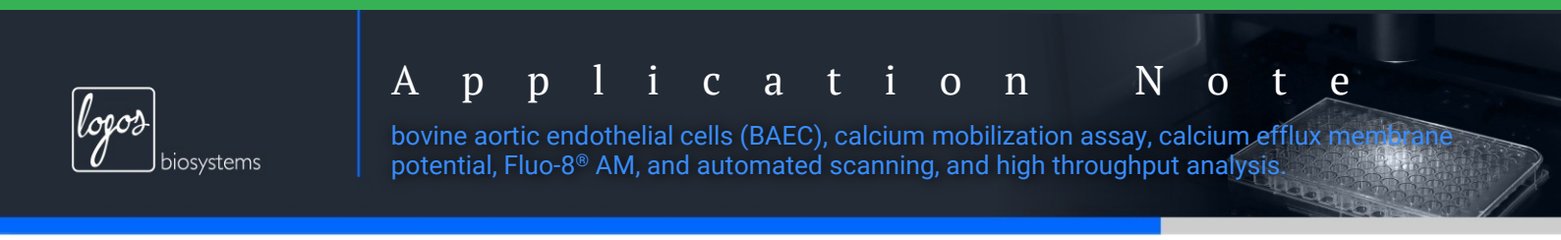


Figure 1. *A montage and a chart after 16 hours of Thymidine or Paclitaxel treatment. (A and B) Color composite images showing from EdU (green), pHH3 (red) and nuclei (blue). (C and D) Dose response curves show the EdU and pHH3 positive cells after different concentrations of Thymidine or Paclitaxel treatment.*

CONCLUSION

Here, we have shown how the automated CELENA® X High Content Imaging System may be used in a multivariate drug screening process. Using CELENA® X Cell Analyzer software with laser autofocus and a data analysis pipeline, the CELENA® X can provide highly efficient workflow and data-driven analysis in accelerating high throughput screening efforts. In conclusion, we showed that the CELENA® X High Content Imaging System, and the CELENA® X Cell Analyzer software is an excellent system for drug screening targeting the cell cycle.



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Application Note

bovine aortic endothelial cells (BAEC), calcium mobilization assay, calcium efflux membrane potential, Fluo-8® AM, and automated scanning, and high throughput analysis.

Fluorescence-based Calcium Mobilization Assay with the CELENA® X High Content Imaging System

KEYWORDS

bovine aortic endothelial cells (BAEC), calcium mobilization assay, calcium efflux membrane potential, Fluo-8® AM, automated scanning, and high throughput analysis.

Key features

- High throughput screening of membrane potentials with the CELENA® X automated microscope system.
- ATP inducible Fluo-8® AM esters into live cells for Kinetic live cell imaging
- The optimized analysis pipeline to evaluate ATP-induced intracellular calcium flux.

INTRODUCTION

Calcium is an essential element found in all living organisms and is necessary for many functions at the cellular level. Calcium migrates across cell membranes and permits the activation and inhibition of a variety of enzymes through embedded calcium ion channels [1-3]. These calcium ions are responsible for facilitating muscle relaxation and contraction, as well as, vascular contraction and vasodilation, neuronal signal transmission, and hormone secretion [4-6]. This assessment evaluates the changes of intracellular calcium flux conditions on bovine aortic endothelial cells (BAEC) by adding ATP agonist [7]. The Fluo-8® AM, a acetoxymethyl ester of fluorescent indicator dye, is hydrolyzed intracellularly in the presence of calcium ions. Thus, the combinations of Fluo-8® AM and ATP agonist were used to detect changes in intracellular calcium efflux levels in the live cells. A 3-minute time-lapse was set for monitoring the BAEC cellular calcium efflux activated by the ATP stimulant forced the intercellular G protein-coupled receptors to release intracellular calcium ions [8].

APPLICATION

The application used combined bovine aortic endothelial cells (BAEC) with Fluo-8® AM, which is a cell-permeable calcium indicator assay. BAEC is a reputable endothelial cell line that efficiently provides a model system to measure calcium permeability of the cellular characteristics. To evaluate the calcium ion efflux, the healthy BAEC were first seeded in the media of DMEM with 10% fetal bovine serum and penicillin-streptomycin overnight at 20,000 cells/100 µl/well in a 96-well plate (SPL, 30296) for 16 hours. The culture media were aspirated, and the cells were incubated with 5µM of Fluo-8® AM and 2.5 mM ReadiUse™ probenecid in Hanks and Hepes buffer (HHBS) at room temperature (protected from light) for one-hour incubation (AATbio.com Cat. 21080 & Cat. 20062). Then, the media was removed, and the wells were washed with 100µL DPBS according to the manufacturer's instructions. The 500µM stock solution of ATP in DPBS was prepared in advance, and the 20 µl of ATP stock was ready to add on each well of 96-well plate to make ATP final concentration of 20µM (Fishersci, Cat AC1028001000).

For acquisition of images, the cell plate was placed on the stage of the CELENA® X system and set CELENA® X Explorer time-lapse function for 3-min scanning of images and capturing speed of 4 images in a second (4 fps, as fast as possible) with 10X objective. The scanning started immediately after gently applying the 20µl of the prepared ATP stock to each well of the plate. Next, the analysis was performed through the pipeline with the six following modules

to quantify membrane potential of calcium mobilization; ①AddsingleImage module to select an image(Time 0) that will be used to create a mask image, ②IdentifyprimaryObjects module to find the individual cells in Time 0 image by the boundary of fluorescent signals. Next ③MaskImage module to mask image using the object identified in the previous step, ④MeasureObjectIntensity module to measure and quantify the intensity of the fluorescent, ⑤ GrayToColor module to convert mono images to green pseudocolor images, and ⑥OverlayOutlines module to draw the outlines each cell. They are using the result file saved as .CSV format, the calcium mobilization of the total and several represented cells were plotted over time (Fig1). The selected time-lapse images of intracellular calcium changes were presented at 0 s, 10 s, 20 s, 40 s, and 160 s, and they were exhibited the Fluo-8® signals elicited higher levels of transient during 10 to 40 second in intracellular calcium ion responding fluorescent, and the highest total intensity was plotted on the 35 s (Fig. F).

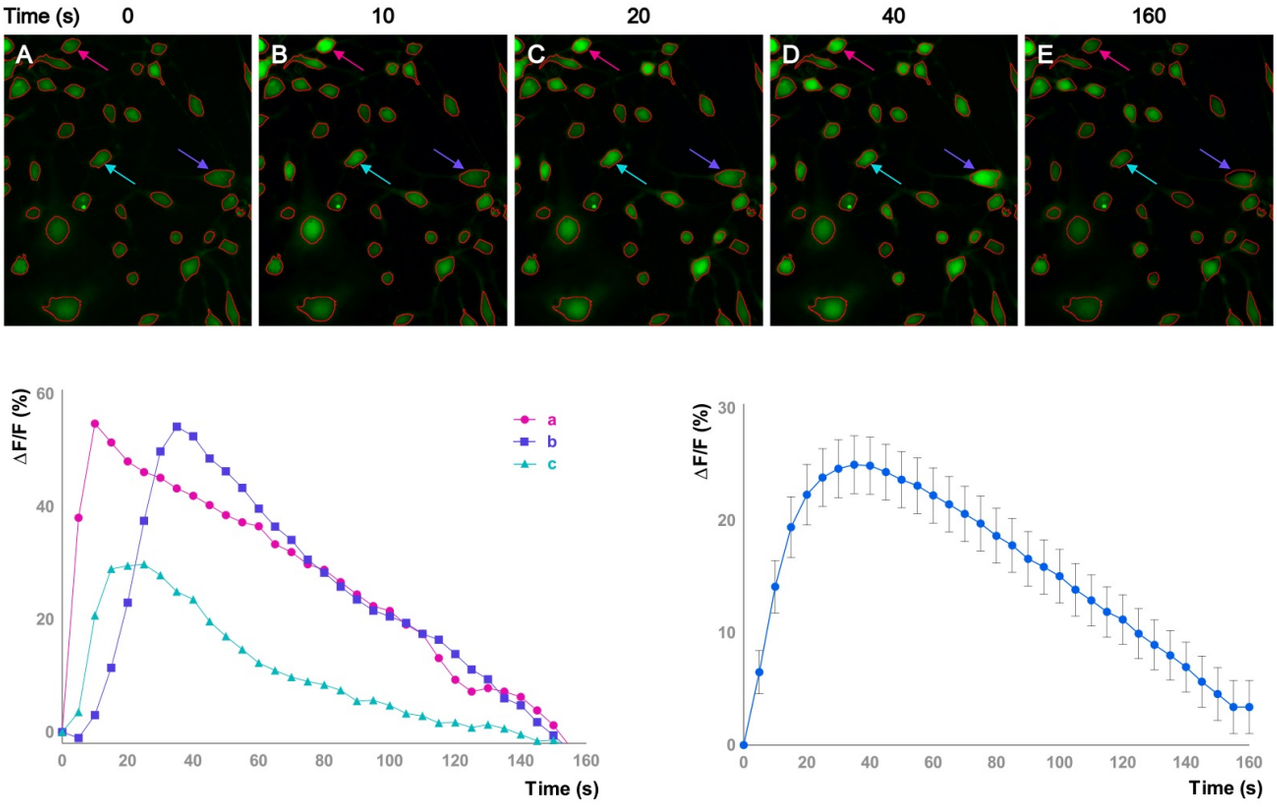

Figure 1. *Time-lapse measuring of ATP-dependent calcium efflux. The represented time-lapse images of Fluo-8® fluorescent signals by calcium mobilization after treatment of 20μM ATP reagent (A-E). The changes of the total cell fluorescent signal were plotted in (F), and the three instance cells have individually plotted the changes of calcium flux by the ATP ageists. The color-coordinated arrows were represented on the plot (G) based on the images (A-E).*

CONCLUSION

In the presence of ATP, intracellular calcium ion was instantly augmented and then gradually returned to baseline levels over 2-3 minutes from the intensity of the total fluorescent. In the color coordinated three cases, the individual cells may not correctly synchronize their intensity on the 35s but scuttled between 15s to 45s. Thus, the results based on the measurement of the intensity over 3 minutes represented the calcium values of changes over time and peak heights in 10-40 second within 180 seconds [9]. Since the reaction occurs in such a short time, delivering the agonist stimulator to wells should be synchronized among the wells by using multi-channel pipet. The calcium flux assay with Fluo-8® AM was perceived as the green fluorescence as the intensity at Ex/Em = 490/525. The [calcium flux measurement] pipeline was built in CELENA® X Analyzer and applicable to measure total fluorescent and to trace specific cells' intensity out of the multiple sequential images of either z-stack or time-lapse in CELENA® X imaging system of live cell fluorescent measurement.

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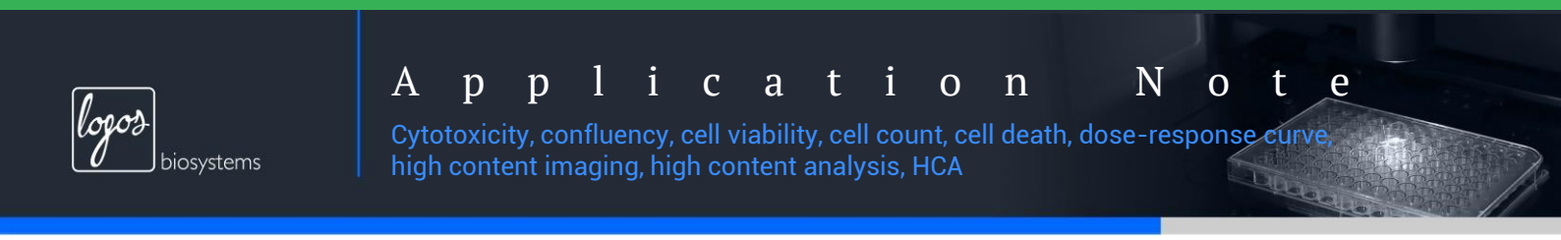

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Application Note

Cytotoxicity, confluency, cell viability, cell count, cell death, dose-response curve, high content imaging, high content analysis, HCA

A multiparameter assessment of the dose-dependent cytotoxicity of Camptothecin on HeLa cells

INTRODUCTION

High content cytotoxicity assays are in high demand for the cell-based analysis of anticancer drugs. In this study, we demonstrate an automated, multi-parameter assessment of dose-dependent Camptothecin cytotoxicity using the CELENA® X High Content Imaging System. The CELENA® X is a simple solution for the quantitative analysis of cytotoxicity because it can rapidly capture multi-channel fluorescence images from a multi-well plate and the integrated analysis software can accurately analyze multiple cellular parameters.

APPLICATION

Cell preparation

To study cytotoxicity *in vitro*, we treated HeLa cells with different concentrations of Camptothecin (CPT; Sigma-Aldrich, C9911). HeLa cells were counted with the LUNA-II™ Automated Cell Counter, seeded at a density of 1 × 10^4 HeLa cells/well on a 96-well plate, and cultured overnight. Cells were then treated with serial dilutions of Camptothecin (DMSO, 0.0625 µM, 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, 2 µM, 4 µM, 8 µM, 16 µM, 32 µM, 64 µM) at four wells per condition with 95% humidity and 5% CO_2 at 37°C for 20 hours. To distinguish dead cells, cells were stained with propidium iodide (PI; Logos Biosystems, F23003). To assess total cell numbers, nuclei were counterstained with Hoechst 33342 (Life Technologies, H3570).

Imaging and analysis

Dead and total cells were visualized using the CELENA® X High Content Imaging System. Images were acquired using image-based autofocusing and a 4X LWD high NA objective in combination with filters for Hoechst 33342 (DAPI filter cube: Ex375/28, Em460/50) and propidium iodide (RFP filter cube: Ex530/40, Em605/55). One image field was acquired per well from 48 wells. Acquired images were analyzed using CELENA® X Cell Analyzer software.

For quantitative analysis of cytotoxicity, the integrated CELENA® X Cell Analyzer software was used to batch process and analyze images automatically. Two pipelines, *Cytotoxicity_DAPI_HeLa_4X* and *Cytotoxicity_RFP_HeLa_4X*, were established to determine the effects of Camptothecin (Table 1). Both pipelines, *Cytotoxicity_DAPI_HeLa_4X* and *Cytotoxicity_RFP_HeLa_4X*, employed the *IdentifyPrimaryObjects* module to identify individual cells based on Hoechst 33342 or propidium iodide staining, respectively. Both used the *MeasureImageAreaOccupied* module measured the surface area occupied by the fluorescence-stained cells. In the *Cytotoxicity_RFP_HeLa_4X* pipeline, propidium iodide-stained cells were further analyzed with the *MeasureObjectSizeShape* module to quantify their size and shape, followed by the *MeasureObjectIntensity* module to measure propidium iodide signal intensity.

Table 1. *Pipelines used to assess transfection efficiency with CELENA® X Cell Analyzer*

Pipeline	Order	Module	Use
Cytotoxicity_DAPI_HeLa_4X	1	IdentifyPrimaryObjects	To find Hoechst-stained nuclei
	2	MeasureImageAreaOccupied	To quantify the surface area occupied by the Hoechst-stained nuclei
	3	GrayToColor	To produce color images from grayscale images
	4	OverlayOutlines	To place outlines around identified nuclei
Cytotoxicity_RFP_HeLa_4X	1	IdentifyPrimaryObjects	To find PI-stained nuclei
	2	MeasureImageAreaOccupied	To quantify the surface area occupied by the PI-stained nuclei
	3	MeasureObjectSizeShape	To measure the size and shape of the PI-stained nuclei
	4	MeasureObjectIntensity	To measure RFP intensity
	5	GrayToColor	To produce color images from grayscale images
	6	OverlayOutlines	To place outlines around identified nuclei

To visualize the data, both pipelines used the *GraytoColor* module to apply pseudocolor to the grayscale images of each channel and then the *OverlayOutlines* module to outline the identified cells. GraphPad Prism® software (GraphPad Software, Inc.) was used to plot sigmoidal dose-response curves and calculate IC_{50} values.

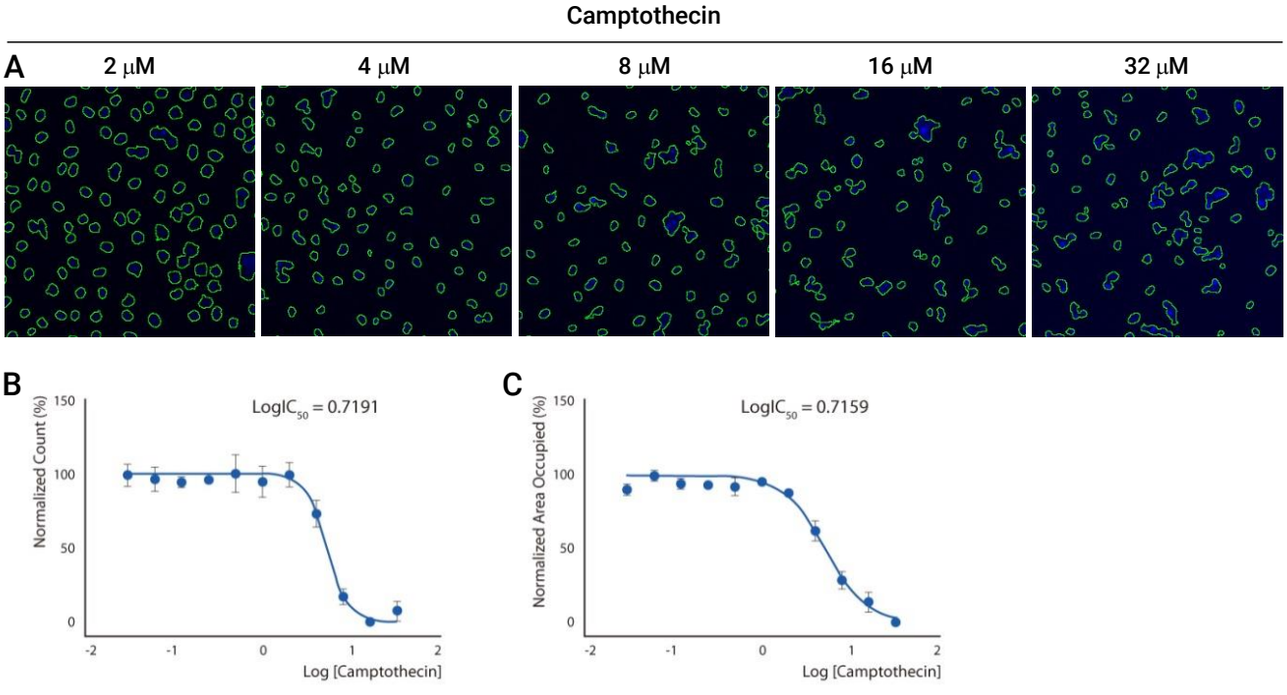

Figure 1. *Hoechst-stained HeLa cells after 20 hours of Camptothecin treatment. (A) Image analysis of Camptothecin-induced cytotoxicity with green borders outlining Hoechst-stained nuclei. Dose-response curves show the cytotoxic effects of Camptothecin on Hoechst-determined (B) cell count and (C) occupied surface area.*

Figure 1 showed that higher doses of Camptothecin exposure decreased the number of Hoechst-stained nuclei and the area that they occupied. The IC_{50} of Camptothecin in relation to these two parameters was 5.2180 μM on average. Figure 2 shows that higher Camptothecin concentrations lead to an increase in clumpy cellular aggregates (Fig. 2A, B), propidium iodide signal size (Fig. 2C), and signal intensity (Fig. 2D). These data demonstrate that cell death leads to the formation of clumpy aggregates caused by the presence of cell debris and free DNA from dead cells. The IC_{50} of Camptothecin upon assessing these parameters was 4.8591 μM on average. Combined, these results indicate a Camptothecin dose-dependent increase of cell death.

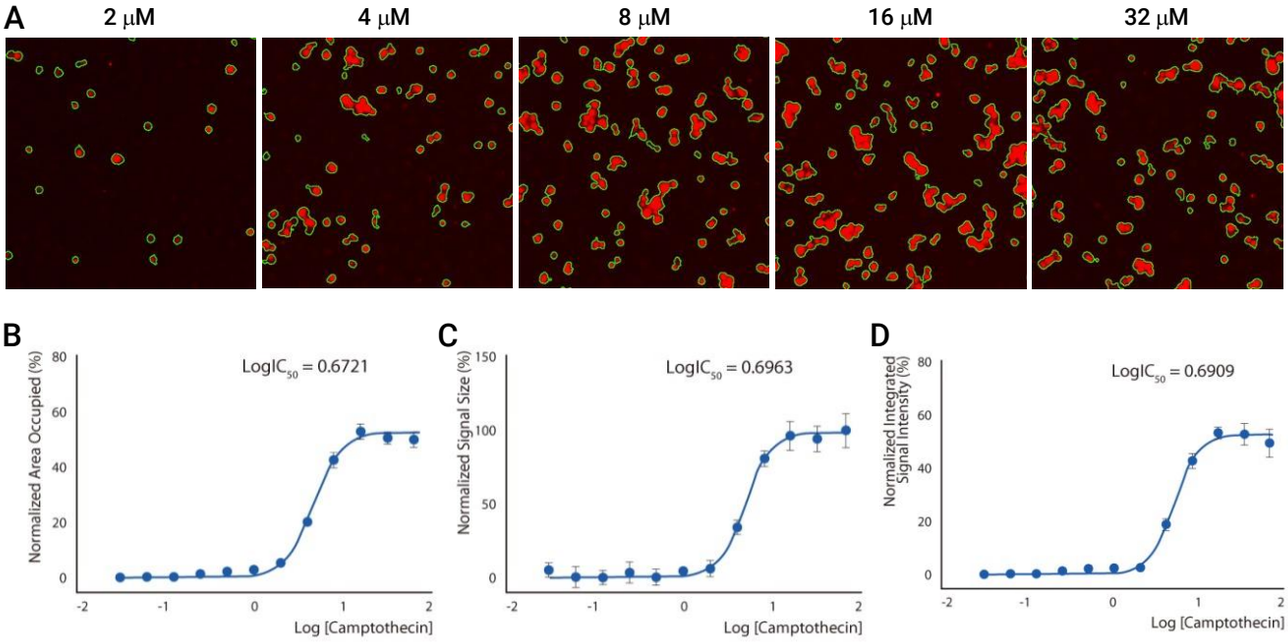

Figure 2. *Propidium iodide-stained HeLa cells after 20 hours of Camptothecin treatment. (A) Image analysis of Camptothecin-induced cytotoxicity with green borders outlining propidium iodide-stained nuclei. Dose-response curves show the cytotoxic effects of Camptothecin on (B) occupied surface area, (C) size, and (D) integrated intensity of the propidium iodide signal.*

CONCLUSION

In this study, we used the CELENA® X to evaluate the dose-dependent cytotoxicity of Camptothecin in a simple, effective, and highly reproducible way. The CELENA® X allowed us perform a multi-parameter assessment on the effects of Camptothecin on cell viability. Using the CELENA® X High Content Imaging System and CELENA® X Cell Analyzer software, the same experimental conditions as well as the identical analysis pipeline can be reused to verify results.

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Application Note

Transfection efficiency, Hoechst, cell counting, GFP expression, GFP-positive, high content imaging, high content analysis, HCA

Evaluating transfection efficiency using the CELENA® X

INTRODUCTION

Transfection is a common technique used to introduce nucleic acids into cells and is important to the study of the function and regulation of genes and their products. Establishing a reproducible way to assess transfection efficiency in a high-content capacity accurately is critical. In this study, the effects of plasmid concentration on transfection efficiency were investigated using the CELENA® X High Content Imaging System.

APPLICATION

Cell preparation

HeLa cells were counted with the LUNA-II™ Automated Cell Counter, seeded at a density of 1×10^4 HeLa cells/well on a 96-well plate, and cultured overnight. To evaluate transfection efficiency, we transfected HeLa cells with different concentrations of the GFP-expressing pCG-HttQ103 plasmid (control, 0.05 μg, 0.1 μg, and 0.2 μg) using Lipofectamine 2000 (Invitrogen, 11668) according to the manufacturer's protocol. 24-hours post-transfection, cells were fixed in 100 μL 4% PFA for 15 minutes at room temperature. To count total cell numbers, nuclei were counterstained with Hoechst 3342 (Life Technologies, H3570).

Imaging and analysis

GFP expression and cell nuclei were visualized using the CELENA® X High Content Imaging System. Images were acquired using image-based autofocusing and a 10X LWD high NA objective in combination with filters for Hoechst 33342 (DAPI filter cube: Ex375/28, Em460/50) and GFP (EGFP filter cube: Ex470/30, Em530/50). One image field was acquired per well from 12 wells.

For quantitative analysis of transfection efficiency, the integrated CELENA® X Cell Analyzer software was used to batch process and analyze images automatically. Individual cells were segmented based on Hoechst 3342 nuclear staining using the *IdentifyPrimaryObjects* module, while GFP-expressing cells were identified using the *IdentifySecondaryObjects* module. GFP intensity was quantified within the Hoechst-defined boundaries for each cell with the *MeasureObjectIntensity* module. A fluorescence intensity threshold was set using the *FilterObjects* module to eliminate false-positives, identify the transfected cells, and determine transfection efficiency. The threshold was defined as the maximum value of the median intensity of the GFP-channel images of the negative control group of cells.

Table 1. *Description of the pipeline used to assess transfection efficiency with CELENA® X Cell Analyzer*

Order	Module	Use
1	*IdentifyPrimaryObjects*	To find Hoechst-stained nuclei
2	*IdentifySecondaryObjects*	To find GFP-expressing cells
3	*MeasureObjectIntensity**	To measure GFP intensity
4	*FilterObjects*	To filter out non-transfected objects based on GFP intensity
5	*GrayToColor*	To produce color images from grayscale images
6	*OverlayOutlines*	To place outlines around identified nuclei and transfected areas

To visualize the data, the *GraytoColor* module was used to apply colors to the grayscale images of each channel, and the *OverlayOutlines* module was used to overlay the images with the outlines of the identified cells and transfected areas to confirm segmentation precision (Figure 1).

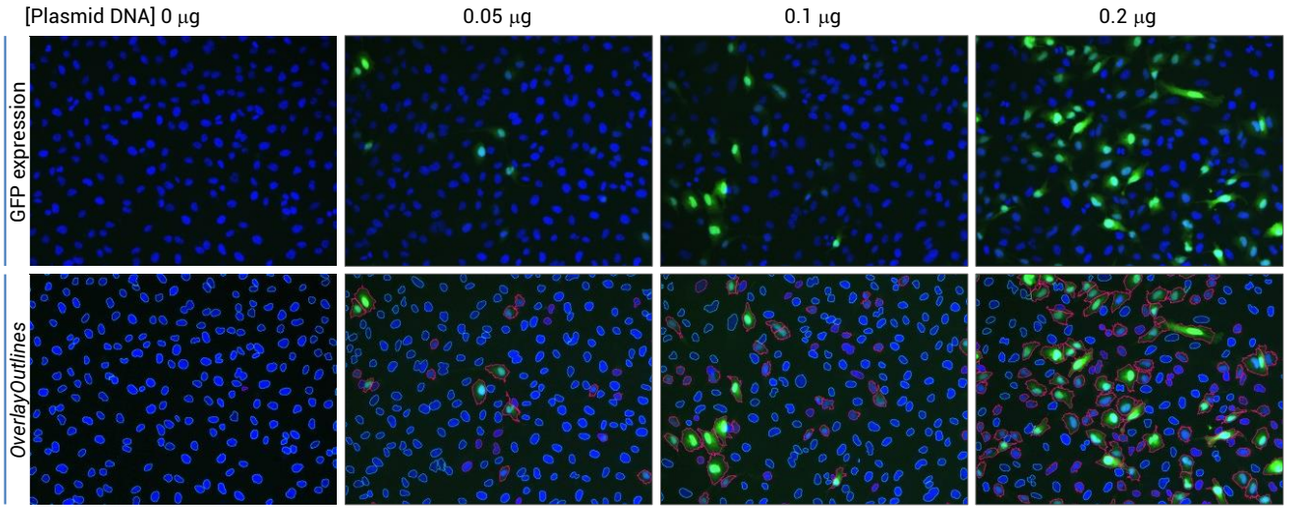

Figure 1. *Image analysis of GFP expression in HeLa cells 24 h post-transfection. Higher plasmid concentrations correlated with increased numbers of GFP-positive cells (green). Blue lines indicate control Hoechst-stained nuclei (blue) and magenta lines outline GFP-positive cells.*

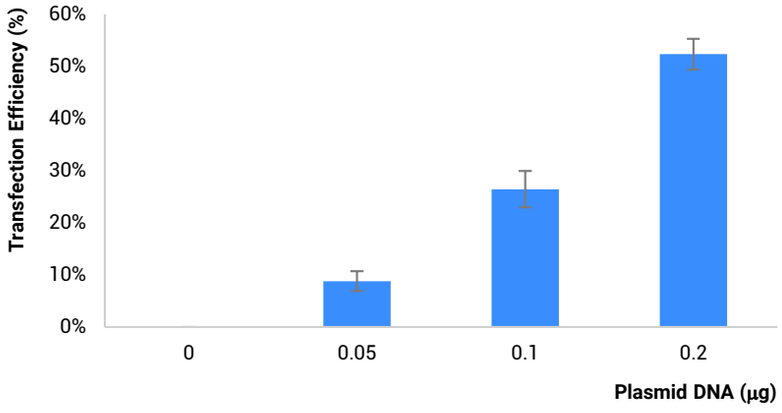

Figure 2. *Quantification of transfection efficiency. The number of GFP-positive cells were compared to the number of Hoechst-stained nuclei. Transfection efficiency improved with increasing concentrations of the GFP-expressing pCG-HttQ103 plasmid.*

CONCLUSION

In this study, we analyzed the effects of plasmid concentration on transfection efficiency using the CELENA® X High Content Imaging System. Images of the adherent HeLa cells in a multi-well plate were captured automatically and batch processed to identify objects, measure fluorescence intensity, apply a fluorescence intensity threshold, and even label images to help visualize data. As shown in Figure 1 and 2, transfection efficiency increased with plasmid concentration.

Using the CELENA® X for fluorescence cell imaging and to quantitatively analyze multiple cellular features from each image automatically gives researchers a simple way to monitor transfection-based cell assays. The same analysis pipeline can easily be reused to verify or compare results from subsequent experiments.

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Application Note

Cell counting, acridine orange, Hoechst, AO/HO staining, high content imaging, high content analysis

Counting adherent cells in multi-well plates with the CELENA® X

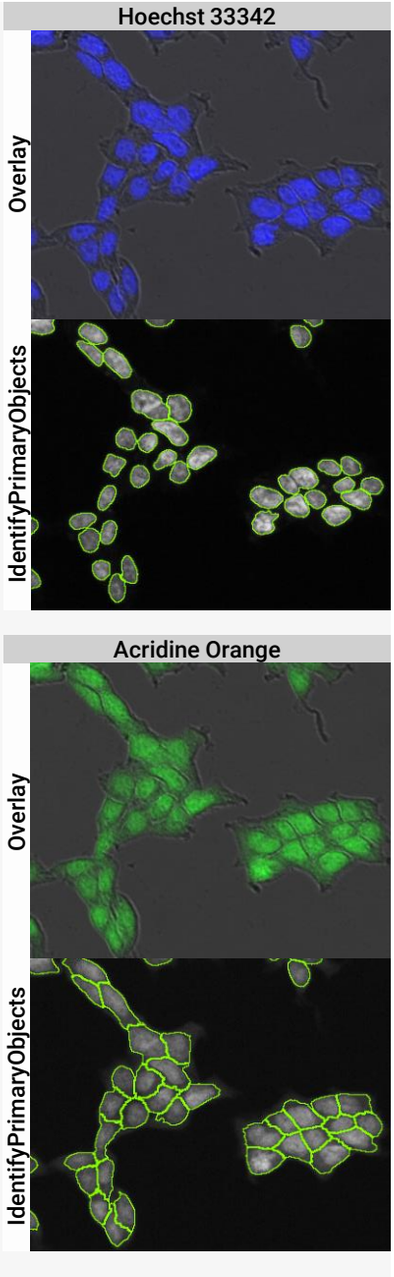

Figure 1. *Identification of adherent cells for high content cell counting. Example images of HEK293 cells with nuclei labeled with Hoeschst 33342 and acridine orange and then segmented with Cell Analyzer software using the* IdentifyPrimaryObjects *module.*

INTRODUCTION

Cell-based assays are foundational to drug discovery and development studies. Serving as a way to normalize experimental conditions and data as well as monitor cell health, cell counting is an important quality control parameter for such assays.

The CELENA® X High Content Imaging System is convenient for capturing and analyzing images of adherent cells directly from well-plates to determine cell counts in a high content capacity. The CELENA® X can be set up to image multi-well plates in up to four fluorescence channels and is integrated with CELENA® X Cell Analyzer software that can assess captured images for multiple parameters and cellular phenotypes. In this study, we investigated cell counting capabilities of the CELENA® X by double staining HEK293 cells with acridine orange and Hoechst 33342.

APPLICATION

Cell preparation

HEK293 cells were counted with the LUNA-II™ Automated Cell Counter, seeded at a density of 1x 10^4 cells/well into a 96-well plate, and cultured overnight. Cells were washed with PBS, fixed in 100 µL 4% PFA for 15 minutes at room temperature, and then washed twice with PBS. For cell staining, 100 µL acridine orange (Logos Biosystems, F23002) was added to each well and incubated for 15 minutes at room temperature. After washing each well twice with PBS, 100 µL of 4 µg/mL Hoechst 33342 (Life Technologies, H3570) was added to each well and incubated for 15 minutes at room temperature. Cells were washed twice in PBS to remove excess stain before imaging.

Imaging and analysis

Brightfield, GFP, and DAPI images were acquired from 60 wells of the 96-well plate with the CELENA® X High Content Imaging System using a 10X LWD high NA objective and image-based autofocusing.

Cell counting with CELENA® X Cell Analyzer software is based on identifying nuclei or cells with the *IdentifyPrimaryObjects* module from a designated channel. Nuclei are easily identifiable with this module as they are uniform in shape, have a high contrast relative to its background once stained, and are well-spaced apart from adjacent nuclei. Although both

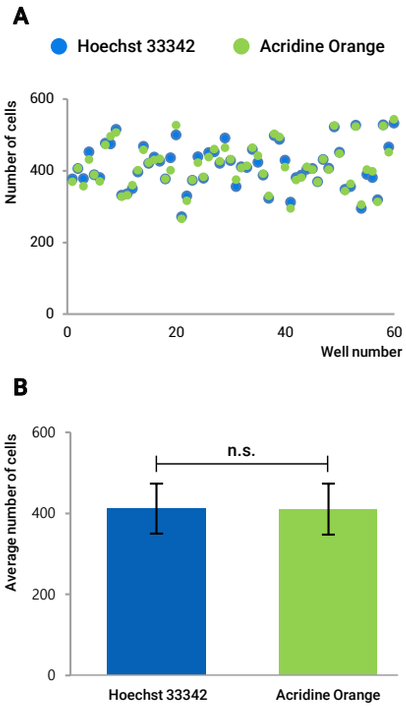

Figure 2. *Cell numbers produced by the* IdentifyPrimaryObjects *module. (A) Hoechst 33342 and acridine orange staining resulted in the same or a similar number of cells being counted in each well. (B) When the number of cells in each well were averaged, there was no significant difference seen for the two staining methods (n.s., not significant).*

acridine orange (GFP) and Hoechst (DAPI) are fluorescent dyes that stain nuclei, Hoechst distinctly stained nuclei whereas acridine orange was also seen diffusely throughout the cytoplasm. To identify the cells stained with each dye, two *IdentifyPrimaryObjects* modules were used in sequence. Table 1 shows the recommended adjustments to make to the *IdentifyPrimaryObjects* module for each dye.

Table 1. *Description of the* IdentifyPrimaryObjects *advanced settings used to identify cells and determine cell number.*

	Hoechst 33342	Acridine Orange
Channel	DAPI	GFP
Threshold strategy	Adaptive	Adaptive
Threshold method	MCT	Otsu
Size of smoothing filter	15	14
Suppress local maxima	5	10

CONCLUSION

In this study, we demonstrated how simple it is to count adherent cells with the CELENA® X High Content Imaging System. Using the CELENA® X for fluorescence cell imaging paired with automated object identification and quantification gives researchers a simple and flexible way to monitor diverse cell-based assays. The same analysis pipeline can be reused to verify or compare results, making the CELENA® X a powerful tool for cell-based assays.

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Application Note

Wound healing assay, scratch test, collective cell migration, time lapse imaging, live cell imaging, high content imaging, high content analysis

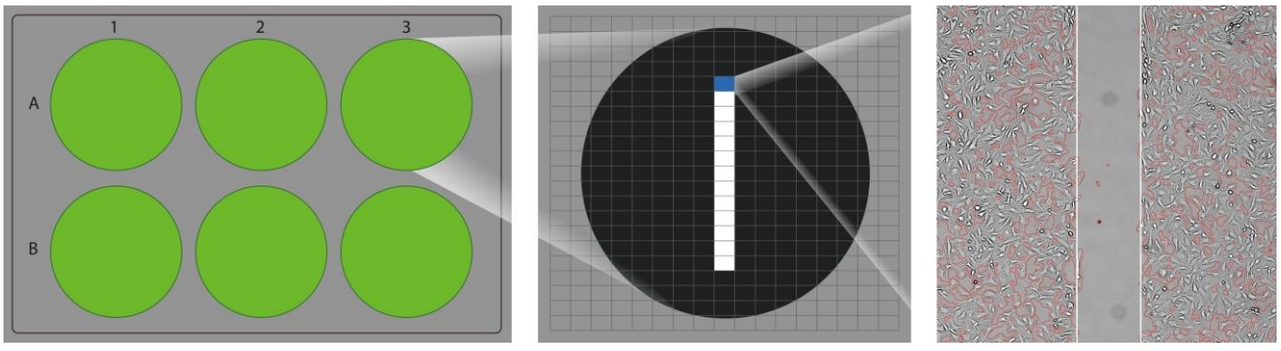
Wound healing analysis based on image segmentation

INTRODUCTION

Collective cell migration is the coordinated movement of a group of cells that maintain intracellular connections and is crucial to various biological processes including embryo development, the immune response, and cancer metastasis. The wound healing assay, also known as the scratch assay, is a simple and cost-effective way to measure collective cell migration *in vitro*. A uniform 'wound' or 'scratch' is created on a cell monolayer and the migration of the cells around the wound edge toward the cell-free space is observed via time-lapse imaging. Here, we describe a way to perform a multi-well plate wound healing assay that produces quantitative data using the CELENA® X High Content Imaging System.

APPLICATION

Bovine aortic endothelial cells (BAEC) were counted with the LUNA-II™ Automated Cell Counter, plated in a 6-well plate (SPL, 30006) at a density of 1 x 10^6 cells/well, and cultured for 24-48 hours until confluent. Scratches were made using SPLScar Scratcher (SPL, 201906). The plate was placed in the CELENA® X onstage incubator with 95% humidity and 5% CO_2 at 37°C and automated kinetic imaging was set up with the CELENA® X High Content Imaging System. Cells were imaged at the start of the experiment (t=0) and at two-hour intervals for 16 hours. 10 consecutive fields-of-view were captured per well using a 4X LWD objective and image-based autofocusing.



To quantify wound closure over time, images captured at the same time point were processed with CELENA® X Cell Analyzer software. The strategy to quantify wound closure is based on distinguishing cells from the background and measuring the change in the occupied surface area. A pipeline was created in Cell Analyzer to automatically batch process and analyze images. To do this, the *EnhanceEdges* module was used to create binary images that distinguished the foreground (the cells) from the background. The *Smooth* module was used to reduce intensity irregularities, which homogenized the cells, smoothed edges, and removed debris from the background. The resulting segmented areas were identified as cells using the *IdentifyPrimaryObjects* module, making sure that declumping was not selected so that the cell mass, not individual cells, would be identified. The identified areas were then measured using the *MeasureImageAreaOccupied* module to quantify the surface area occupied by the cells within the field. The *OverlayOutlines* module was used to overlay the original brightfield images with the outlines of the segmented areas to visually confirm segmentation precision.

Figure 1 shows one field-of-view at each time point, demonstrating wound closure over time. Evidence of cell migration is seen as early as 2 hours post-wound creation and the cell-free space steadily decreases over time as the cells move to close the space completely. Image segmentation allowed quantification of the surface area occupied by cells (Figure 2).

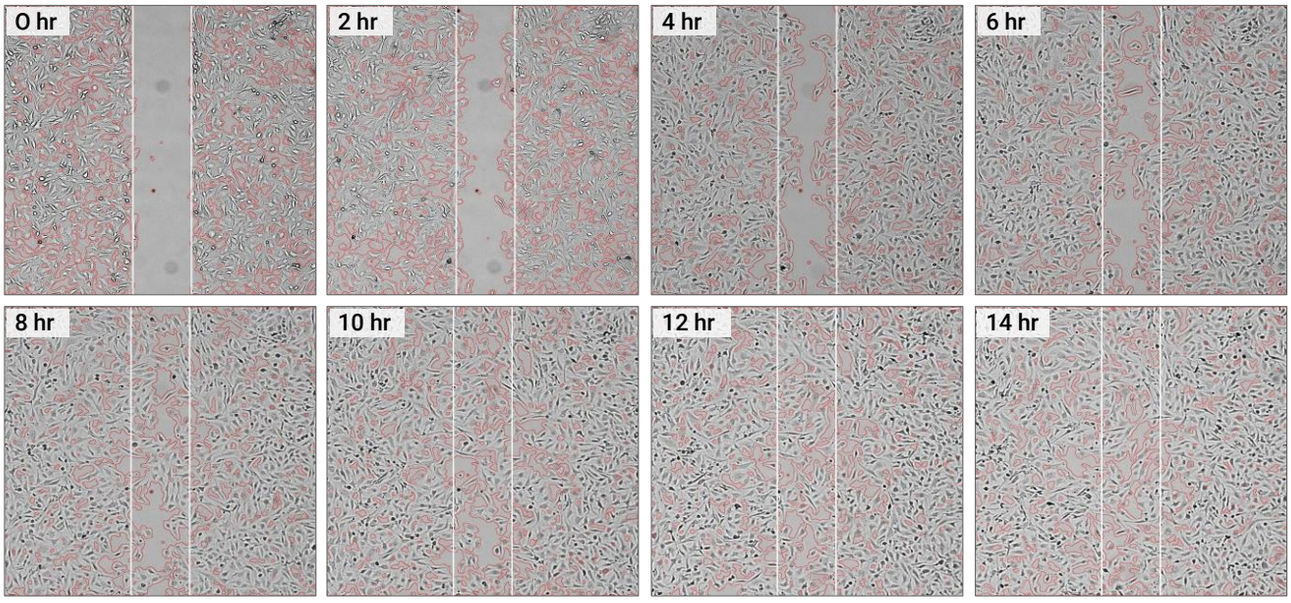


Figure 1. *Image analysis of BAEC cells showing wound closure over time. Red borders illustrate the separation between background and the areas covered by intact cells. The white boxes outline the initial wound site.*

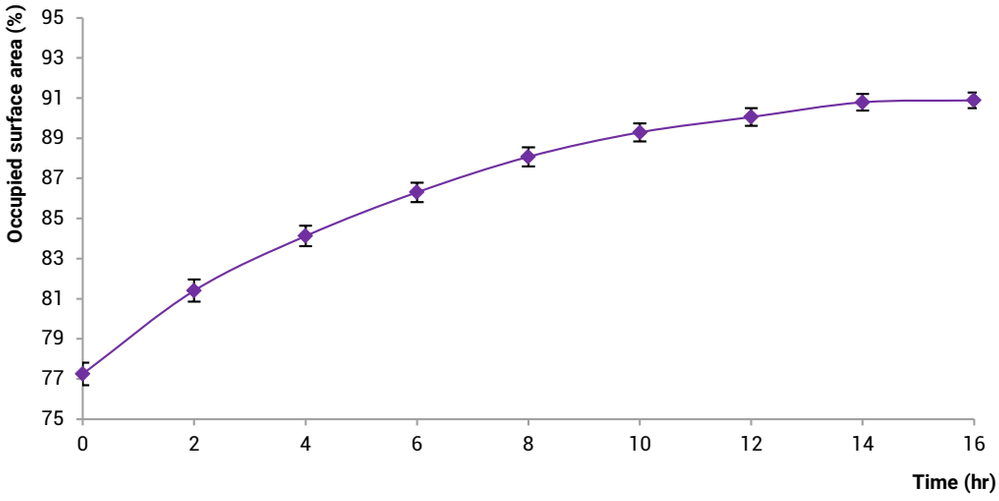


Figure 2. *Quantification of* in vitro *wound healing in BAEC cell images deduced from surface area occupied at the start and end of the experiment. N = 6 wells, 10 fields-of-view per well*

CONCLUSION

In this study, we defined a method to measure cell migration using the CELENA® X High Content Imaging System. Kinetic live cell imaging combined with quanitifcation of the change in cell surface area provides an easy-to-use, reproducible, and objective method to generate accurate cell migration data. Batch processing images for image segmentation analysis on Cell Analyzer is faster and more accurate than traditional image analysis methods. The method presented here can be modified for a variety of diverse experimental conditions and different multi-well plates, making it a high-throughput method for applications such as drug screening.

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Application Note

Cytotoxicity, confluency, high content imaging, high content analysis

Non-destructive quantification of cytotoxicity in live HeLa cells using the CELENA® X

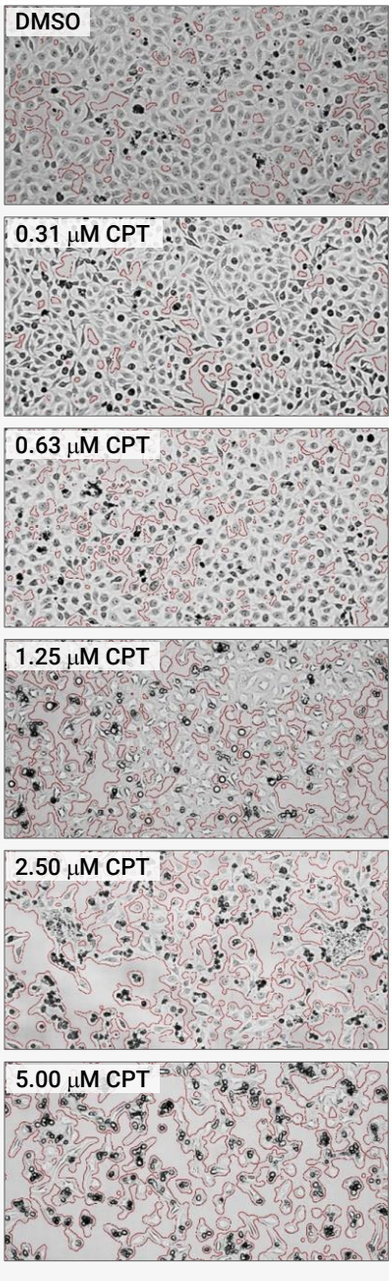

Figure 1. *Image analysis of HeLa cells after 16 hours of CPT treatment. Red borders illustrate the separation between background and the areas covered by intact cells. Image processing and object identification modules were applied to determine confluency.*

INTRODUCTION

Cytotoxicity assays are a crucial step in screening for and developing therapeutic anti-cancer drugs. Most assays designed to measure cytotoxicity *in vitro* evaluate cell membrane integrity or metabolic activity after exposure, but are typically based on studying a single time point and require disturbing the growth of cells in culture.

Here, we demonstrate an automated, non-destructive method to monitor and quantify cytotoxicity based on its effects on confluency. This method uses brightfield imaging, which avoids the use of fluorescent stains that can have toxic effects in and of themselves over long incubation times. In this study, a combination of digital brightfield imaging with high content analysis using the CELENA® X High Content Imaging System provides quantitative readouts for assessing and comparing confluency changes over time.

APPLICATION

To study cytotoxicity *in vitro*, we treated HeLa cells with different concentrations of Camptothecin (CPT; Abcam, ab120115). HeLa cells were counted with the LUNA-II™ Automated Cell Counter, seeded at a density of 1x 10^4 cells/well of the 96-well plate, and cultured overnight. Cells were then treated with serial dilutions of Camptothecin (DMSO, 0.3125 µM, 0.625 µM, 1.25 µM, 2.5 µM, and 5 µM) at eight wells per condition and placed in the CELENA® X onstage incubator with 95% humidity and 5% CO_2 at 37°C. Cells were imaged automatically at two-hour intervals for 16 hours with the CELENA® X High Content Imaging System using a 4X LWD objective and image-based autofocusing. Acquired images were analyzed using CELENA® X Cell Analyzer software.

The strategy for confluency analysis with the Cell Analyzer software is based on identifying cells and measuring the total area occupied by these objects. A pipeline was created in Cell Analyzer to automatically batch process and analyze images. To do this, the *EnhanceEdges* module was used to create binary images that distinguished the foreground (the cells) from the background. The *Smooth* module was used to reduce intensity irregularities, which homogenized the cells, smoothed edges, and removed debris from the background. The resulting segmented areas were identified as cells using the *IdentifyPrimaryObjects* module and

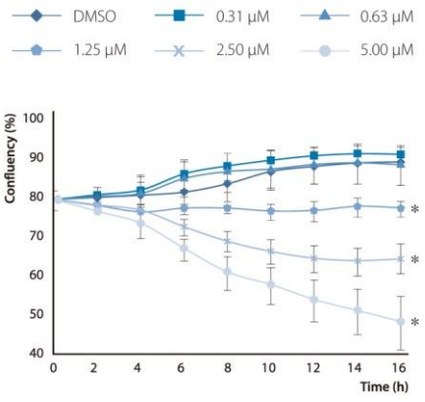

Figure 2. *Dose-response curves deduced from confluency. A significant loss of cells was observed with increasing concentrations of CPT. N =8 wells. * p : <0.05.*

then measured using the *MeasureImageAreaOccupied* module to quantify the surface area occupied by the cells within the field. The *OverlayOutlines* module was used to overlay the original brightfield images with the outlines of the segmented areas to visually confirm segmentation precision.

Figure 1 shows the segmentation of brightfield HeLa cell images after 16 hours of CPT treatment. Image segmentation using the created Cell Analyzer pipeline showed that compared to the control group, there was a 12.8%, 27.1%, and 44.6% reduction in confluency after 16 hours of incubation with 1.25 µM, 2.5 µM, and 5.0 µM CPT, respectively, whereas incubation with 0.3125 µM and 0.625 µM had negligible results (Figure 2).

CONCLUSION

The determination of cytotoxicity is essential to drug development. In this study, we analyzed the effects of Camptothecin on confluency using the CELENA® X High Content Imaging System. Live cell imaging combined with analysis of cell surface area occupying each well is a reproducible and quantifiable approach for measuring cytotoxicity. The easy-to-use, powerful building blocks of the Cell Analyzer software provides a simple way to quantify confluency objectively.

For the cytotoxicity study here, the measurement of confluency as an analysis tool for cytotoxicity was effective, simple, and most importantly, highly reproducible. Using the CELENA® X High Content Imaging System and CELENA® X Cell Analyzer software, the same experimental conditions as well as the identical analysis pipeline can be reused to verify results.

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CELENA® X High Content Imaging System
by Logos Biosystems

"Great to use, inexpensive - watch out big microscopy companies..."

Ease of use: ★★★★★ **After sales service:** ★★★★★ **Value for money:** ★★★★★

Rating: ★★★★★

Application Area: Cell Culture

"The Celena-X is so easy to use, it has huge flexibility and it is oh so affordable making the 'big name brands' look like a rip off!"

Peter Davis, ATA Scientific

"Perfect combination of high content and high quality imaging."

Ease of use: ★★★★★
After sales service: ★★★★★
Value for money: ★★★★★

Rating: ★★★★★

Application Area: High-throughput image acquisition with high quality pictures

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