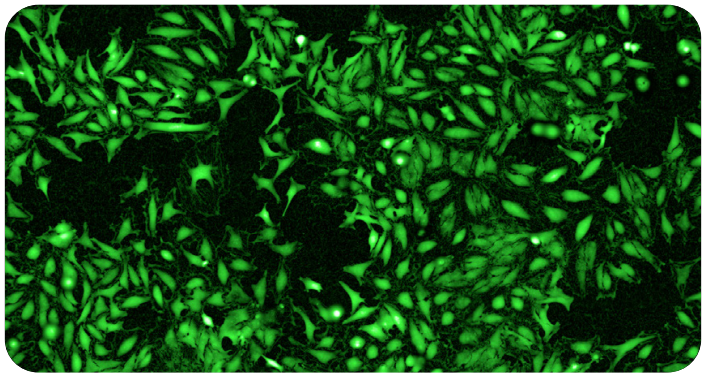
How to perform long term live cell imaging in a high-content analysis system.



Key points

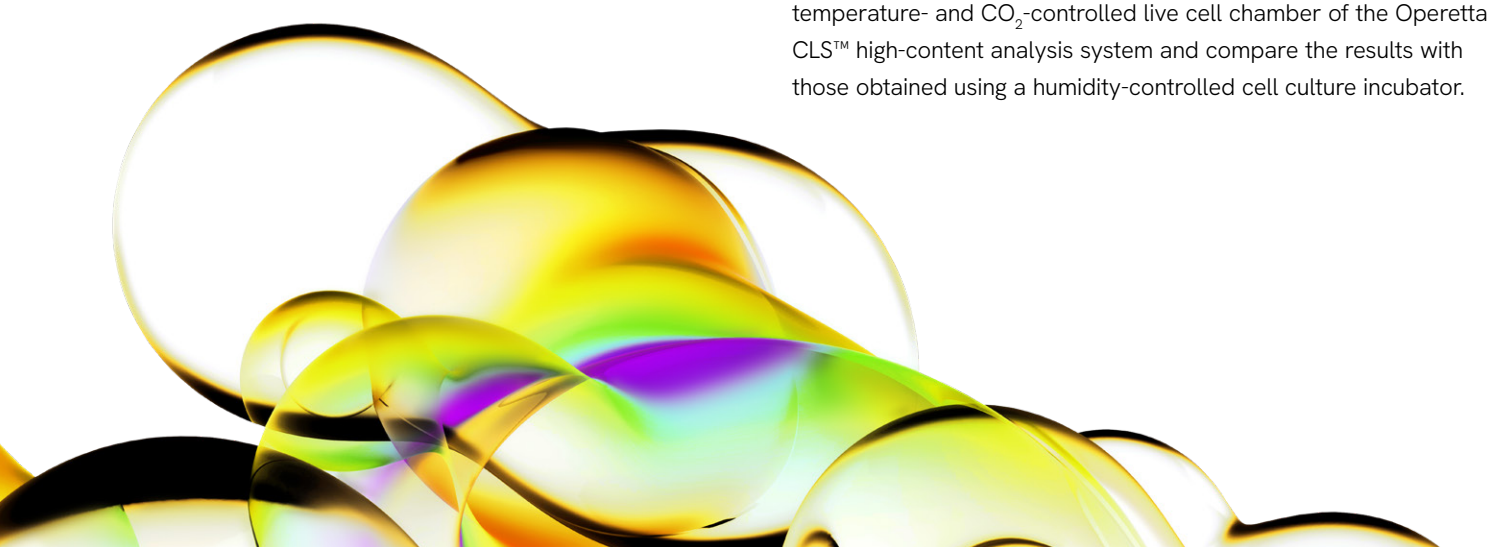
- Successful five day live cell imaging on Operetta CLS and Opera Phenix high-content systems
- Avoidance of phototoxicity with gentle digital phase contrast imaging
- Analysis of cell growth and morphology on a single cell level without any fluorescence staining

Introduction

Live cell imaging has gained importance within drug discovery over recent times, as researchers look for more meaningful insights into cellular behavior and function. When combined with the ability to phenotypically analyze individual cells on a high-content screening system, live cell imaging allows analysis of events such as proliferation, cellular movement, cell signaling, and morphological changes over time, or the dynamics of apoptosis and cytotoxicity.

However, setting up live cell experiments can be challenging, as temperature, CO_2 and evaporation need to be controlled to ensure optimal cell growth conditions. In principle, there are two options for overcoming these challenges: either the imaging device is placed inside a conventional cell-culture incubator, or the growth conditions are controlled by the imaging instrument.

Both ways can be problematic since controlling evaporation by increasing the humidity can cause microbial contamination inside the instrument and can have a detrimental effect on the performance of imaging equipment. In this technical note, we monitor evaporation rates, cell growth and morphology inside the temperature- and CO_2-controlled live cell chamber of the Operetta CLS™ high-content analysis system and compare the results with those obtained using a humidity-controlled cell culture incubator.



Kinetic analysis of cell growth

As the majority of live cell experiments are performed in 96-well format, HeLa cells were seeded into 96-well PhenoPlate™ microplates. Revvity PhenoPlate microplates for high-content imaging benefit from a low profile lid to reduce evaporation. To analyze cell proliferation in the Operetta CLS system over time, a time-course experiment over five days was set up. Digital phase contrast (DPC) images were acquired every 30 min, using a 20x high NA objective. To analyze cell growth, individual cells were segmented in the digital phase contrast images (Figure 1A) using Harmony® high-content analysis software and the average number of cells per well

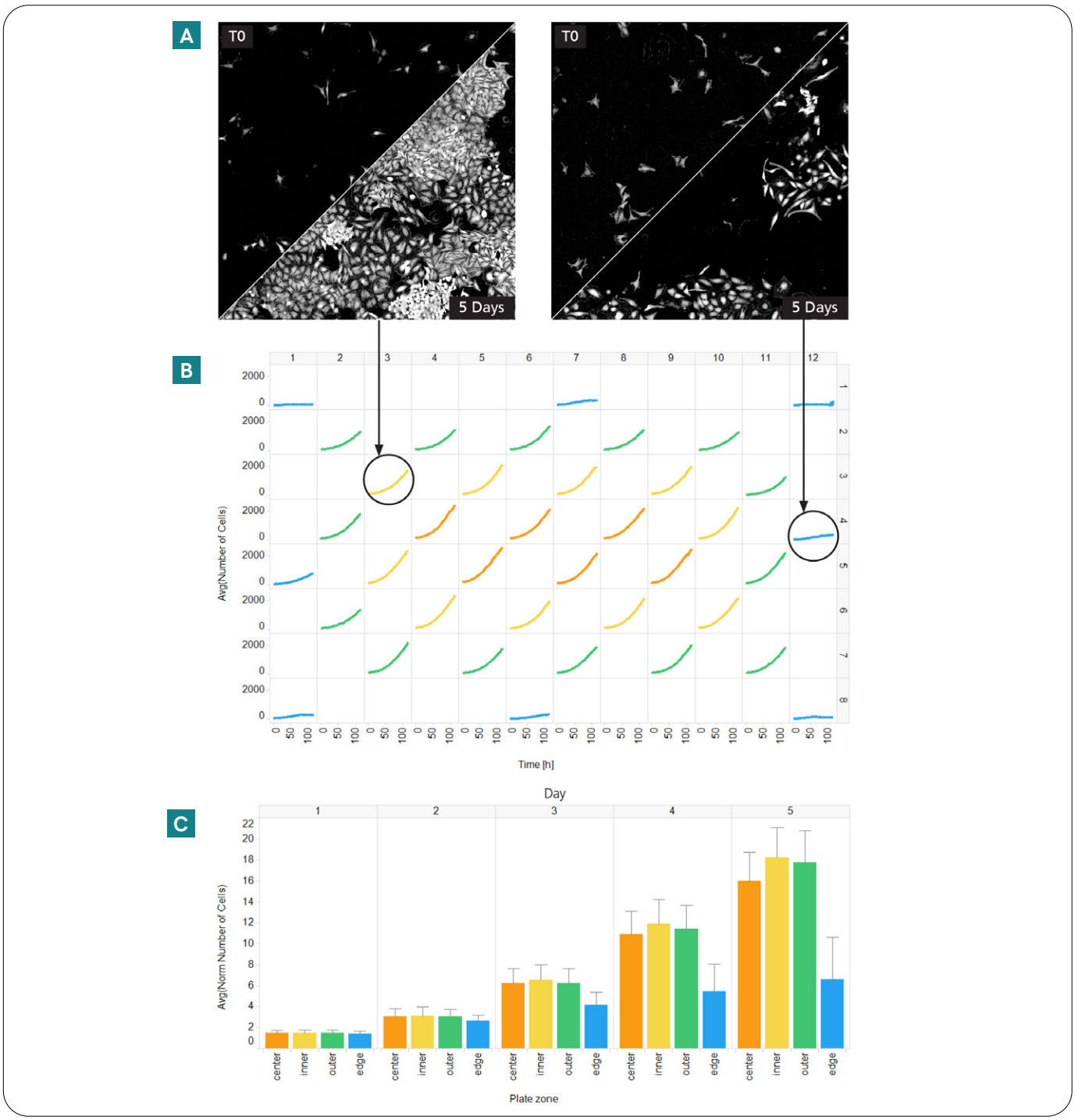


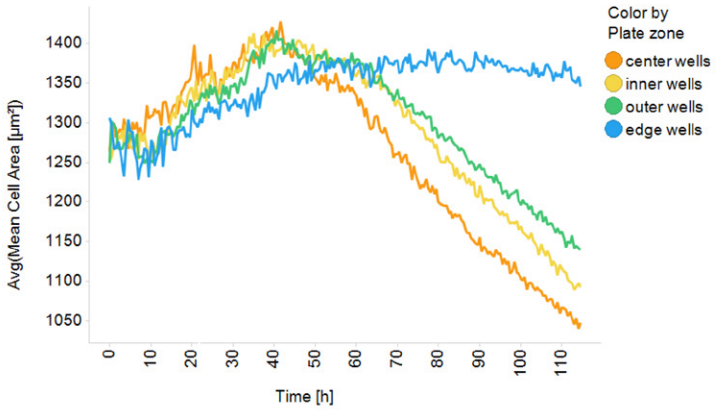
Figure 1: Analysis of cell growth over five days in the Operetta CLS system. (A) DPC images at the beginning (T0) and at the end (five days) of the time-course measurement. Shown are example images of the inner- and edge well zone. (B) Kinetic plate view showing the number of cells per well over time. (C) Normalized average number of cells per well and day in respective plate zones.

was plotted against time to generate growth curves (Figure 1B). If the plate is divided into different zones, i.e. center wells (orange), inner wells (yellow), outer wells (green) and edge wells (blue), it can be seen that over the five days, the center, inner and outer wells show comparable growth curves, while proliferation is reduced in the edge wells. Analysis of the average cell number per plate zone and day reveals that the reduced proliferation in the edge wells starts from day two onwards (Figure 1C)

Kinetic analysis of cell morphology

The ability to segment individual cells not only allows the counting of cells but also the analysis of morphological parameters, such as cell area. Figure 2 shows how the cell area changes over time. As the cells proliferate and the cell number increases, individual cells have less space and the cell area decreases. This can be observed for the center, inner and outer wells. The average cell size first increases and then decreases again, once the cells become more confluent. However, the average area of cells in the edge wells does not decrease, which is possibly due to their slower proliferation.

A



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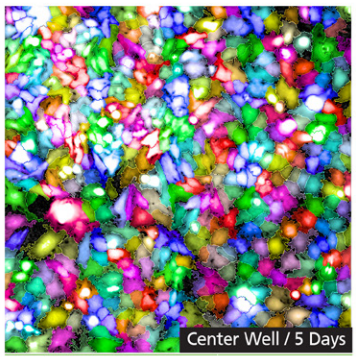

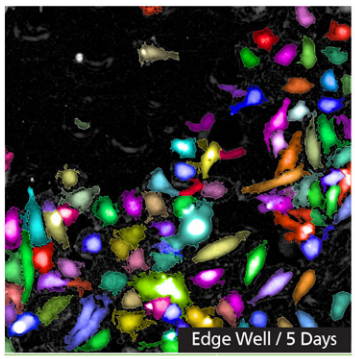

Population: Cells	Value
Number of Objects	424
Property	**Mean**
Area [µM²]	906.821
Area [px²]	2557.44
Intensity Digital Phase Contrast	967.547

Population: Cells	Value
Number of Objects	106
Property	**Mean**
Area [µM²]	130.539
Area [px²]	3681.51
Intensity Digital Phase Contrast	638.633

Figure 2: Analysis of cell area over five days in the Operetta CLS system. (A) Mean cell area in different plate zones over time. (B) Example images showing single cell segmentation in the DPC channel for center and edge wells. Below each image, a results table with calculated cell properties is shown. Area of cells growing within center, inner and outer wells decreases after the second day of growth.

Analysis of evaporation rate

To better understand the reasons for reduced cell proliferation in the edge wells, a set of control plates was analyzed for evaporation over the five days. These plates were seeded with the same number of HeLa cells and had identical starting volumes (250 µL medium/ well). One control plate was kept inside a temperature, CO_2 and humidity-controlled cell culture incubator. Two control plates were kept in a temperature and CO_2-controlled Operetta CLS system. One of these plates had the inter-well space filled with water to test for the impact on evaporation rate. To calculate the evaporation rate per well, the amount of medium at the beginning and at the end of the five day live cell experiment was determined. Additionally, the number of cell divisions was calculated by counting single cells at the beginning and after five days of incubation. The plate kept in the cell culture incubator shows a very low and uniform evaporation rate of 0.01 - 0.1 µL/h (Figure 3, right panel). Considering the starting volume of 250 µL per well, this corresponds to a total loss of about 0.5 - 5.0% after five days of incubation. The medium evaporation in the instrument is less uniform if only the wells are filled and the inter-well space is kept empty (Figure 3, left panel). Under this condition, the highest evaporation (1.3 µL/h, 61% after five days) was measured in the edge wells and the least evaporation in the center wells (0.02 µL/h, 1% after five days). The results for the center and inner well zone are both within the range of the plate kept in the incubator. However, if the inter-well space is filled with water, the evaporation rate is strongly reduced across the whole plate to 0.02 - 0.2 µL/h, which is comparable to the incubator conditions (Figure 3, middle panel).

Number of cell divisions

To analyze whether evaporation has an effect on the number of cell divisions, individual cells were segmented and counted in the digital phase contrast images using Harmony software. Based on the starting and final cell number, the number of cell divisions was calculated (Figure 4). In the incubator plate, HeLa cells divided, on average, 4.1 times during the five days of the experiment, without any distinctive edge effects. Similar cell division rates were achieved in the center, inner and outer wells of the plate with empty inter-well space, incubated and imaged on the Operetta CLS system. In the edge wells, HeLa cells divided, on average, only three times on the imaging instrument. These wells are impacted by the higher evaporation rate (1.3 µL/h) and should not be used for more than two days of live cell imaging. However, when the inter-well space of the 96-well PhenoPlate is filled with water, the number of cell divisions in the Operetta CLS system is comparable to the plate kept in the cell incubator (Figure 4, middle panel). With the inter-well space filled, the whole plate can be used for live cell experiments with up to five days of continuous imaging.

The Operetta CLS and Opera Phenix™ high-content systems' live cell chambers are identical in construction. Nonetheless, the experiments were repeated on the Opera Phenix system, whereby no differences were detected between the two instruments (data not shown).

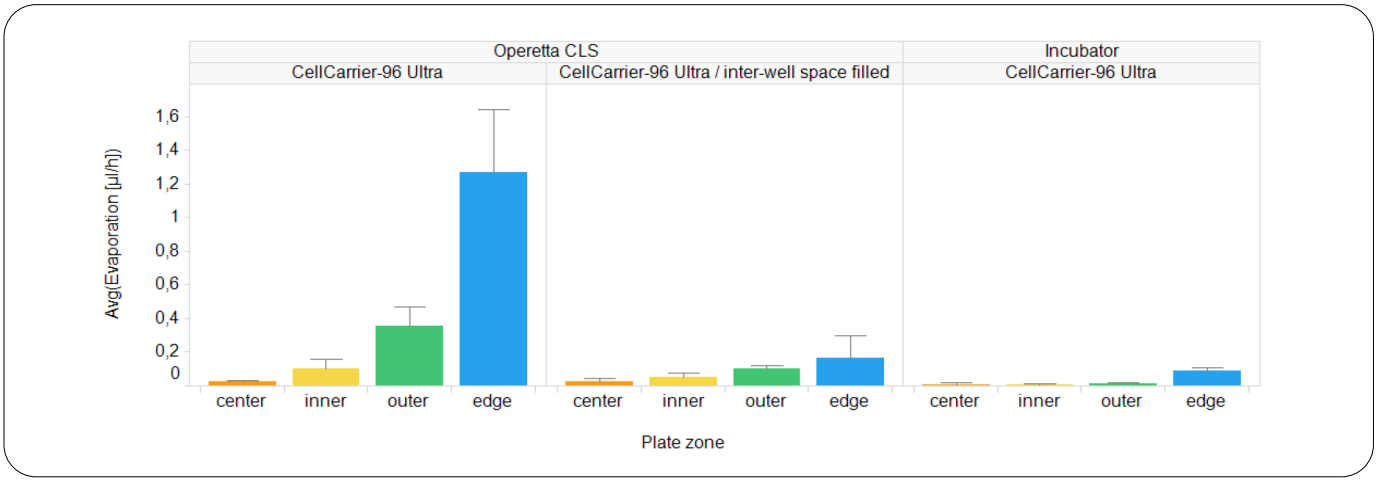

Figure 3: Evaporation rates per well across the 96-well PhenoPlate. The average evaporation rate [µL/h] in each plate zone is plotted. If the inter-well space of the 96-well PhenoPlate is empty, the mean evaporation is up to 1.3 µL/h in edge wells (left panel). If the inter-well space is filled with water, the mean evaporation is reduced to 0.2 µL/h in edge wells (middle panel). These rates are comparable to the low evaporation rates in a cell culture incubator (right panel).

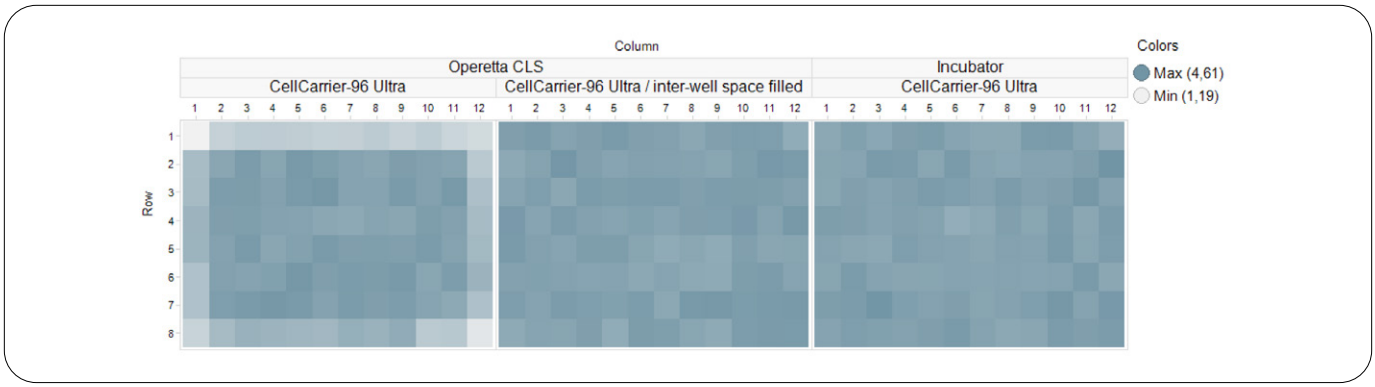

Figure 4: Heat map of the average number of cell divisions per well. The 96-well PhenoPlate, with inter-well space filled, allows five days of live cell imaging on the Operetta CLS system. Cells grow equally well in all wells of the incubator plate (right panel). Light grey values indicate decreased cell growth in the edge wells of the Operetta CLS plate, when the inter-well space is empty (left panel). With the inter-well space filled (middle panel), the plate kept in the Operetta CLS system shows an equally distributed cell growth that is comparable to the cell incubator.

Conclusions

Taken together, our experiments with HeLa cells and 96-well PhenoPlates show that the environmental conditions inside the Operetta CLS or Opera Phenix systems favor live cell imaging, even for longer term experiments (five days). The design of the 96-well PhenoPlate benefits live cell imaging, as it provides a low profile lid to reduce evaporation and allows the space between wells to be filled with water to generate incubator-like conditions during live cell imaging. A great advantage of using an Operetta CLS or Opera Phenix system for live cell imaging is the gentle, label-free digital phase contrast mode, and the ability to use these high-quality images to segment and analyze individual cells within the Harmony software. Even a morphological analysis of individual cells is possible without any staining, providing advantages over simple confluency measurements. Based on confluency alone, compounds affecting the cell area can be misinterpreted as anti-proliferative or even cytotoxic. Single cell morphological readouts provide a much deeper understanding of the underlying biological effect, whether this is induced by addition of compounds, or by genetic perturbation.

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