Determination of Cell Proliferation Using the CELLCYTE X Live-cell Imaging System

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Abstract

Live-cell imaging is a powerful tool that can provide researchers with direct quantitative data on cell proliferation, using acquired phase contrast and fluorescent images analyzed with detection software. In this application note, we explore the use of live-cell imaging to determine proliferation of label-free cells as well as fluorescently labeled cells. The CELLCYTE X live-cell imaging system was used to acquire images of A549 (lung carcinoma) and HepG2 (hepatocellular carcinoma) cells, and the detection software determined cell proliferation by measuring either cell confluency or by counting cells and nuclei. In the case of cell confluency, phase contrast images were used to study cell proliferation under normal growing conditions as a quality control; and cells under treatment were used to observe the effect of the drug. Cell and nucleus count were analyzed from fluorescently labeled cells cultured under normal conditions to observe the growth curve of A549. The results support the use of live-cell imaging to measure cell proliferation in label-free cells, using cell confluency, and in fluorescently labeled cells, using cell and nucleus count.

Introduction

Cell proliferation, a widely used parameter to evaluate the capacity of cells to grow and reproduce, reflects the dynamic process between cell division and cell death in a population. The significance of cell proliferation lies in its ability to indicate the status of a cell culture—for example, it can reveal the cytotoxic or cytostatic effect of a drug treatment (Aysun, 2016).

There are a variety of methods to determine cell proliferation through indirect and direct measurements, which can be performed as time-course or end-point experiments. Conventional techniques, like manual cell count using permeable viability dyes, are useful but present disadvantages because the user’s experience plays an important role in the results, and the sample is usually damaged during the readings, making it impossible to use the same cell population over time. Additionally, some cell proliferation techniques cannot be modified to perform high-throughput experiments therefore they become very time consuming when handling a large number of samples. To overcome the mentioned obstacles, researchers have developed techniques to directly measure cell proliferation using microscopy (Drey, 2013; Jaccard, 2014). Among the parameters used are cell confluence, cell count and nucleus count.

Cell confluence measures the area covered by cells in a given field of view observed under an inverted microscope. This parameter has been used for several years on a daily basis while working with adherent cell cultures and is a useful way to determine when cells are ready to perform an experiment or need to be split, as well as to evaluate growth, behavior and health. Typically, it has been performed by estimating a subjective value for cell confluence, making it non-ideal and inaccurate because individuals estimate based on their own perception. The advancement of computer programs has made it possible for researchers to develop methods to obtain accurate cell confluence measurements (Busschots, 2015), eliminating the varying perceptions of
individual users. The more precise the detection of the program is, the more accurate the results will be. Cell confluence can be measured in phase contrast images in label-free cells and in fluorescent images using whole-cell staining. Nowadays, cell confluence determined by software applications is an accepted direct method to monitor cell proliferation in adherent cells since an increase in cell number correlates with an increase in cell confluence.

Other direct methods to determine cell proliferation are cell and nucleus count, commonly they require fluorescent labeling of the respective cell components. Currently, an increasing number of fluorescent reagents are available, but their use can have a disruptive impact on cells (Purschke, 2010); therefore, it is important to corroborate that the dye is not affecting the outcome of results. Cell proliferation determined by fluorescent live-cell imaging is an elegant and informative technique. Using fluorescent images to study cell proliferation is common in cells modified to express fluorescent proteins as well as in cells in co-culture where either whole-cell or nucleus labeling aids to distinguish between the two cell types.

In this application note, we show how the CELLCYTE X system can determine cell proliferation by measuring cell confluency as well as by counting cells and nuclei.
Materials and methods

Cell lines and cell culture conditions
A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. HepG2 cells were cultured in Minimum Essential Medium supplemented with 10% FBS, 1% GlutaMAX, 1% antibiotic/antimycotic, 1% non-essential amino acids and 1% sodium pyruvate. All the cell lines were cultured at 37°C, 5% CO2 and <95% humidity.

Quality control of A549 and HepG2 cell cultures
Per well, 2x10^5 A549 cells and 3x10^4 HepG2 cells were seeded into 6-well plates. The medium was replaced on Day 2 of the experiment. The cells were monitored for 96 hours and 25 phase contrast images per well were acquired every 6 hours using the 10X objective of the CELLCYTE X live-cell imaging system. A549 proliferation curve was stopped after 72 hours since it reached high confluency at this point. The images were analyzed using the CELLCYTE Analysis software to create an accurate detection mask for cell confluency for each of the cell lines. The experiments were performed in triplicate.

Treatment of A549 with an EGFR inhibitor
A total of 2x10^5 A549 cells were seeded into 6-well plates and allowed to attach overnight. The next day, the cells were treated with increasing concentrations of an EGFR inhibitor and imaged immediately after addition of the treatment. Using the CELLCYTE X system, 25 phase contrast images per well in a 10X magnification were acquired every 3 hours. The images were analyzed using the CELLCYTE Analysis software to generate a cell confluence detection mask. The experiments were performed in duplicate in three independent experiments.

Cell proliferation using cell and nucleus count
A total of 6x10^5 A549 cells were stained using green whole cell and blue nuclear fluorescent dyes. The cells were washed with PBS three times to remove any dye excess. The cell pellet was resuspended and divided into three different wells of a 6-well plate to achieve 2x10^5 cells per well. Image acquisition of the green and blue fluorescent channels started after allowing the cells to attach. The images were obtained under a 10X magnification using the CELLCYTE X system and analyzed using the CELLCYTE Analysis software. Each channel was analyzed individually to create detection masks for either whole cell count in green or nucleus count in blue. The experiments were done in triplicate in three independent experiments.

Results and discussion

Monitor cell proliferation for quality control of cell cultures
Live-cell imaging has emerged as a helpful technique in cell culture, and together with accurate software, it can provide precise quantitative data. One common application for this technique is to aid in the routine work in cell culture by serving as a quality control that includes detailed information about the growth curve, morphology and growing patterns of the cultures. In this application note, we performed cell proliferation experiments using high resolution phase contrast images of two different cell lines (A549 and HepG2) to demonstrate the CELLCYTE X system’s ability to measure cell proliferation in non-stained cells. Figure 1 shows the steps of image analysis in the CELLCYTE Analysis software used to detect HepG2 cells. For more detailed information on how the software acquires these images, we recommend reading “Cell proliferation using live cell imaging: A technical note.” To further test cell detection under different experimental conditions, both cell lines were seeded at low confluency and monitored over time until the cells reached high confluency. Figure 2 shows the cell confluency graphs for A549 and HepG2 cells. Each cell line presents its own growth curve, reflecting the nature of its growing patterns (Giard, 1979; Aden, 1979). A549 cells grow in monolayers that...
spread uniformly, while HepG2 cells aggregate to form islands and grow in this way until they form a confluent monolayer. A549 cells have a faster doubling time compared to HepG2 cells, explaining why they reached high confluency after three days, while HepG2 cells required more time. Our results demonstrate phase contrast images of label-free cells acquired and analyzed using the CELLCYTE X system are a useful tool to monitor cell proliferation and the general health of the cell cultures by detecting cell morphology and growing characteristics under different experimental environments. The growth curves obtained using label-free cells are a good indication of the behavior of the cell culture. Well-reported parameters such as doubling time can be compared and used to determine if cells are changing over passages, helping to maintain good practice in cell culture as well as to detect discrepancies before performing experiments.

Figure 1. Representative images obtained and analyzed using the CELLCYTE X system. A) Original phase contrast image of HepG2 cells acquired using a 10X objective, B) cell detection after applying a detection filter using a cell proliferation recipe, C) cell detection after refining the subset filtering, and D) recipe mask applied to the original image.

Figure 2. Confluency graph following A549 and HepG2 cell proliferation. Both cell lines were seeded at low cell confluence and monitored over time using 10X objective in phase contrast images. A549 cells reached high confluency faster than HepG2 cells, therefore the A549 proliferation curve was stopped at 72 h while HepG2 cells were allowed to grow for 24 h more. Cell proliferation was determined by analyzing cell confluency percentage in three different experiments performed in triplicate, data in this experiment represents the mean ±SD.
Label-free determination of cell confluence during drug treatment

As mentioned previously, cell proliferation is a commonly used parameter in drug discovery. In this application note, we used live-cell imaging in phase contrast channel to study cell proliferation of A549 cells treated with different concentrations of an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). A549 have been reported to be resistant to EGFR TKI in colony formation (Ono, 2004) and cell proliferation assays; this resistant phenotype is linked to a wild type status of the EGFR exon 19 (Zhang, 2018). Cell proliferation was measured based on the metabolic activity of the cells, dismissing important intrinsic characteristics related to cell health upon drug treatment such as changes in morphology. In our study, we monitored the effect of the drug on cell proliferation by measuring cell confluency and registering general changes in cell health; no dyes were used to eliminate variables while observing the effect of the drug. Figure 3 shows the results of A549 treatment with different concentrations of the drug over 60 hours. Our data clearly shows how the drug exhibited a cytostatic effect over time. With the lower concentration of the drug, the cells recovered and resumed proliferation. Despite the lack of cytotoxic effects with the drug treatment, the cells presented obvious changes in morphology. Figure 4 contains representative images of the cells after 48 hours of treatment with different drug concentrations; and the observed morphological changes indicate impaired cell health. Altogether, our data represents an example of more complete drug treatment data obtained with a simple, user-friendly technique: phase contrast live-cell imaging.

![Figure 3](image-url) Monitoring of treatment of A549 cells with an EGFR inhibitor over time. The cells were seeded at low confluency and treated with different concentrations of an EGFR inhibitor or control (vehicle) for 60 h. Phase contrast images were acquired every 3 h. Cell proliferation was determined by analyzing the percentage of cell confluency of three different experiments performed in triplicate, data in this figure represents the mean ±SD.
Cell proliferation using cell and nucleus count

Depending on the complexity of the experiment performed, researchers might use different parameters to study cell proliferation, including cell and nucleus count (Purschke, 2010). To obtain these measurements, the respective organelles have to be stained so they become visible in order to be counted. In our study, we analyzed cell proliferation by counting cells and nuclei simultaneously (Figure 5). The data shows how both counts follow the same growth curve, validating mutually throughout the different time points, and both measurements had a strong correlation of $R^2 = 0.99$ (data not shown). It is also important to note the detection of a short lagging time at the beginning of the curve, this phenomenon is due to disturbance of the exponential growth phase of cell cultures when they are seeded into a new vessel.

Figure 5. Whole cell count and nucleus count proliferation of A549 cells. The cells were stained using whole-cell dye in green and nucleus staining in blue. Image acquisition was performed every 6 h for the green and blue fluorescent channels. Cell proliferation was measured by counting whole cells and nuclei separately, using the CELLCYTE Analysis software. The data represents the mean of three independent experiments ± SD.
Conclusions

Cell culture is a crucial pillar in different research fields, consistency and reliable data are basic requisites to guarantee reliable results. Routine quality control of cell cultures should be performed to ensure the cells being cultured behave as expected, but these quality controls should not disturb or destroy the samples. Taking this into account, live-cell imaging of label-free cells emerges as an ideal technique to accomplish this task. In this application note, phase contrast live-cell images acquired by the CELLCYTE X system were analyzed to measure cell proliferation over time; as a quality control for general health of the culture as well as during drug treatment experiments, providing evidence of how label-free cells can be used to determine cell proliferation using cell confluency.

When basic parameters to determine cell proliferation like cell confluency do not provide sufficient information to answer the scientific question being investigated, it is necessary to use additional strategies. Fluorescent live-cell imaging is a quantitative method for studying cell proliferation by counting whole cells and/or nuclei. Our results show how we were able to obtain accurate counts for both parameters by using the CELLCYTE X system, therefore proving the ability of the system to perform cell proliferation assays using cell and nucleus count.

Altogether, the present application note demonstrates live-cell imaging using the CELLCYTE X system is able to detect cells with different morphologies and growth patterns in phase contrast and fluorescent channels under diverse experimental conditions, providing accurate quantitative measurements of cell proliferation.
References


