# Live-cell Assays Measuring Complex Compound Effects in Real Time

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## INTRODUCTION

There is an increased need for expanding the variety and complexity of cell-based assays for biologic research and drug discovery. Live-cell assays allow monitoring of cell responses in real time and provide important insights about compound treatment effects, biological complexity, and physiological relevance of assay results. We used real-time quantitative cell imaging assays with the ImageXpress Pico system to monitor complex effects of anti-cancer drugs or cardio-active compounds in the appropriate cell models.

To characterize the effects of anti-cancer compounds we monitored the time-dependent effects on cell adhesion, proliferation, cell cycle, and apoptosis, for several cancer cell lines. An environmental control chamber was used to provide desired $CO_2$ and $O_2$ concentrations, including hypoxia conditions. Time-lapse imaging in transmitted light was used to quantify cell number, confluency, and cell area at different time points during the experiment. In addition, time-courses of the compounds' effects on cell cycle versus apoptosis were monitored using appropriate fluorescent markers. Effects of staurosporine, mitomycin C, and several other anti-cancer compounds were measured and found to be consistent with their known mechanisms of action.

The effects of selected compounds on cardiac physiology were monitored by measuring spontaneous contractions of stem cell derived cardiomyocytes. Kinetic patterns were characterized by measurements of calcium ion flux using time-lapse recording of the fluorescence intensity of calcium ion sensitive dyes. In addition, the effects on cell viability and mitochondria integrity were monitored at the end-point using additional fluorescence readouts. We demonstrated the effects of several cardio-active and cardio-toxic compounds on amplitude and the frequency of calcium oscillations and determined $IC_{50}$ values for the effects on calcium oscillations, as well as $IC_{50}$ values for cell viability and mitochondria potential.

Overall, our results demonstrate how a combination of assays can be utilized for quantitative evaluation of compound effects for multiple readouts and enable multidimensional biological profiling of complex biological effects.

## INSTRUMENT

Cell-based assays were performed using the ImageXpress® Pico Automated Cell Imaging System in combination with the CellReporterXpress™ Image Acquisition and Analysis Software. The imager provides 6 fluorescence channels plus transmitted light time-lapse capacity to enable automatic monitoring of cell proliferation, differentiation, compound toxicity, and a variety of other cell-based assays.

- **ImageXpress Pico** Automated Cell Imaging System
- Equipped with 6 fluorescence channels plus transmitted light; 4x-63x objectives; EC chamber and Z-stack capabilities
- **CellReporterXpress** Image Acquisition and Analysis Software
- Includes 20+ preconfigured assay protocols

## METHODS

### Environmental Control and Time-lapse monitoring:

ImageXpress Pico System has an optional environmental control (EC) chamber that enables control and monitoring the temperature, $CO_2$ and $O_2$ content, as well as humidity. In combination with time-lapse imaging it provides an efficient tool to perform live experiments, including hypoxia conditions.

**Cell Culture:** HeLa Cells (from ATCC) were plated into 384-well black clear bottom plates at a density of 3,000 cells per well and cultured in the environmental control chamber for 24h at 37˚ C and 4% CO2. Treatment with compounds was performed for 24h.

**Cell Imaging:** To visualize cells and monitor cell proliferation and death, 10x images were taken in transmitted light every 1-2 hours. Image analysis was performed during the experiment using cell count in transmitted light protocol. The analysis allowed us to determine numbers of cells, average cell area, and total cell covered area.

## Time-Lapse Monitoring of Cell Proliferation

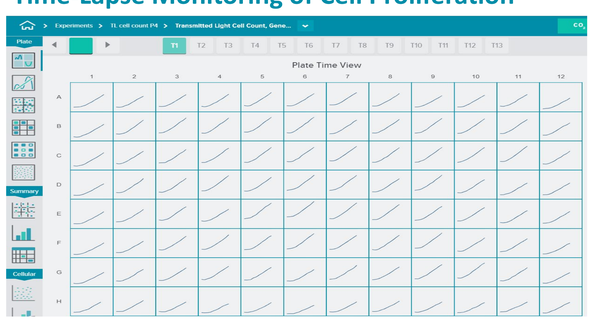



Figure 1. Cell proliferation and compound effects were monitored in real time over 24h experiment. Cells were plated into 96w or 384w format and placed into the EC chamber. Transmitted light images were taken every 1-2 hour using 10x magnification, 4 sites per well for 96w, or 1 site per well for 384 plates. Automated image analysis was performed during acquisition. Note – no plate edge effect was present in IX-Pico with EC.

## RESULTS

## Assessing Compound Effects on Cell Viability and Proliferation Using Cell Count in Transmitted Light

Phenotypic changes in cell number and morphology can be monitored using transmitted light or fluorescent markers. Effects of anti-cancer compounds were evaluated using transmitted light. Inhibition of cell proliferation by compounds was observed as a decrease in cell number or cell covered area.

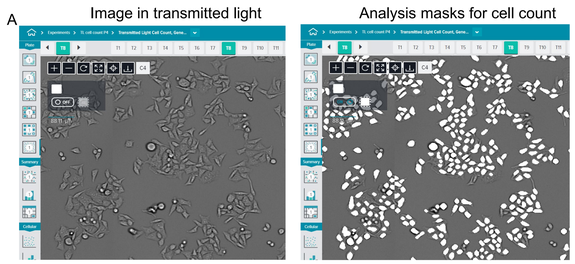



Figure 2. A. Images of HeLa cells taken with 10x magnification in transmitted light and the analysis masks in while shown for cell count (20% of the full image presented).

B. Time –course data visualization presented for cell count for control wells vs wells treated with increasing concentrations of indicated compounds (duplicate wells).

C. Concentration-dependencies presented for the 24h time point for mitomycin C (in red; IC50 0.27 µM) and staurosporine (in green, 0.05 µM).

## Identification of Mitotic Cells

We monitored cell division in HeLa cells stably expressing mCherry-H2B and Tubulin-GFP. By using fluorescently labelled DNA and the cytoskeleton protein tubulin it was possible to follow individual cells progressing through the cell cycle. Figure 3 shows 5 cells which divide within 12 h. The intercellular bridge and also the forming spindle poles are visible (white arrows). By obtaining both, total cell number and small and bright objects, it was possible to identify mitotic cells (Figure 6).

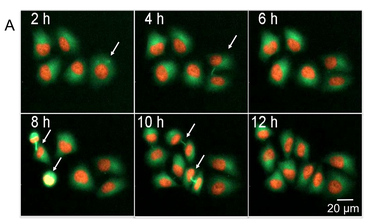
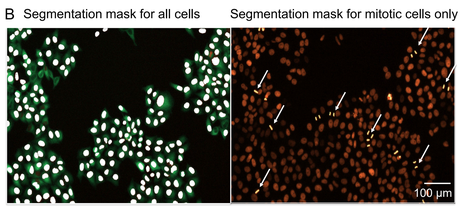



Figure 3 Live cell microscopy of mitotic cells. A. Image series showing HeLa cells stably expressing mCherry-H2B, Tubulin-GFP undergoing cell division. Mitotic cells are marked with arrows, images were acquired every 2 h for 25 h using 10x objective and FITC and TRITC illumination. B. Mitotic cell identified with a Cell Scoring Module by nuclear shape/intensity.




## Identification of Apoptotic Cells using live cell time-lapse microscopy and phenotypic analysis

Phenotypic changes over time can be used to monitor cell parameters like cell division or apoptosis. We aimed to identify apoptotic cells by phenotypic analysis of nuclear and cytoskeletal marker proteins over time. Apoptotic cells were identified by acquiring images of HeLa cells stably expressing mCherry-H2B and Tubulin-GFP. Apoptosis was induced by adding staurosporine (2 µM – 0.009 µM) to the media.

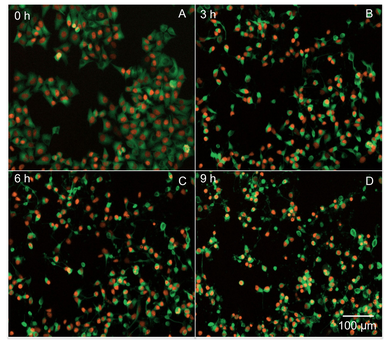
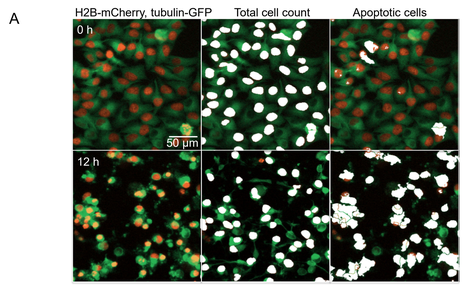



Figure 4: HeLa cells stably expressing mCherry-H2B and Tubulin-GFP were imaged after staurosporine addition (0.66 µM). A-D Image series showing different time points 0 h – 9 h (A-D). Cell undergo dramatic morphological changes after drug addition, roundness and brightness are increasing. 10x magnification, FITC and TRITC channels.



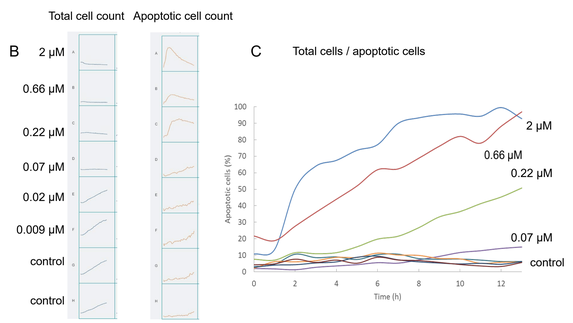
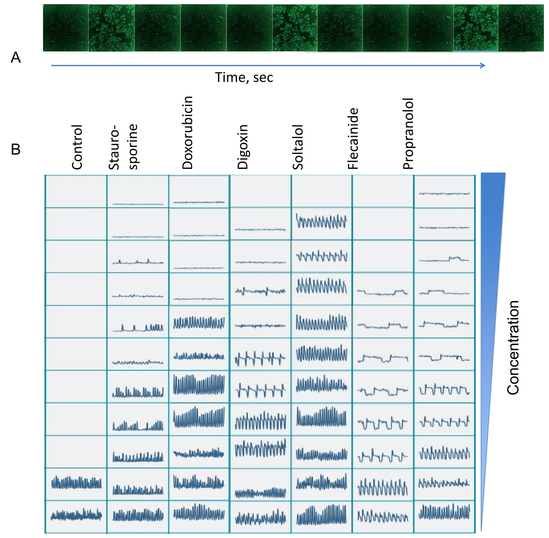




Figure 5. A, B. Apoptotic cell identification. Apoptotic cells were identified by segmenting and counting total nuclei (middle lane) and specifically apoptotic nuclei (third column), By exploiting intensity and size changes (small and bright) it was possible to quantify apoptotic cells. C. Plot showing apoptotic cells over time for different staurosporine concentrations (2-0.009 µM). The data shows a concentration dependent increase in apoptosis over time. 10x magnification, FITC and TRITC channels.

## Assessing Compound Effects on Calcium Oscillations in Cardiomyocytes

iPSC-derived cardiomyocytes are a very attractive *in vitro* model as they form a synchronously beating monolayer that can be used to reliably reproduce drug-associated cardio-physiologic phenotypes using a fast kinetic fluorescence assay that monitors changes in intracellular Ca2+ flux (Grimm et al. 2016; Sirenko et al. 2013). In this work, we have adapted a calcium oscillation assay for a new imaging system, the ImageXpress Pico system, that allows live cell assays including time-lapse imaging and environmental control.



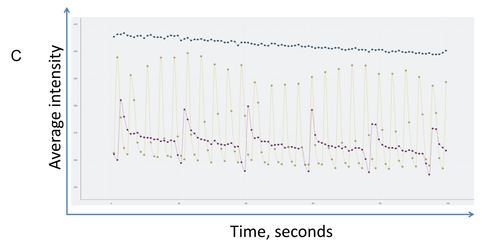




Figure 6. To measure Ca oscillations, iPSC cardiomyocytes were loaded with EarlyTox Calcium dye then imaged using the ImageXpress Pico system in the FITC channel using a time-lapse mode. A. The panel above presents a series of images taken with 0.5 sec intervals. B. Traces of calcium oscillations are shown for increasing concentrations of indicated compounds. C. Traces compared for control (0.1% DMSO, yellow), doxorubicin (1 mM, blue), or flecainide acetate (1 mM, purple). The numbers of peaks were counted manually and plotted against the compound concentrations using a 4-parametric curve fit. $EC_{50}$ values can be calculated using SoftMax® Pro Software or other programs.

## SUMMARY

- We presented methods for complex multi-parametric assays utilizing live time-lapse imaging and on-the-fly image analysis
- Multiple readouts from analysis allows characterization of phenotypic effects of different compounds and also assessment of effective concentrations
- The study demonstrates the breadth of imaging assays available with using the ImageXpress Pico Automated Cell Imaging System and environmental control unit for evaluation of various biological effects

MOLECULAR DEVICES