MULTIPLEXED AUTOMATED IMAGING ASSAYS FOR COMPOUND TESTING USING INDUCED PLURIPOTENT STEM CELL-DERIVED CELL MODELS

Oksana Sirenko, Felix Spira, Ryan Gordon*, Grischa Chandy
Molecular Devices LLC, San Jose, California, USA ; *StemoniX Inc., Maple Grove, MN

INTRODUCTION

There is an increased need for expanding variety and complexity of cell-based assays for biologic research and drug discovery. Stem cell-derived cells and tissues become an increasingly attractive alternative to traditional *in vitro* and *in vivo* testing in pharmaceutical drug development and toxicological safety assessment. In this study, we used human iPSC-derived cardiomyocyte and neuronal cell models to develop functional and morphological readouts for testing effects of different compounds in a multi-parametric assay format.

We used automated cell imaging and analysis with the ImageXpress® Pico Automated Cell Imaging System to simultaneously determine calcium oscillation frequency, cell viability, cytoskeletal integrity, apoptosis, and mitochondrial function. Effects on cardiomyocyte beating frequency were characterized by measurements of calcium oscillations. For neuro-spheroids automated imaging was used to evaluate the number of live cells and monitor calcium oscillations using time-lapse imaging. Multiplexed assessment of different readouts provides additional insight of the mechanisms of action of various compounds. The methods were characterized using a set of cardio-active drugs, neurotransmitters, and selected neurotoxic or cardiotoxic compounds.
Overall, our results demonstrate how a variety of assays can be utilized for quantitative screening of chemical effects in iPSC cardiomyocytes and neuronal models and enable rapid and cost-efficient multidimensional biological profiling.

INSTRUMENT

Cell-based assays were performed using the ImageXpress Pico system in combination with the CellReporterXpress™ Image Acquisition and Analysis Software. The imager provides 4 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 4 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 equipped with environmental control chamber enables the control and monitoring of 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: Human iPSC-derived cardiomyocytes or hepatocytes and the appropriate media were purchased from Cellular Dynamics International, Fujifilm Co. Cells were plated into 384-well black clear bottom plates at a density of 10,000 cells per well and cultured as recommended by protocols from CDI. Treatment with compounds was performed for 1h or 24h, or as indicated in the figures. Neurospheroids were obtained from StemoniX Inc (Maple Grove, MN).

Cell Staining: To visualize Ca^{2+} oscillations, cells were loaded with the Calcium 6 dye (Molecular Devices). To assess phenotypic changes, live cells were stained using a mixture of three dyes: viability dye Calcein AM (1 mM), mitochondria potential dye MitoTracker Orange (0.2 mM), and nuclear dye Hoechst (2 mM) (all from Life Technologies). For visualizing the actin cytoskeleton, cells were fixed with 4% formaldehyde (Sigma) and stained with AlexaFluor 488 (AF488) labeled phalloidin stain. Neurons were stained with anti-TuJ-1 antibodies (BD Biosciences).

Stem Cell-Derived Cell Models

There is an increasing interest in using stem cell-derived cell models in assay development. Stem cell-derived cardiomyocytes, liver cells, and neurons provide very useful models for compound testing and toxicity assessment. Cardiotoxicity and hepatotoxicity remain among two of the main reasons for drug attrition during clinical trials and post-marketing. In addition, a significant percentage of cardiovascular diseases are believed to be due to environmental exposures. Accordingly, an important target area for assay development for *in vitro* screening includes the detection of potential toxic effects.

RESULTS:

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 adopted calcium oscillation assay for a new imaging system, the ImageXpress Pico system, that allow live cell assays including time-lapse imaging and environmental control.

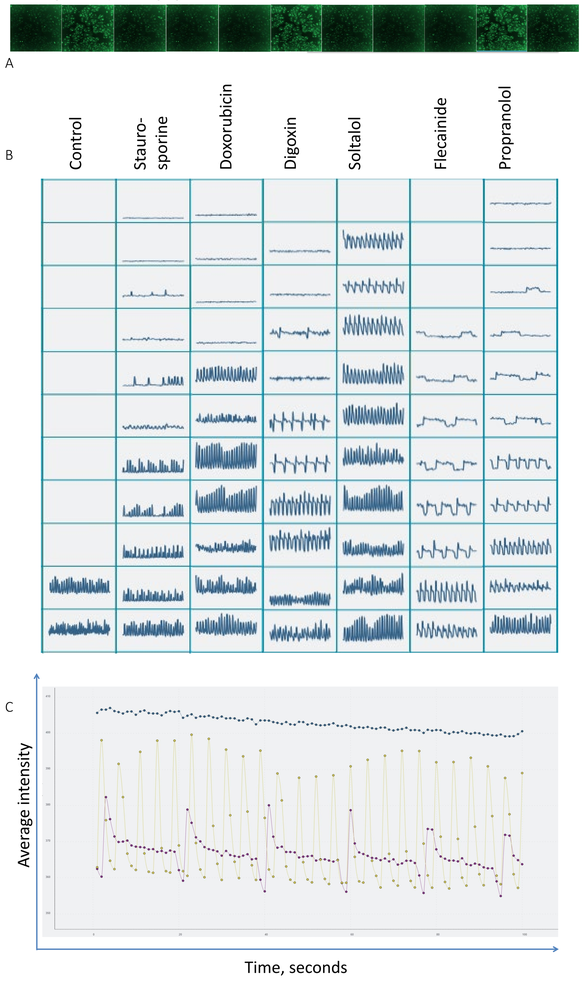


Figure 1. To measure Ca oscillations, iPSC cardiomyocytes were loaded with EarlyTox Calcium dye then imaged using the ImageXpress Pico system in the FITC channel using a time-lapse mode. A. The panel above presents a series of images taken with 0.5 sec interval. B. Traces of calcium oscillations shown 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).

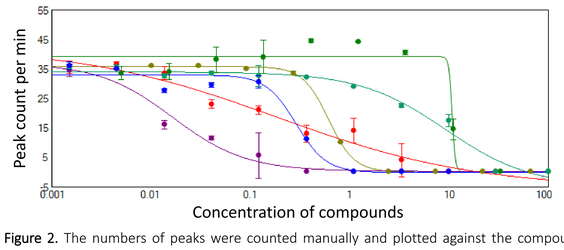
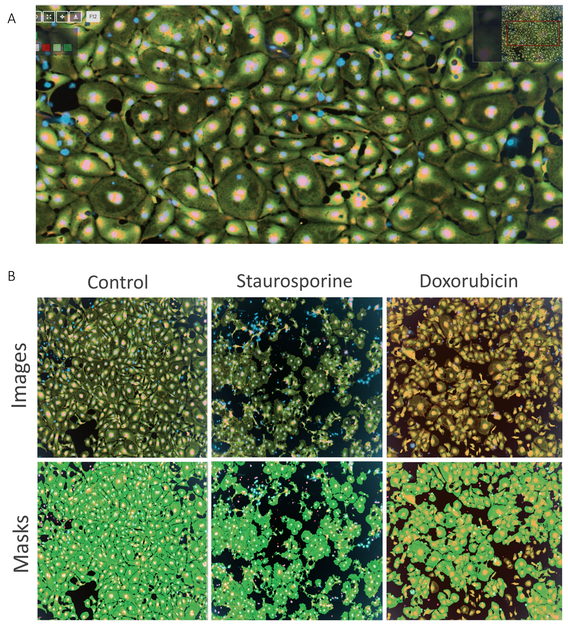


Figure 2. The numbers of peaks were counted manually and plotted against the compound concentrations using 4-parametric curve fit. EC_{50} values were calculated using SoftMax® Pro Software. Dose-dependent decreases in peak count shown for cisapride (purple), haloperidol (red), staurosporine (blue), imatinib (yellow), doxorubicin (dark green), flecainide acetate (green).

Assessing Compound Effects on Cell Viability and Morphology

While the evaluation of changes in beating profiles is very important for detection of functional effects, high content imaging is an essential complimentary assay for monitoring morphological changes and cytotoxicity effects of compounds. Imaging and analysis methods provide efficient tools for characterization of multiple readouts including kinetics of calcium oscillations, cell viability, characterization of cell shape, cell adhesion and spreading, cytoskeleton integrity (morphology), and mitochondria potential.



C

EC50, µM	Peak rate	*Number of live cells	Total area of live cells	Average intencity (Calcein AM)
Cisapride	0.017	0.22	0.13	0.15
Propranolol	0.038	**		
Rotenone	0.1	0.2	0.1	0.1
Haloperidol	0.19			
Staurosporine	0.39	3.1	1.2	1.5
Digoxin	0.61	10	9	11.2
Imatinib	0.62	2.1	0.83	0.81
Flecainid acetate	2.7			
Sotalol	3.4			
Doxorubicin	16	10	12	5.8

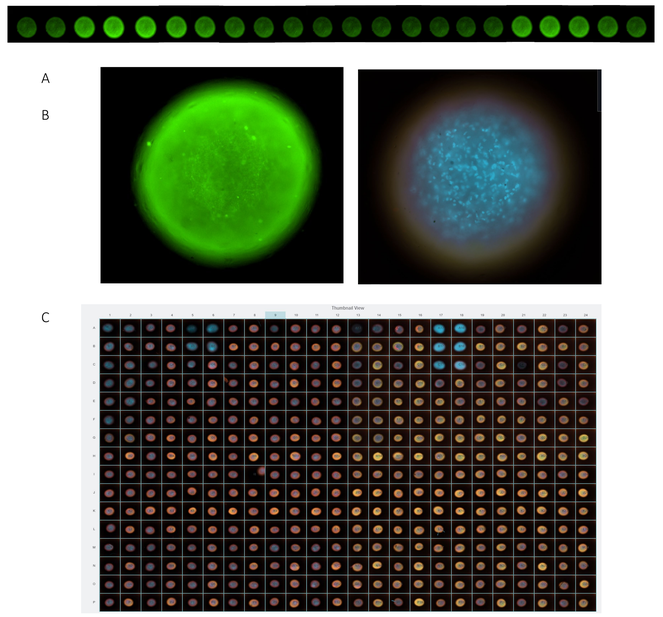
* Numbers of live cells (positive for Calcein AM stain), also the total area covered by live cells, and average intensities of Calcein AM stain were measured using image analysis
** Blank cells indicate no effect at highest concentration tested (50µM)

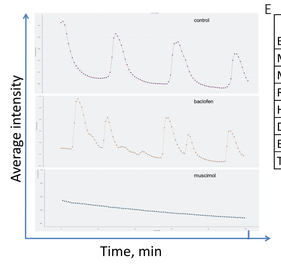
Figure 2. A. Images of cardiomyoctes (20x magnification) stained with viability dye Calcein AM, Mitotracker Orange dye for detection of mitochondria with intact membrane potential, and Hoechst nuclear dye (all from Thermo Fisher Scientific, 0.5 µM, 0.2 µM, and 0.5 µM respectively). B. Images and the analysis masks for multi-parametric analysis. I-Cell derived cardiomyocytes were treated with compounds for 24 hours and then stained with a nuclear stain (Hoechst 33342), Calcein AM, and MitoTracker Orange CMTMRos. Images and analysis masks were compared for control cells and cells treated with 0.1 µM staurosporine and 10 µM doxorubicin. Cells were imaged with DAPI, FITC, and TRITC using a 10x Plan Fluor objective. The images show nuclei (blue), Calcein AM stain (green), mitochondria (orange). Images were analyzed using the Cell Scoring analysis modules optimized for quantitation of Calcein AM positive cells or MitoTracker Orange positive cells. The analysis masks: light green - positive nuclei, red- negative nuclei, green-actin cytoskeleton. C. EC_{50} values for indicated measurements defined from concentration –dependence curves presented in the table.

Notably, most compounds demonstrated cardiac specific toxicity, with IC_{50} value concentrations for inhibition of beating rate ½ log or lower than concentrations for cytotoxic effects measured by a decrease in numbers of Calcein AM positive cells. In contrast, the cytotoxic effect of doxorubicin was observed at a similar concentration as the inhibition of the beating rate.

Assessing Compound Effects on Neuro-spheroids

To evaluate cytotoxicity effects, microBrain® 3D cells from StemoniX Inc were treated with various neurotoxic compounds for 24 hours. For evaluation of calcium oscillations cells were loaded with calcium 6 dye. Calcium oscillations were visualized using time-lapse imaging, then images were analyzed with Cell Count analysis using entire spheroid as a "cell" object. Average intensities were then plotted against time that allowed to visualize the patterns of calcium oscillations. For cytotoxicity assessment cells were stained with Hoechst nuclear stain, MitoTracker Orange dye, and Calcein AM for detection of cell viability.





E

EC50, µM	Peak rate	*Number of live cells	Average intencity
Methyl Mercury	3.5	32	18
Muscimol	0.02	**	
Rotenone	0.03		
Haloperidol	0.1		
Deltamethrin	1.2	26	13
Baclofen	0.6		
Tetrodotoxin	0.02		

Figure 3. A. Time-lapse string of images shown for neurospheroids. To measure Ca oscillations, iPSC-derived neurospheroids (StemoniX) were loaded with Calcium 6 dye then imaged using ImageXpress Pico system in the FITC channel using a time-lapse mode. Images shown were taken with 1 sec interval. B. Images shown for spheroid stained with Calcium 6 dye (Molecular Devices, stained for 2h, left); or the combination of 3 viability dyes: Hoechst, Calcein AM, and MitoTracker Orange as indicated below (right). C. For cytotoxicity assessment spheroids were stained for 2h with a combination of Hoechst (2 µM), MitoTracker Orange (0.2 µM), and Calcein AM (2 µM, all from Thermo Fisher Scientific). Images were taken with the ImageXpress Pico system, using a 10x Plan Fluor objective, and the DAPI, TRITC, and FITC channels. Images were processed using the Cell Scoring analysis algorithm. Decreases of Calcein AM or MitoTracker stains were observed for neurospheroids treated with various compounds. D. Traces of calcium oscillations shown for control (0.1% DMSO), baclophen (1 µM), and muscimol (1 µM). E. EC_{50} values for indicated measurements defined from concentration –dependence curves are presented in the table.

SUMMARY

- We presented methods for complex multi-parametric assays utilizing iPSC-derived human cardiomyocytes and neurospheroids
- Multiple readouts allows characterization of phenotypic effects of different compounds and also assessment of effective concentrations
- The study demonstrates the breadth of imaging assays possible using the ImageXpress Pico system and CellReporterXpress software for evaluation of various biological effects

MOLECULAR DEVICES