Predictive Assays for High Throughput Assessment of Cardiac Toxicity and Drug Safety

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Introduction

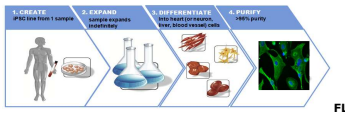
A large percentage of new drugs fail in clinical studies due to cardiac toxicity. Therefore development of highly predicative in vitro assays suitable for high throughput screening (HTS) is extremely important for drug development. Human cardiomyocytes derived from stem cell sources can greatly accelerate the development of new chemical entities and improve drug safety by offering more clinically relevant cell-based models than those presently available. Stem cell derived cardiomyocytes are especially attractive because they express ion channels and demonstrate spontaneous mechanical and electrical activity similar to native cardiac cells. Here we demonstrate cell based assays for measuring the impact of pharmacological compounds on the rate of beating cardiomyocytes with different assay platforms. Cardioactive compounds are used in clinical treatment of heart failure, arrhythmia or other cardiac diseases. Cardiac toxicity can cause arrhythmias or heart failure. We have shown dose-dependent atypical patterns caused by several cardiotoxic compounds and ion channel blockers. Also we have demonstrated effects of several positive (epinephrine, etc.) and negative (α and β blockers) chronotropic agents on cardiac rates and determined EC50s.

We developed methods for the ImageXpress® Micro and the FLIPR® Tetra systems that enable determination of beating rate from changes in intracellular Ca^{2+} that are synchronous with beating. The protocols use induced pluripotent stem cell (iPSC) derived cardiomyocytes loaded with a calcium sensitive dye and allow monitoring of drug impact on the beat rate and amplitude in 96 or 384 well formats. Peak parameters are automatically measured using the Screenworks® Peak Pro™ software and proprietary analysis algorithms. We have demonstrated use of both systems for two important applications: screening compounds for cardiac toxicity, and pre-clinical testing of potential cardiac drug candidates. These methods are well suited for safety testing and can be used to estimate efficacy and dosing of drug candidates prior to clinical studies.

Methods

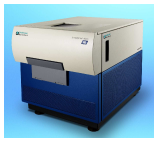
Cell Preparation

- iCell® Cardiomyocytes were received frozen from Cellular Dynamics International (CDI). Cells were thawed and plated according to recommended protocol.
- Cardiomyocytes were plated 20K/96well plate or 4 K/384 well on gelatin coated plates and incubated for 3-5 days. The presence of spontaneous contractions indicated that the cells had appropriately matured.



(figure courtesy of Cellular Dynamics International)

Figure 1. Steps in creation of cardiomyocytes and other cell types from iPS cells.



ImageXpress® Micro XL Automated Imaging System

High Content Imaging

- Image acquisition was done on the ImageXpress Micro XL System using time-lapse images at 20x or 10x magnification using FITC excitation and emission filters.
- Data was acquired at up to 100 frames per second (fps)
- Cells were kept under environmental control (37C, 5% CO_2).

FLIPR® Tetra Cellular Screening System

FLIPR Tetra System Ca^{2+} Flux Assay

- Reagents from FLIPR® Calcium 5 Assay Kit (Molecular Devices) were added to the plates and incubated 1 hour at 37C in 5% CO_2.
- Compound plates were pre-warmed to 37C inside the FLIPR Tetra instrument and compound addition was done simultaneously to all wells
- Experimental plates were loaded and a pre-drug read was acquired of changes in intracellular Ca^{2+} concentration.
  - Acquisition was set up for Calcium 5 (Ex 485nm, Em 530nm)
  - Data was acquired at ~8 fps
- FLIPR system reads were acquired during compound addition and at prescribed times after addition (read time ~ 2 min)

References

1. Functional cardiomyocytes derived from human induced pluripotent stem cells.
Zhang J, Wilson G, F Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. Circ Res. 2009 Feb 27;104(4):e30-41.
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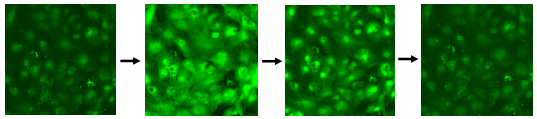
Imaging & Analysis of Beating Cardiomyocytes

Cardiac toxicity is a serious drug safety concern because it can cause arrhythmias or heart failure. We have developed methods for the ImageXpress Micro system that enables image acquisition and determination of beating rate of live cardiomyocytes from a series of time-lapse images. Beating cardiomyocytes were loaded with the Calcium 5 reagents and imaged using a 10X objective at up to 100 frames per second (fps) to allow visualization of Ca^{2+} fluxes that occur synchronous with beating. The total fluorescence intensity was integrated from each frame and then plotted as a function of time. The number of peaks within each plot were counted and used to determine a beat rate. The system allows saving data as a video, presenting intensity curves, and automatic analysis of beat rates.

Visualizing beating cardiomyocytes by Ca^{2+} fluxes



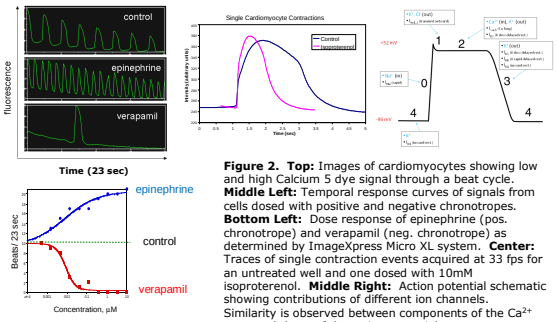


Figure 2. Top: Images of cardiomyocytes showing low and high Calcium 5 dye signal through a beat cycle. **Middle Left:** Temporal response curves of signals from cells dosed with positive and negative chronotropes. **Bottom Left:** Dose response of epinephrine (pos. chronotrope) and verapamil (neg. chronotrope) as determined by ImageXpress Micro XL system. **Center:** Traces of single contraction events acquired at 33 fps for an untreated well and one dosed with 10mM isoproterenol. **Middle Right:** Action potential schematic showing contributions of different ion channels. Similarity is observed between components of the Ca^{2+} trace and those of the action potential.

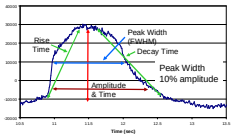
Screenworks® Peak Pro™ Software Automated Peak Analysis

Automated data analysis is required in order to make the FLIPR Tetra system practical for running assays on a large numbers of compounds. Manual counting of 384 traces is time consuming and subject to human error. Exporting the data to a separate program in order to analyze the traces removes subjectivity, but adds additional steps that compromises the usefulness for environments that require true automation and high throughput such as screening labs.

An automated peak detection and analysis algorithm was added to the Screenworks software to process the data and provide user selectable outputs.

First, signal peaks are detected based on a dynamic thresholding and derivative analysis. The peaks are then fit with a binomial function to provide amplitude, time, and width values. The data analysis occurs immediately after acquisition and result are presented on a well-by-well basis to the user. The results can be exported to a standard comma-separated-variable (csv) file for further analysis.

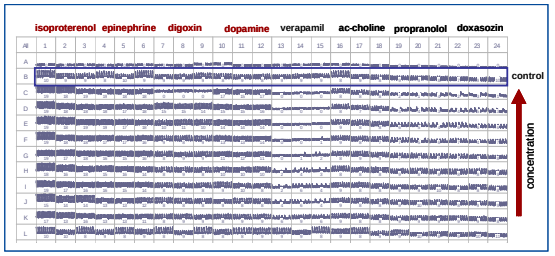
Output Parameters Available from Screenworks Peak Pro Software



- Peak Count & Frequency
- Peak Position (time) and Amplitude
- Peak Width (FWHM)
- Rise Time (10% to 90%)
- Decay Time (90% to 10%)

High Throughput FLIPR® Tetra System Cardiac Beating Assay

A method complementary to imaging uses the FLIPR Tetra system to monitor changes in intracellular Ca^{2+} fluxes associated with cardiomyocyte contractions using the FLIPR Calcium 5 Assay Kit. The FLIPR system allows automatic addition of reagents and compounds simultaneous with reading from 96, 384, or 1536 wells. This has been found to be advantageous for the cardiac beating assays because it reduces well-to-well variability caused by reading at different time points. The absolute beat rates were found to be similar to those measured by imaging methods. Temporal response curves for analysis and visualization of beating can be acquired in ~ 2 min per plate making this assay suitable for high throughput screening of compound libraries.



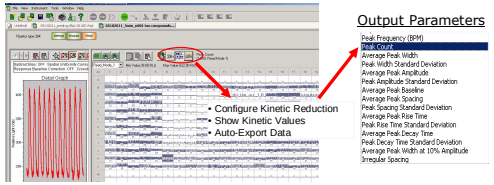
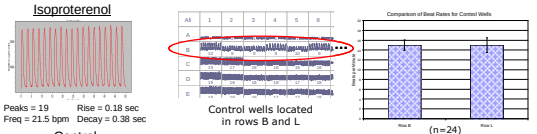


Figure 3. Top: ScreenWorks Peak Pro Software Output for 384 Well Plate 10 Minutes After Dose. **Bottom:** Output parameters available to the user.

FLIPR Tetra System Assay Capabilities



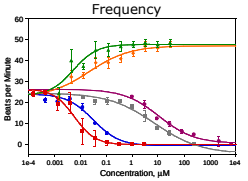
Statistics	Exp1 24 wells			Exp2 200 wells			Exp3 24 wells		
	average	stdev	% stdev	average	stdev	% stdev	average	stdev	% stdev
Peak Count	8.8	0.7	8.2	8.5	1.4	16.6	15.9	2.5	15.8
Average Peak Width	1.0	0.2	20.9	2.3	0.3	12.0	3.0	0.4	14.5
Average Peak Amplitude	18.0	5.0	27.7	2249.3	530.3	23.6	195.1	31.8	16.3
Average Peak Baseline	59.4	5.5	9.2	2805.6	199.7	7.1	265.1	23.4	8.8
Average Peak Spacing	4.1	0.3	8.3	6.9	1.0	15.1	6.4	1.0	16.4
Average Peak Rise Time	0.4	0.1	12.7	0.4	0.1	20.2	0.8	0.1	15.6
Average Peak Decay Time	1.0	0.1	13.5	1.4	0.2	16.6	1.0	0.1	13.5
Average Peak Width at 10% Amplitude	1.7	0.2	9.1	2.7	0.4	15.0	3.6	0.5	14.7

Figure 4. Left: Cardiomyocyte contraction parameters calculated by Screenworks Peak Pro software for three different wells. **Top:** Typical reproducibility of Control Wells (CVs <= 10%) **Middle:** Statistics for various parameters measured for control wells for three different experiments.

Predictive HTS Cell-Based Assay

Development of new, more potent and safer drugs requires an in vitro system where efficacy and safety can be tested. Positive and negative inotropes are used in clinics to treat heart failure, tachycardia, arrhythmia or other cardiac diseases. We have demonstrated effects of several positive (isoproterenol, dopamine, etc.) and negative (α– and β–blockers) chronotropes on cardiac rates and determined EC50s at the expected ranges. Image based assays using calcium flux and iPSC derived cardiomyocytes are suitable for this task and could be used to estimate efficacy and approximate dosing prior to pre-clinical studies. We have also examined effects of known ion channel blockers (Na^+ and hERG) and found that they have a negative effect on beat rate and prolong repolarization times. Results from assays run on the FLIPR Tetra system are shown below.

Effects of β-Adrenergic Receptor (AR) Agonists/Antagonists and Ion Channel Blockers



	IC50 (µM)
Epinephrine – β-AR agonist	0.005
Isoproterenol – β-AR agonist	0.55
Propranolol – β-AR antagonist	7.6
Lidocaine – Na^+ channel blocker	11.3
Cisapride – hERG Channel Blocker	0.023
Astemizole – hERG Channel Blocker	0.006

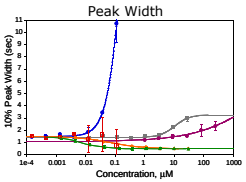


Figure 5. Dose response of six different compounds as measured by the FLIPR Tetra system in Calcium 5 loaded iPSC derived cardiomyocytes. **Top:** Change in frequency of contractions with dose. **Bottom:** Change in average peak width with dose. Positive chronotropes were found to increase beat rate and decrease peak widths as expected. Ion channel blockers were found to decrease beat rate. The hERG channel blocker cisapride was found to have a significant effect on repolarization times as indicated by an increase in peak width.

Summary

- We demonstrate live-cell assays for measuring the impact of pharmacological compounds on the rate and magnitude of beating cardiomyocytes using ImageXpress Micro Automated Microscope for high content imaging and FLIPR Tetra Cellular Screening System. Intracellular Ca^{2+} transient fluxes underlying cell contractions were monitored by using FLIPR Calcium 5 Assay Kit readout.
- Peak parameters were automatically measured on the FLIPR Tetra System using the Screenworks Peak Pro software and proprietary analysis algorithms. The easy-to-use interface and intuitive setup provide fast and reproducible results that agree well with manual measurements.
- We demonstrate applications of these assays for prospective toxicity screening using iPS-derived cardiomyocytes by measuring the impact of various chronotropes and ion channel blockers on the beating rate and Ca^{2+} transient fluxes. Tested reagents modulated the frequency of beating in line with their mode-of-action showing the functional expression of ß-adrenergic and acetylcholine receptors.

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