# High-throughput assessment of compound-induced pro-arrhythmic effects in human iPSC-derived cardiomyocytes

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## Overview

Development of biologically relevant and predictive cell-based assays for compound screening and toxicity assessment is a major challenge in drug discovery. The focus of this study was to establish high-throughput, compatible cardiotoxicity assays using human induced pluripotent stem cell (iPSC)-derived cardiomyocytes. Using human iPSC-derived cardiomyocytes as an *in vitro* model, we evaluated the responses and concentration dependence to 28 drugs linked to low, intermediate, and high torsades de pointes (TdP) risk categories. The impact of various compounds on the beating rates and patterns of cardiomyocyte spontaneous activity was monitored by changes in intracellular $Ca^{2+}$ oscillations measured by fast kinetic fluorescence with calcium-sensitive dyes.

We describe a method for the complex analysis that allows detection and multi-parametric characterization of calcium oscillations. The method allows characterization of complex patterns, secondary peaks, waveform irregularities, and more than 20 other important readouts. We characterized the concentration-dependent effects of 28 compounds on different readouts and demonstrated that the presence of EAD-like events, peak prolongations, and pattern irregularities detectable in the assay were comparable with similar Cmax concentrations found in blood. They can be used as a strong predictive indicator of cardiac arrhythmia *in vivo*.

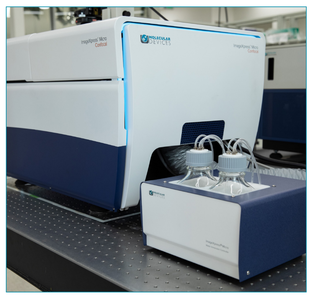
In addition, cellular and mitochondrial toxicities were evaluated by imaging methods. Alpha-actinin patterns were characterized by using high-content imaging with water immersion objectives. Image analysis identifying numbers and length of patterns (fibers, segments) was used to characterize the patterns and effect of tested compounds on cytoskeleton arrangements.

## Calcium oscillations evaluated by the FLIPR Penta system

The iPSC-derived cardiomyocytes generate spontaneous synchronized calcium oscillations. We used high speed fluorescence imaging on the FLIPR® Penta High-Throughput Cellular Screening System to measure the patterns and frequencies of the $Ca^{2+}$ oscillations in cardiomyocytes as monitored by changes in intracellular $Ca^{2+}$ levels with the EarlyTox™ Cardiotoxicity Kit (Molecular Devices). A set of 28 known cardiotoxic compounds, plus several benchmark compounds and negative controls, were tested in the assay.

## Materials and methods

We used a high speed EMCCD camera on the FLIPR Penta system to measure the patterns and frequencies of the $Ca^{2+}$ oscillations of neuro-spheroids as monitored by changes in intracellular $Ca^{2+}$ levels with the EarlyTox Cardiotoxicity Kit. The instrument equipped with new ScreenWorks® Peak Pro 2 software allows analysis and characterization of the primary and secondary peaks and complex oscillation patterns.



Water immersion option for the ImageXpress® Micro Confocal High-Content Imaging System

FLIPR Penta system with high speed EMCCD camera and ScreenWorks Peak Pro 2 software

**iPSC-derived cardiomyocytes:** Cryopreserved human iPSC-derived cells from Fujifilm Cellular Dynamics International (CDI), iCell® Cardiomyocytes$^2$, were used for experiments. Cells were thawed and plated at 20,000/well (96-well format) or 10,000/well into 384-well format plates (Corning) and incubated for seven days in maintenance media. The presence of strong synchronous contractions in the 3D cultures was confirmed visually prior to running experiments.

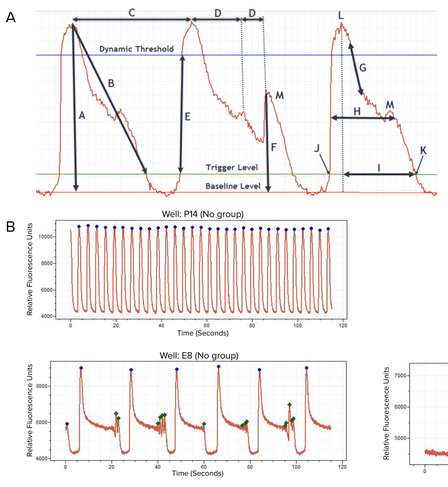
**Calcium oscillation assay:** The intracellular $Ca^{2+}$ oscillations were assessed using the EarlyTox Calcium dye (Molecular Devices) according to regular protocol; cells were loaded with dye for two hours before measurements.

**Cell staining:** To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), the mitochondrial membrane potential dye MitoTracker Orange (0.2 µM), and the Hoechst nuclear dye (2 µM) (all from Thermo Fisher Scientific). Cytoskeleton proteins alpha-actinin and troponin were visualized by using appropriate antibodies (Sigma, 1:100 dilutions).

## Results

### Recording and analysis of kinetic patterns

iCell Cardiomyocytes$^2$ were loaded with EarlyTox Cardiotoxicity Kit and treated with compounds for 15, 30, 60, 90 min, and 24 hours. Cell viability was assessed at 24-hour endpoint. Spontaneous calcium oscillations were recorded using 30–50 frames per second that allowed resolution of the complex oscillation patterns. Advanced analysis methods implemented to provide multiparametric characterization of the $Ca^{2+}$ flux oscillation patterns. This phenotypic assay allows for the characterization of readouts such as oscillation frequency, amplitude, peak width, peak raise and decay times, and irregularity. In addition, the appearance of EAD-like (early-depolarization like event) patterns, peak prolongation, and peak irregularity were evaluated. The effects of cardiotoxic compounds on cardiac activity were evaluated by several measurements.

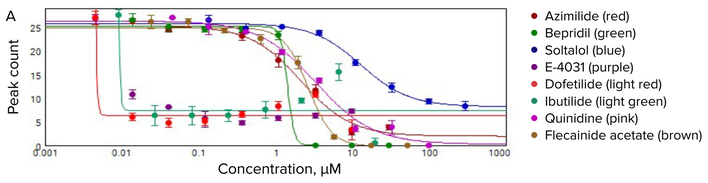



Descriptors:
A. Main peak amplitude
B. Linear decay slope
C. Main peak interval
D. Early-after depolarization-like event (EAD)
E. Rise slope
F. EAD-like event amplitude
G. Decay slope
H. Calcium transient duration (CTD)
I. Calcium transient duration from peak position
J. Start of event
K. End of event
L. Main peak
M. Peak count
N. Irregularity of peak frequency

**Figure 1.** ScreenWorks Peak Pro 2 software equipped with additional tools for peak analysis using >20 descriptors for observed phenotypic changes (A). The intracellular $Ca^{2+}$ oscillations were assessed using the EarlyTox Cardiotoxicity Kit. Trace examples shown for control pattern, E-4031, dofetilide (B).

### Concentration response evaluation of compound effects

Multiparametric analysis was used for evaluation of compound effects. Several readouts like peak count, peak frequency, amplitude, and spacing showed concentration-dependent monotonous response; and $EC_{50s}$ were calculated. Other readouts like peak width, rise, and decay times, EAD-like events (secondary peaks) were nonmonotonous, but effective concentrations can be calculated by benchmark concentrations or other methods.




- Azimilide (red)
- Bepridil (green)
- Soltalol (blue)
- E-4031 (purple)
- Dofetilide (light red)
- Ibutilide (light green)
- Quinidine (pink)
- Flecainide acetate (brown)

B

Toxicity Assessment Using iCell Cardiomyocytes$^2$, CDI

| High TdP risk | Peak count | Amplitude | Peak width increase | Viability decrease | Alpha- Actinin |
|---|---|---|---|---|---|
| Azimilide | 2.1 | 15.3 | 5.9 | 30* | 30* |
| Bepridil | 1.4 | 1.3 | ^0.1 | 30* | 30* |
| Sotalol | 13.3 | ^100 | 25.4 | no effect | no effect |
| E-4031 (+ cont) | <0.01 | 0.011 | 0.012 | no effect | no effect |
| Dofetilide | 0.004 | ^0.3 | 0.1 | no effect | no effect |
| Ibutilide | 0.007 | ^0.6 | 0.1 | no effect | 0.01 |
| Quinidine | 3.36 | ^30 | 10 | 7 | 6.4 |
| Vandetanib | 9.73 | 9.64 | ^3 | 30* | no effect |
| Haloperidol (+ cont) | <0.1 | 0.69 | 0.1 | 0.1 | 10* |
| Flecainide (+ cont) | 2.64 | 7.1 | 5 | 5 | no effect |
| Amoxicillin (- cont) | no effect | no effect | no effect | no effect | no effect |
| Biotin (- cont) | no effect | no effect | no effect | no effect | no effect |
| Intermediate TdP risk | Peak count | Amplitude | Peak width increase | Viability decrease | Alpha- Actinin |
| Astemizole | <0.1 | 0.65 | 0.1 | 10* | 1.1 |
| Lidocaine (+ cont) | 19.1 | 19.5 | no effect | no effect | no effect |
| Cisapride | <0.1 | 10 | ^1 | no effect | no effect |
| Clarithromycin | 38 | ^100 | ^100 | 120* | no effect |
| Clozapine | 5.2 | 2.6 | no effect | no effect | 20* |
| Domperidone | <0.1 | 5 | ^1 | no effect | no effect |
| Droperidol | 28 | >50 | 2.7 | no effect | no effect |
| Ondansetron | 6.1 | >100 | 3.3 | no effect | no effect |
| Pimozide | 0.48 | 0.7 | 1.1 | no effect | no effect |
| Risperidone | 0.2 | 3 | ^10 | no effect | no effect |
| Terfenadine | 0.54 | ^0.6 | no effect | 0.61 | 0.43 |
| Acetaminophen (- cont) | no effect | no effect | no effect | no effect | 100* |
| Low TdP risks | Peak count | Amplitude | Peak width increase | Viability decrease | Alpha- Actinin |
| Diltiazem | 0.7 | 0.068 | decrease | no effect | no effect |
| Loratadine | >10 | 23.8 | decrease | no effect | no effect |
| Metoprolol | no effect | no effect | no effect | no effect | 208 (increase) |
| Mexiletine | 4.7 | 4.89 | decrease | no effect | no effect |
| Nifedipine | 0.1 | 0.021 | decrease | no effect | 0.038 |
| Nitrendipine | 0.1 | 0.003 | decrease | no effect | no effect |
| Ranolazine | no effect | >100 | decrease | no effect | no effect |
| Tamoxifen | 1.3 | 3.7 | decrease | 80* | ^20 |
| Verapamil | 0.1 | 0.07 | decrease | no effect | no effect |
| Isoproterenol (+ cont) | 0.1 | 3 | decrease | no effect | no effect |
| cloropromazin (+ cont) | 0.1 | 0.007 | decrease | 3* | 1.16 |
| DMSO | no effect | no effect | no effect | no effect | no effect |

**Figure 2.** (A).The patterns of intracellular $Ca^{2+}$ oscillations were assessed using ScreenWorks Peak Pro 2 software. Concentration-dependent decrease of oscillation rate shown for high TdP risk group, 30 min treatment. (B). $EC_{50}$ values for indicated readouts summarized in the table. Oscillation readouts for 30 min, viability for 24 hours. Asterisk means that effect was observed only at highest concentration tested (indicated).

## Results

### Assessment of cell viability by high-content imaging

Confocal imaging and image analysis methods were used to characterize compound effects on viability and integrity of cytoskeleton in cardiomyocytes. To evaluate cytotoxicity effects, cells were imaged after treatment with compounds for 24 hours using the ImageXpress Micro Confocal system. Images were analyzed using a cell scoring algorithm for detection of cell numbers for all cells, live cells (Calcein AM positive cells), and cells with intact mitochondria (MitoTracker Orange positive cells).

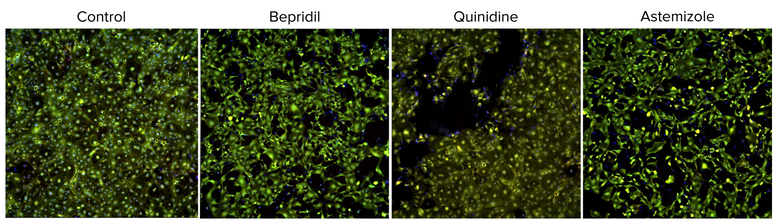



**Figure 3.** Composite images of cardiomyocytes treated with 10 µM of indicated compounds for 24 hours, then stained with a nuclear stain (Hoechst 33342, blue), viability stain (Calcein AM, green), and mitochondrial membrane potential dye MitoTracker Orange CMTMRos (red) for two hours (2 µM, 1 µM, and 0.5 µM respectively). Cells were imaged with the DAPI, FITC, TRITC, and 10X Plan Fluor objective. Cytotoxicity was observed after 24 hours for the following compounds at concentrations of 10 µM or greater: azimilide, bepridil, quinidine, vandetanib, astemizole, clarithromycin, terfenadine, and tamoxifen.

### Assessment of cytoskeleton arrangements

We have also characterized compound effects on integrity of cytoskeleton in cardiomyocytes. After 24 hours of compound treatment, cells were fixed and stained with anti alpha-actinin antibodies. The patterns of alpha actinin were visualized by 40X or 60X magnification using water immersion objectives. While control cells showed extensive patterns of parallel structures of alpha-actinin, some compounds resulted in cell damage and disappearance of the pattern. Interestingly, decrease of intensity of cytoskeleton pattern was observed with several compounds that have not shown changes in viability at tested concentrations. To quantitate changes in cytoskeleton, images were analyzed using custom module editor using “fibers” finding feature. Readouts like fibers, segments, and fiber length sum were used to quantitate dose responses and $EC_{50}$ values.

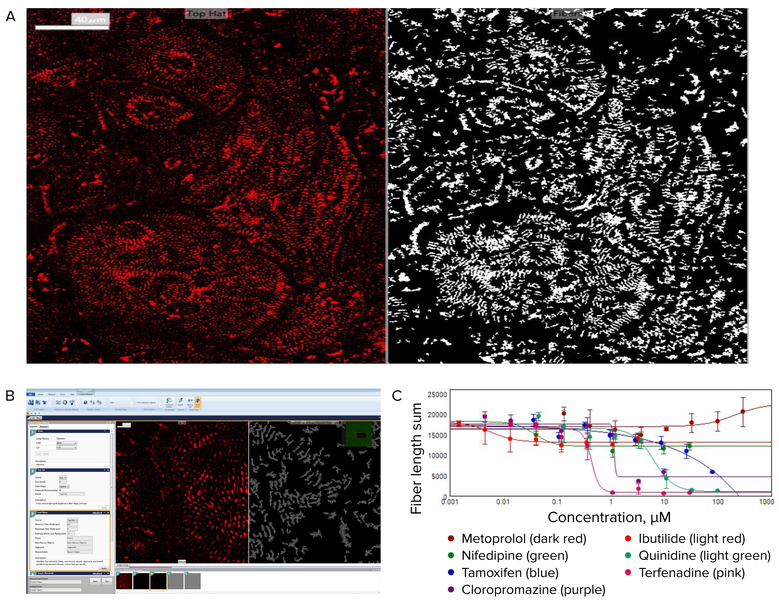



**Figure 4.** Cardiomyocytes were stained with anti alpha-actinin antibodies and imaged with 40X water immersion objectives (A). Images were sharpened by using the Top Hat feature, then fibers and segments were found by “find fibers” algorithm (B). Numbers of fibers and segments as well as their length sum demonstrated dose-dependent decrease and $EC_{50s}$ were quantitated for compound effects (C).

## Conclusion

- We presented methods for high-throughput evaluation of cardiotoxicity effects of various compounds by using the FLIPR Penta system and the ScreenWorks Peak Pro 2 software
- We have also developed methods for cytotoxicity evaluation and changes in cytoskeleton by using high-content imaging
- Multiparametric assessment of various phenotypic effects allows better evaluation of potential toxicity and provides additional information about potential mechanisms of toxicity