Measuring Cardiac Activity:

Intracellular Calcium Flux Detection on the FLIPR Tetra System

Introduction

iCell® Cardiomyocytes are human cardiomyocytes that recapitulate the electrophysiological, biochemical, mechanical, and pathophysiological characteristics of native human cardiac myocytes. Their derivation from human induced pluripotent stem cells, high purity, functional relevance, and ease of use, make iCell Cardiomyocytes an optimal in vitro test system for interrogating cardiac biology in basic research and many areas of drug development.

The FLIPR Tetra High Throughput Cellular Screening System (FLIPR Tetra System) is a kinetic plate-based, cellular assay screening system that utilizes calcium fluorescent dyes to measure calcium flux and detect cardiomyocyte beating activity (1, 2). iCell Cardiomyocytes can be cultured on 96- or 384-well plates where they form a stable, electrically and mechanically active syncytium amenable to measuring drug-induced perturbations. Together, iCell Cardiomyocytes and the FLIPR Tetra System offer a high-throughput platform for in vitro screening of compound efficacy and toxicity in human cardiac myocytes.

This Application Protocol describes how to handle iCell Cardiomyocytes for use on the FLIPR Tetra System and provides basic instructions for compound treatment, data acquisition, and analysis.

Required Equipment, Consumables, and Software

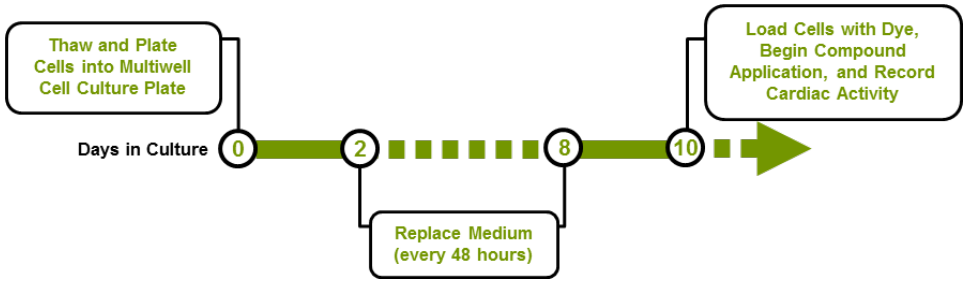
The following equipment, consumables, and software are required in addition to the materials specified in the iCell Cardiomyocytes User's Guide.

Item	Vendor	Catalog Number
Equipment		
FLIPR Tetra High Throughput Cellular Screening System (FLIPR Tetra System)	Molecular Devices	
Consumables		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
EarlyTox Cardiotoxicity Kit	Molecular Devices	R8210
Flat Clear Bottom Black Polystyrene TC-treated Microplate (Cell Culture Plate)*	Multiple Vendors	
FLIPR Tetra System Pipette Tips, Black, Non-sterile (Pipette Tips)*	Molecular Devices	9000-0762 (96-well) 9000-0764 (384-well)
Software		
ScreenWorks Peak Pro	Molecular Devices	

* Order the format compatible with the pipette head of the FLIPR Tetra System.

Workflow

iCell Cardiomyocytes are thawed and plated into either a 96- or 384-well cell culture plate previously coated with gelatin. On days 2, 4, 6, and 8 post-plating, spent medium is replaced with iCell Cardiomyocytes Maintenance Medium (Maintenance Medium). From days 10 - 14 post-plating, cardiomyocytes can be loaded with calcium fluorescent dye, treated with compounds, and assayed for cardiac beating activity.



Methods

The following procedure describes culturing iCell Cardiomyocytes in 96-well cell culture plates. Instructions for 384-well format are provided in the notes.

Thawing iCell Cardiomyocytes

1. Coat a 96-well cell culture plate with 100 µl/well of 0.1% gelatin solution at 37°C for at least 1 hour according to the iCell Cardiomyocytes User's Guide.

   Note: *For 384-well format, coat with 25 µl/well of 0.1% gelatin solution*.

2. Thaw iCell Cardiomyocytes according to the User's Guide. Dilute the cardiomyocyte suspension in iCell Cardiomyocytes Plating Medium (Plating Medium) to 200,000 plated cardiomyocytes/ml. See the User's Guide for instructions to calculate the *Target Plating Density* based on *Plating Efficiency*.

   Note: *For 384-well format, dilute the cardiomyocyte suspension to 125,000 plated cardiomyocytes/ml.*

3. Aspirate the gelatin solution. Immediately add 100 µl/well of the cardiomyocyte suspension (20,000 plated cardiomyocytes/well).

   Note: *For 384-well format, add 40 µl/well of the cardiomyocyte suspension (5,000 plated cardiomyocytes/well).*

4. Culture iCell Cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2 for 48 hours.

   Note: *iCell Cardiomyocytes culture has been performed at both 5% and 7% CO_2 with no functional impact detected.*

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Maintaining iCell Cardiomyocytes

1. On day 2 post-plating, aspirate the spent Plating Medium and replace with 100 µl/well of iCell Cardiomyocytes Maintenance Medium (Maintenance Medium).

   Note: *For 384-well format, replace with 25 µl/well of Maintenance Medium.*

2. Maintain the cardiomyocytes for at least 10 days, replacing medium every 48 hours.

Data Acquisition and Analysis

The following section details loading cardiomyocytes with Ca^{2+}-sensitive dye, data acquisition, and basic data analysis. Cardiac beating activity typically stabilizes approximately 10 - 14 days post-plating. Be aware that iCell Cardiomyocytes beat rate is highly temperature-sensitive. Avoid temperature fluctuations by maintaining the cell culture plate containing iCell Cardiomyocytes and the plate containing test compounds dilutions (compound plate) at 37°C throughout the assay.

ScreenWorks Peak Pro Software provides a variety of parameters to measure and characterize the calcium oscillations of iCell Cardiomyocytes. See the manufacturer's instructions for specific guidelines on using ScreenWorks Peak Pro Software for data acquisition and analysis.

Adding the Dye and Applying Compounds

1. Prepare the Cardiotox Dye loading solution according to the manufacturer's instructions.

   Note: *The Cardiotox Dye is provided in the EarlyTox Cardiotoxicity Kit.*

2. Remove the 96-well cell culture plate containing iCell Cardiomyocytes from the cell culture incubator and replace the spent medium with 100 µl/well of fresh Maintenance Medium.

   Note: *For 384-well format, replace with 25 µl/well of fresh Maintenance Medium.*

3. Add an equal volume (100 µl/well) of Cardiotox Dye loading solution to each well of the 96-well cell culture plate to reach a final volume of 200 µl/well.

   Note: *For 384-well format, add 25 µl/well of Cardiotox Dye loading solution to reach a final volume of 50 µl/well.*

4. Incubate the cell culture plate containing iCell Cardiomyocytes and Cardiotox Dye loading solution (assay plate) in a cell culture incubator at 37°C, 5% CO_2 for 2 hours. Do not remove the plate from the incubator until ready to transfer to the FLIPR Tetra System deck equilibrated to 37°C for recording.

5. Equilibrate the FLIPR Tetra System deck to 37°C.

6. Prepare test compound dilutions in Maintenance Medium at 5X the final concentration in a clean 96-well plate (compound plate). Incubate the compound plate at 37°C until ready to apply compounds onto iCell Cardiomyocytes.

   Note: *Final DMSO concentrations above 0.1% should be used with caution. Therefore, if test compounds are dissolved in DMSO, the 5X compound solutions should not exceed 0.5% DMSO.*

7. After incubation, place a new box of pipette tips onto the FLIPR Tetra System deck and immediately transfer the assay plate and the compound plate from the cell culture incubator directly to the FLIPR Tetra System deck equilibrated to 37°C.

   Note: *Do not rinse the cells after adding the Cardiotox Dye loading solution.*

8. Record a baseline measurement (240 reads in 30 seconds) according to the FLIPR Tetra System and the EarlyTox Cardiotoxicity Kit guides. See the settings in the table below:

Parameter	96-well Format		384-well Format	
	EMCCD Camera	ICCD Camera	EMCCD Camera	ICCD Camera
Excitation LED (nm)	470 - 495		470 - 495	
Emission Filter (nm)	515 - 575		515 - 575	
Exposure (sec)	0.05		0.05	
Read Time Interval (sec)	0.125		0.125	
Camera Gain	130	2000	130	2000
LED Intensity (%)	80	50	80	50
Camera Gate (%)	N/A	20	N/A	20
Addition Volume (µl)	50		12.5	
Addition Height (µl)	160 - 180		30 - 40	
Addition Speed (µl/sec)	10 - 20		10 - 20	
Tip-up Speed (10 mm/sec)	10		10	

   Note: *See the FLIPR Tetra System or EarlyTox Cardiotoxicity Kit guide for specific instrument settings and recommendations on how to adjust parameters to optimize the cellular response.*

9. Transfer 50 µl/well of the 5X compound dilutions from the compound plate to the assay plate, maintaining both plates on the FLIPR Tetra System deck equilibrated to 37°C. Record a simultaneous compound treatment measurement to detect immediate/short-term responses.

   Note: *For 384-well format, transfer 12.5 µl/well of the 5X compound dilutions.*

10. After the compound treatment measurement, return the assay plate to the cell culture incubator. Intermediate/long-term responses can be detected with subsequent measurements (i.e. 20 and 60 minutes post-compound treatment).

Example Data

Results displayed in Figure 1 and 2 were obtained 20 minutes post-compound treatment. The following figures illustrate representative calcium oscillation waveforms and the effects of modulating crucial ion channel and GPCR activity through compound addition.

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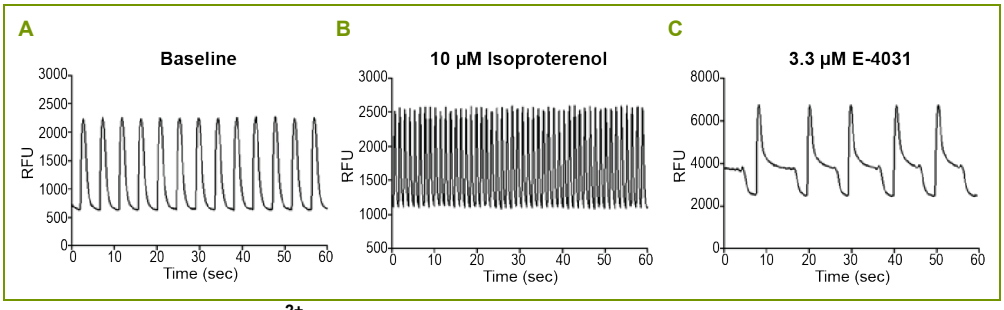

Figure 1: Intracellular Ca^{2+} Oscillations Provide a High-throughput Biomarker for Ion Channel and GPCR Activity

Electrical activity at the membrane is controlled by ion channels and GPCRs. This activity drives intracellular Ca^{2+} oscillations, which can thus be used as a high-throughput biomarker for membrane protein activity. Panel A shows calcium oscillation waveforms under control conditions (baseline). Panels B and C show the effect of the GPCR-agonist isoproterenol or the I_{Kr} channel blocker E-4031, respectively, on the calcium oscillation waveforms. iCell Cardiomyocytes were exposed to the indicated compounds at the concentrations listed.

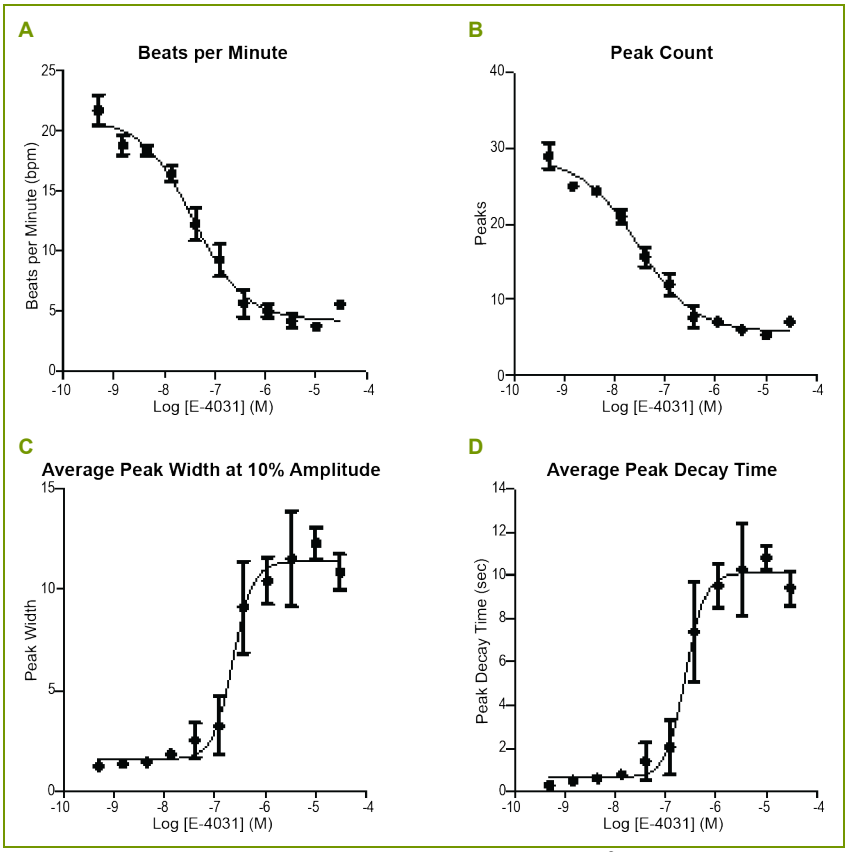

Figure 2: Pharmacological Effects on Intracellular Ca^{2+} Oscillations Are Characterized Using Different Parameters

Peak frequency (beats per minute, bpm), peak count, average peak width at 10% amplitude, and average peak decay time were calculated with the ScreenWorks Peak Pro Software to analyze pharmacological modulation on the calcium oscillation waveforms of iCell Cardiomyocytes after treatment with the I_{Kr} channel blocker E-4031.

Summary

iCell Cardiomyocytes provide an in vitro test system that recapitulates native human cardiac myocyte physiology and function while the FLIPR Tetra System provides a high-throughput platform for measuring cardiomyocyte behavior. The methods and results presented here highlight the ease of use with which robust and relevant data can be generated on human cardiomyocyte contractile activity. Together, these tools bring 96- and 384-well based predictive assessments of compound efficacy and toxicity on human cardiomyocytes to the drug development process.

References

1. Sirenko O, Crittenden C, Callamaras N, et al. (2013) Multiparameter In Vitro Assessment of Compound Effects on Cardiomyocyte Physiology Using iPSC Cells. J Biomol Screening.
2. Sirenko O, Cromwell EF, Crittenden C, et al. (2013) Assessment of Beating Parameters in uman Induced Pluripotent Stem Cells Enables Quantitative In Vitro Screening for Cardiotoxicity. Toxicol Appl Pharmacol.

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