Using LEAP to Measure Cardiac Action Potential Morphology

Introduction

iCell® Cardiomyocytes² are cardiac myocytes derived from human induced pluripotent stem cells (iPSC). These cardiomyocytes, which are an extension of the well-characterized iCell product line, have been optimized for faster recovery from cryopreservation and can be used in a myriad of functional applications. Additionally, iCell Cardiomyocytes² are the featured cell model in the Comprehensive in vitro Proarrhythmia Assay (CiPA) initiative for cardiac safety testing.

Multielectrode array (MEA) technology is the standard for plate-based, multi-well measurements of cardiac electrophysiology. MEA assays with iPSC-derived cardiomyocytes enable label-free detection of extracellular field potentials from a population of electrically active cells. Analysis of the recordings yields metrics such as spontaneous beat rate, sodium spike amplitude, field potential duration (FPD), and more. The assay feature called Local Extracellular Action Potential (LEAP) was developed to measure the cardiac action potential on MEA. Induction of LEAP increases coupling between the cells and the electrodes, resulting in a larger assay signal (millivolts vs. microvolts) that is more robust against pharmacological modulation. Importantly, LEAP not only enables quantitation of compound effects on action potential morphology, such as triangulation and action potential duration (APD90), but it also offers automated detection and classification of arrhythmias.

iCell Cardiomyocytes² can be maintained in MEA plates to form an electrically stable and mechanically active syncytium amenable to electrophysiological examination and pharmacological interrogation. Taken together, these technologies provide an excellent platform for studying the activity of ion channels in cardiac myocytes, as well as for in vitro screening of compound safety and efficacy in human cells.

This Application Protocol describes how to handle iCell Cardiomyocytes² in a 96-well plate format and perform the LEAP assay on the Maestro Pro MEA system.

Required Equipment, Consumables, and Software

The following equipment, consumables, and software are required in addition to the materials specified in the iCell Cardiomyocytes² User's Guide.

Item	Vendor(s)	Catalog Numbers
Equipment		
12-channel Pipettor, 200 µL	Multiple Vendors	
Maestro Pro MEA System	Axion BioSystems	
Consumables		
iCell Cardiomyocytes² Kit, 01434 or 11713	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1017 or R1218
• iCell Cardiomyocytes Plating Medium, 30 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	(included in kit)
Centrifuge Tubes, 1.5, 15, and 50 ml	Multiple Vendors	
DPBS, no calcium, no magnesium	ThermoFisher	14190-144
Fibronectin Solution, from Human Plasma	FUJIFILM Wako Chemicals U.S.A. Corp	063-05591
Sterile Water	Multiple Vendors	NA

Item	Vendor(s)	Catalog Numbers
BioCircuit MEA 96-well plate (recommended), or		M768-BIO-96
Classic MEA 96-well plate	Axion BioSystems	M768-KAP-96

Software		
AxIS Navigator 2.0 Software	Axion BioSystems	Latest Version
Cardiac Analysis Tool	Axion BioSystems	Latest Version

Workflow

While iCell Cardiomyocytes^2 establish spontaneous and regular beating in culture by Day 4, the recommended timepoint per the CiPA protocol to measure baseline electrical activity and to dose cells with compound(s) is Day 7 in culture. This interval allows for the highest consistency and most flexibility to perform the MEA assay. The experimental workflow is as follows:

- Day 0: Coat 96-well MEA plate with fibronectin, thaw and then plate iCell Cardiomyocytes^2 (CM).
- Day 1: Replace iCell Cardiomyocytes Plating Medium (Plating Medium or iCPM) with iCell Cardiomyocytes Maintenance Medium (Maintenance Medium or iCMM).
- Day 2 – Day 6: Perform complete media changes with iCMM every 2 days; perform baseline recordings to monitor activity of the cells in culture, if desired.
- Day 7: Perform a complete media change on the day of assay, wait 4 hours and record baseline MEA activity (“pre-dose”); apply test compounds to cells and record response (“post-dose”); induce LEAP and record activity (“post-LEAP”).

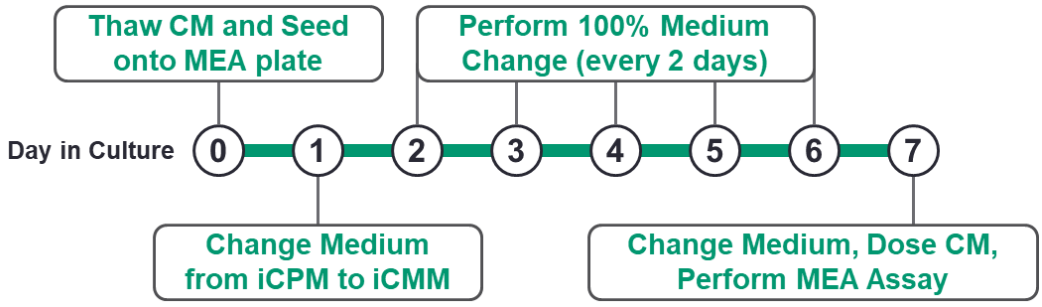

Figure 1: Schematic of the MEA assay workflow.
iCell Cardiomyocytes^2 can be assayed in 7 days by following the steps outlined in this protocol.

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Start TUES	Wed: iCPM-->iCMM	xx	Fri: iCMM	"weekend-free"		Mon: iCMM	Assay
Start WED	Thurs: iCPM-->iCMM	Fri: iCMM	"weekend-free"		Mon: iCMM	xx	Assay
Start THURS	Fri: iCPM-->iCMM	"weekend-free"		Mon: iCMM	xx	Wed: iCMM	Assay

Table 1: Schedules for a “weekend-free” workflow.
iCell Cardiomyocytes^2 are compatible with a cell culture feeding schedule that enables weekend-free work. Example schedules are outlined in the table above.

Tips Before Starting

1. Refer to the iCell Cardiomyocytes^2 User's Guide for information on storage and handling of the cells and media.
2. Read this entire Application Protocol before handling iCell Cardiomyocytes^2 to become familiar with assay workflow.
3. Thaw both bottles of media required for this assay, including iCell Cardiomyocytes Plating Medium (30 ml) and iCell Cardiomyocytes Maintenance Medium (100 ml), overnight at 4°C the day prior to thawing and plating cells.

Methods

Coating the MEA Plate with Fibronectin

1. Prepare a 50 µg/ml working solution of fibronectin by diluting the stock solution 1:10 in DPBS immediately before use.

   Note: *To prepare a fibronectin stock solution, reconstitute fibronectin in sterile water at 0.5 mg/ml according to the manufacturer's instructions. Aliquot and store at -20°C. Approximately one 100 µl aliquot is required to coat an entire 96-well MEA plate.*

2. Tilt the MEA plate at a 30° angle so that the bottom of each well is visible. Dispense an 8 µl/well droplet of fibronectin working solution over the recording electrode area of each well (see Figure 2).

   Note: *Do not touch the surface of the MEA plate with the pipette tip to avoid damaging the electrodes.*

3. Add 2-3 ml sterile water to the area outside of the wells on the MEA plate to prevent fibronectin droplets from evaporating.
4. Replace the lid and incubate the fibronectin-coated MEA plate in a cell incubator at 37°C for at least 1 hour.

   Note: *Longer incubation times may be acceptable; however, the droplet of fibronectin should not be allowed to evaporate to avoid impacting proper cell attachment.*

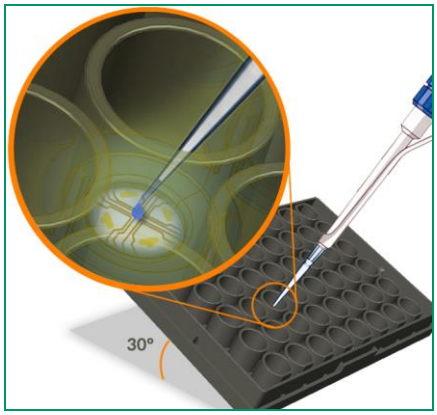

Figure 2: Placement of the droplet.
iCell Cardiomyocytes^2 are seeded on the MEA by tilting the plate approximately 30 degrees and dispensing an 8 µl droplet over the recording electrode area of each well.

Thawing iCell Cardiomyocytes^2 for MEA Assay

1. Obtain the number of viable cells per vial from the Certificate of Analysis for the specific lot of iCell Cardiomyocytes^2.

Note: *Each Certificate of Analysis can be found online here:* fujifilmcdi.com/resources/coa-lookup/

2. Calculate the final volume of Plating Medium needed to resuspend the cell pellet to 6,250,000 viable cardiomyocytes/ml, which yields 50,000 viable cells per 8 µl droplet.

   Note: *Expect this cell suspension volume to be less than 1 ml, typically.*

3. Warm the Plating Medium to 37°C in a water bath prior to use.

4. Thaw cells into a sterile 50 mL centrifuge tube according to the iCell Cardiomyocytes^2 User's Guide.

   Note: *The total volume of cell suspension at thaw is 5 ml (1 ml cryovial contents + 1 ml Plating Medium rinse + 3 ml of additional Plating Medium), which is less than the amount listed in Chapter 5 of the User's Guide.*

   Note: *A sample (up to 250 µL of the 5 ml cell suspension) may be removed to confirm viability and cell number using an automated cell counter or hemocytometer at this step.*

5. Transfer the 5 mL cell suspension to a sterile 15 ml centrifuge tube and spin at 300 x g for 5 minutes.

6. Aspirate the supernatant, being careful not to disturb the cell pellet.

7. Gently resuspend the cell pellet with the final volume of Plating Medium determined in Step 2 above.

8. Transfer the cell suspension to a sterile 1.5 ml centrifuge tube for ease of handling when plating.

Plating iCell Cardiomyocytes^2 onto the MEA plate

The following procedure details plating (or dotting) of the cells into a 96-well MEA plate.

Note: *Timing is critical for plating iCell Cardiomyocytes^2. It is recommended to aspirate fibronectin and dot cells one row (or one column) at a time to avoid evaporation or crystallization of the fibronectin and compromise attachment of the cells.*

1. Remove the fibronectin-coated MEA plate from the cell culture incubator and work in a biological safety cabinet.

2. Aspirate the fibronectin from each well. Additional rinsing is not necessary.

3. Dispense an 8 µl droplet of the cell suspension (approximately 50,000 cells) over the recording electrode area of each well in the MEA plate (Figure 2).

4. Close the lid on the 1.5 ml centrifuge tube and invert the cell suspension after every row (or column) to ensure equal distribution of cells across the plate.

5. Repeat steps 2 through 4 until the entire MEA plate has been dotted with iCell Cardiomyocytes^2.

   Note: Timing is also critical here. Performance of iCell Cardiomyocytes^2 is compromised if the droplets are allowed to evaporate.

6. Cover the MEA plate with the lid and incubate it in a cell culture incubator at 37°C, 5% CO_2 for approximately 60-90 minutes.

7. Equilibrate the Plating Medium in a 37°C water bath for approximately 15 min prior to use.

8. Remove the fibronectin-coated MEA plate from the cell culture incubator and work in a biological safety cabinet.

9. Tilt the MEA plate at 30 degrees. Gently add 100 µl/well of 37°C Plating Medium down the side of the well of the MEA plate one row at a time. Adding the medium too quickly may dislodge the adhered cells.

10. Slowly return the MEA plate to a flat position to allow the medium to gently cover the droplet after media has been added to all rows.

11. Rotate the MEA plate 180 degrees and repeat step 9 gently adding another 100 µl/well of 37°C Plating Medium down the opposite side of the wells.
12. Slowly return the MEA plate to a flat position to allow the medium to gently cover the droplet after media has been added to all rows.

    Note: *The final volume of medium recommended for culturing iCell Cardiomyocytes² on a 96-well MEA plate is 200 µl/well.*
13. Add an additional 2-3 ml of sterile water to the area surrounding the wells of the MEA plate to limit evaporation.
14. Place the MEA plate with iCell Cardiomyocytes² in Plating Medium in a cell culture incubator at 37°C, 5% CO_2 and incubate overnight.

Culturing of iCell Cardiomyocytes² in the MEA Plate

1. Equilibrate Maintenance Medium to 37°C in a water bath prior to use.

   Note: *Aliquots of Maintenance Medium (~20 ml) may be removed from the stock bottle of Maintenance Medium.*
2. Replace 100% of the medium with 37°C Maintenance Medium. Slowly aspirate the Maintenance Medium from the MEA plate using a 12-channel pipettor and replace with 200 µl/well of 37°C Maintenance Medium.
3. Maintain the cardiomyocytes in the MEA plate, replacing 100% of the spent medium with Maintenance Medium every 2 days.

   ***Note*:** It is recommended to work row-by-row (or column-by-column) when aspirating and replacing media.
4. Culture the cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.

Data Acquisition and Analysis

Data Acquisition

Please refer to the AxIS Navigator 2.0 User Guide software manual for the Maestro Pro MEA system for complete instructions on how to acquire data on the instrument.

On the data of the assay, three separate recordings are performed: 1) pre-dose, 2) post-dose, and 3) post-LEAP (see Figure 3 below).

Figure 3: Schematic of recording schedule on the MEA system.
Data from iCell Cardiomyocytes² is acquired on Day 7 according to this schedule. Equilibration periods are pictured in gray, dosing with compounds or inducing LEAP is shown in black, and each of the recording sessions are illustrated in red.

Measuring the Electrical Activity and Compound Response of iCell Cardiomyocytes^2

1. Prepare test compound solutions during this incubation period (see insert below).

Preparing Test Compounds

- Prepare stock solutions of test compounds in DMSO (or appropriate solvent) at a concentration of at least 1000X higher than the final concentration of the top dose required.
- Prepare intermediate solutions of test compounds in Maintenance Medium at 5X the final concentration in a separate 96-well cell culture plate.
- ***Note:*** *For example, 1 µl of 1000X stock solution into 200 µl of Maintenance Medium in the 96-well plate yields a 5X intermediate solution with 0.5% DMSO. iCell Cardiomyocytes^2 can tolerate 0.1-0.5% DMSO in this MEA assay.*
- Equilibrate the cell culture plate containing the 5X compound solutions (covered with lid) in a cell culture incubator at 37°C, 5% CO_2.

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2. To begin the assay, remove the MEA plate from the cell culture incubator and place it on the Maestro Pro MEA system to equilibrate at 37°C, 5% CO_2 for at least 10 minutes.
3. Perform a baseline recording of cardiac electrical activity (“pre-dose”) for 5 minutes using the Field Potentials configuration in the AxIS Navigator software.

   Note: *Refer to the next section for more information on Data Acquisition.*
4. Remove the MEA plate from the instrument. While working in a biological safety cabinet, quickly transfer 50 µl of the 5x compound solutions from the cell culture plate to the MEA plate using a 12-channel pipettor. Gently mix by pipetting 2-3 times.
5. Return the MEA plate to the instrument after dosing and incubate at 37°C, 5% CO_2 for 30 minutes.

   Note: *An incubation period of 30 minutes is recommended per the CiPA protocol.*
6. Record cardiac electrical activity “post-dose” for 5 minutes using the Field Potentials configuration.

   Note: *At this point in the assay workflow, cardiac action potentials can be recorded from the iCell Cardiomyocytes^2 using LEAP mode (see Hayes, et. al.* ***2019*** *Scientific Reports).*
7. Induce LEAP on the instrument according to the instructions provided by the manufacturer. This step takes approximately 10-15 minutes to complete.

   Note: *The BioCircuit MEA plate is recommended for LEAP mode with iCell Cardiomyocytes^2 to achieve the highest percentage of successful LEAP signals when following this Application Protocol. LEAP can be induced on all 8 electrodes in all 96-wells of the BioCircuit MEA plate.*
8. After LEAP induction is complete, immediately record cardiac electrical activity (“post-LEAP”) for at least 10 minutes using the LEAP configuration in AxIS Navigator software.
9. Proceed to data analysis.

Data Analysis

Each recording produces a RAW data file that needs to be batch processed. The first two FPD recordings (“pre-dose” and “post-dose”) are batch processed together under the Field Potentials configuration. The third recording (“post-LEAP”) must be batch processed under the LEAP configuration. The output from batch processing is a Comma Separated Values (.csv) file that contains numerous metrics for cardiac

activity. While iCell Cardiomyoytes[2] were used to help establish default settings in the AxIS Navigator software, supervised data processing with the Cardiac Analysis Tool (CAT) is recommended for the most accurate detection of the T-wave and quantitation of FPD. Additionally, the CAT software is used to visualize cardiac action potential morphology and determine action potential duration (APD) values. Please refer to the AxIS Navigator software User Guide, the LEAP quick start guide, and the CAT User Guide for additional information.

Representative Data

The electrical activity of spontaneously beating iCell Cardiomyocytes[2] typically stabilizes by Day 4 in culture, at which point baseline data can be recorded from the cells. However, for the most consistent assay performance and per the CiPA protocol, it is recommended to perform the MEA assay with dosing of test compounds and inducing LEAP on Day 7.

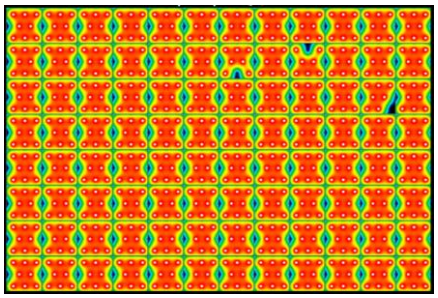

Figure 4. Cardiac Activity Heat Map.
Snapshot from AxIS Navigator software 2.0 of iCell Cardiomyocytes[2], 01434 cultured until Day 7 on a 96-well BioCircuit MEA plate with the maximum plot settings (in red) for Spike Amplitude set at 300 µV. It is evident that 765/768 electrodes (>99%) are detecting activity at this signal threshold.

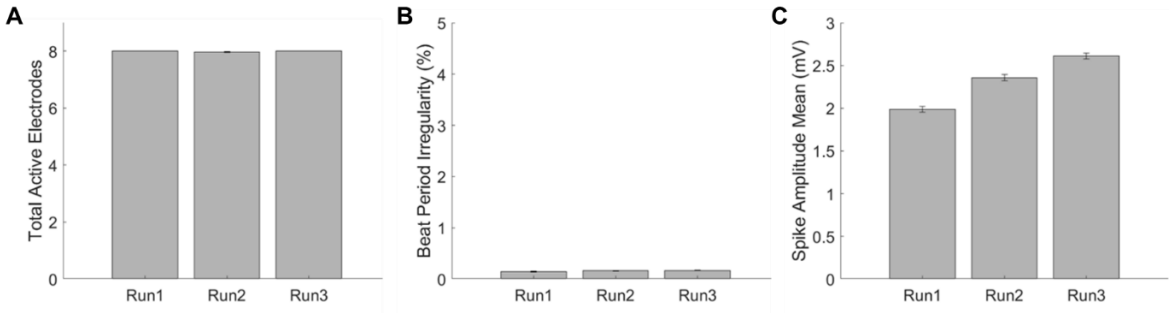

Figure 5. Reproducible Baseline Cardiac Activity.
Three independent assays were performed iCell Cardiomyocytes[2], 01434 maintained in culture on a 96-well BioCircuit MEA plate until Day 7. Baseline activity was recorded on the Maestro Pro and representative data is presented. Panel A shows the total active electrodes essentially reached eight in every well for all 3 runs; Panel B shows the Beat Period Irregularity was below 1% across all assays.Panel C shows the Spike Amplitude Mean reached 2 mV or higher in all cases.

FPDc (Fridericia), msec													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	399	399	385	388	386	391	393	381	386	388	395	394	
B	378	367	383	365	366	371	351	356	366	370	370	369	average
C	371	370	372	370	363	370	367	369	370	365	364	381	373
D	370	353	355	371	370	369	357	362	370	349	351	392	std dev
E	361	373	365	379	358	383	369	373	377	364	353	381	13.5
F	366	370	353	358	363	370	367	343	348	346	361	387	%CV
G	388	374	372	375	377	373	367	363	360	358	375	398	3.61%
H	384	399	378	389	397	380	381	380	392	394	393	381	

APD90c (Fridericia), msec													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	428	439	429	428	433	422	427	434	432	431	437	434	
B	421	410	415	406	397	419	414	N/A	430	406	409	414	average
C	405	404	408	410	402	420	420	405	415	404	402	424	415
D	413	400	398	399	403	403	403	414	414	412	399	426	std dev
E	412	410	403	408	412	408	418	401	413	412	402	416	12.0
F	425	404	409	400	408	399	411	393	401	395	401	424	%CV
G	416	423	412	420	416	415	403	409	411	422	405	422	2.88%
H	424	433	432	430	434	423	425	424	438	434	438	434	

Figure 6. Consistent Spontaneous Activity.
iCell Cardiomyocytes^2, 01434 were maintained in culture in a 96-well BioCircuit MEA plate until Day 7 and baseline activity was recorded. Pre-dose data was analyzed using the Cardiac Analysis Tool for both Field Potential Duration (FPD) and Action Potential Duration (APD)(example beat rate-corrected data (FPDc and APD90c) data is presented). The well-to-well consistency across an entire 96-well plate exhibits coefficients of variation (%CV) below 4% for both metrics.

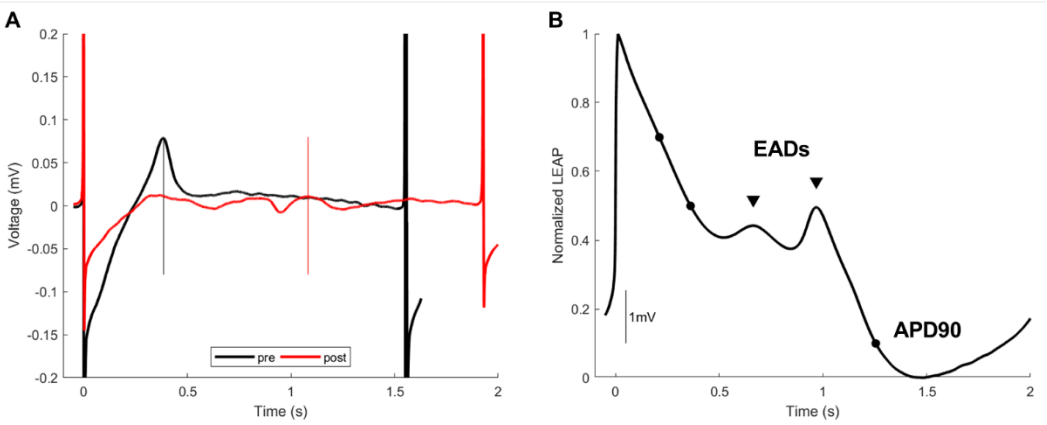

Figure 7. Response to hERG Channel Block by E-4031.
iCell Cardiomyocytes^2, 01434 were maintained in culture in a 96-well BioCircuit MEA plate until Day 7 and treated with 100 nM of E-4031. Panel A shows the “pre-dose” FPD trace (black) and “post-dose” FPD trace (red) are overlaid and the markings for FPD quantitation are indicated. In Panel B, the normalized LEAP trace showing the cardiac action potential from the same well is featured, with quantitation of APD90 and automatic detection of early after depolarizations (EADs) indicated.

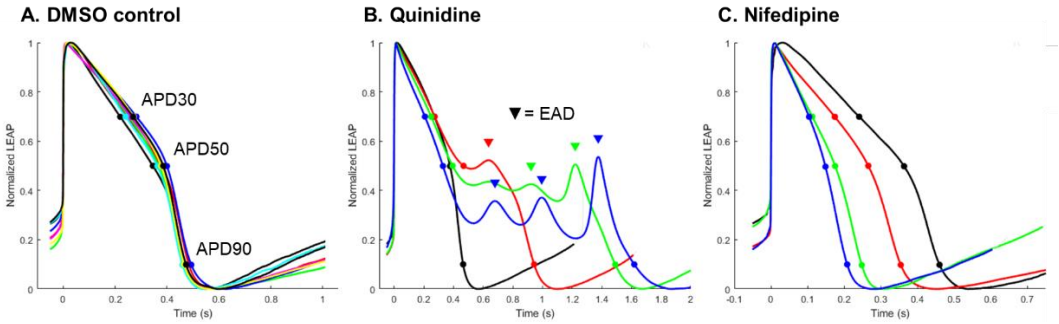

Figure 8. Compound-induced Changes in Cardiac Action Potential Morphology. iCell Cardiomyocytes^2, 01434 were cultured in a 96-well BioCircuit MEA plate until Day 7, treated with test compounds, and MEA data was recorded using LEAP. Panel A shows the classic cardiac action potential traces are maintained under control conditions (0.1% DMSO) and consistent across wells (n=8). Panel B shows that the iCell Cardiomyocytes^2 exposed to quinidine (1 µM in red, 3 µM in green, 10 µM in blue), a drug known to cause increased action potential duration, induced EADs at all 3 concentrations and in 3/3 wells. In Panel C, Treatment of iCell Cardiomyocytes^2 with nifedipine (30 nM in red, 100 nM in green, 300 nM in blue), a calcium channel blocker, resulted in a dose-dependent shortening of the action potential morphology.

Summary

iCell Cardiomyocytes^2 can be reanimated from cryopreservation and plated directly into MEA plates where they rapidly recover to exhibit spontaneous beating and the expected electrical activity. This Application Protocol highlights the ease-of-use for culturing iCell Cardiomyocytes^2 in 96-well format. Furthermore, the consistency of assay performance from well-to-well, plate-to-plate, and lot-to-lot offers a reliable cell-based system for dosing of test compounds in safety / toxicity studies. Implementation of the LEAP assay provides a more robust solution for pharmacological modulation because of the larger assay signal and enables detailed investigation of compound effects on cardiac action potential morphology. Refer to the *Hayes et. al.* ***2019*** *Scientific Reports* publication and Axion BioSystems website for more information on LEAP. Together, these technologies offer a high-throughput in vitro system for gathering physiologically relevant data on the electrophysiological activity of human cardiac cells.

Revision History
Version 1.0: September 2020
AP-CMC2LEAPMEA200915