

Contractility Analysis of iCell Cardiomyocytes²:

Evaluating iCell Cardiomyocytes² in co-culture with iCell Cardiac Fibroblasts on the FLEXcyte 96 System

Introduction

Human induced pluripotent stem cell (iPSC)-derived cardiomyocytes, specifically iCell® Cardiomyocytes², have become an important tool in the safety pharmacology space for monitoring the cardiotoxic effects of compounds. Numerous *in vitro* assays to measure the activity of these cardiac cells using multi-electrode array (MEA) or calcium oscillations have been developed. Next-level approaches to monitor contractility, however, have been limited by throughput and biological relevance. Furthermore, the electromechanical function of cardiac muscle depends on the crosstalk of myocytes with other regenerative non-myocyte cells, specifically cardiac fibroblasts. A major function of cardiac fibroblasts is to produce and secrete growth factors and cytokines, which have specific effects on cardiomyocytes that can impact contractile function.

To enable the isogenic co-culture of iCell Cardiomyocytes² with human iPSC-derived cardiac fibroblasts, FUJIFILM Cellular Dynamics has launched a new cell type with iCell Cardiac Fibroblasts. These cells are differentiated from the same 01434 female donor, are of high purity, have low lot-to-lot variability, and express cardiac fibroblasts specific markers such as POSTN, TE7, and Cx43.

In this application protocol, we provide recommendations for co-culturing iCell Cardiomyocytes² with iCell Cardiac Fibroblasts on the Nanion FLEXcyte 96 system and offer basic instructions for compound dosing, data acquisition, and analysis. This system uses a 96-well plate format with flexible PDMS membranes which enables contractility measurements of cells cultured in a more physiological environment, thus more closely reflecting the mechanical conditions of the native human heart versus overly stiff substrates like glass or plastic. iCell Cardiomyocytes² together with iCell Cardiac Fibroblasts can be co-cultured and maintained on the FLEXcyte 96 system for extended durations allowing for the measurement of acute and sub-acute drug-induced effects, thus offering an excellent platform for *in vitro* screening of compound effects on human cardiac contractility. This Application Protocol describes how to prepare and maintain these cell types together in co-culture for use on the FLEXcyte 96 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 and iCell Cardiac Fibroblasts Quick Guide.

Item	Vendor(s)	Catalog Numbers
Equipment		
FLEXcyte 96	Nanon Technologies GmbH	
12-channel pipettor, 200 µl and 20 µl	Multiple Vendors	
Consumables		
iCell Cardiomyocytes ² Kit, 01434	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1017
• iCell Cardiomyocytes ² , 01434	FUJIFILM Cellular Dynamics, Inc. (FCDI)	(included in kit)
• 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)
iCell Cardiac Fibroblasts Kit, 01434	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1257
• iCell Cardiac Fibroblasts, 01434	FUJIFILM Cellular Dynamics, Inc. (FCDI)	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc. (FCDI)	(included in kit)
FLX-96 Plate	Nanon Technologies GmbH	201010
Geltrex™ hESC-Qualified, Ready-To-Use, Reduced Growth Factor Basement Membrane Matrix	ThermoFisher Scientific	A1569601
DPBS, no calcium, no magnesium	Multiple Vendors	
15 ml Conical Tubes	Multiple Vendors	
50 ml Conical Tubes	Multiple Vendors	
200 µl and 20 µl Pipette Tips	Multiple Vendors	
Sterile Reagent Reservoirs	Multiple Vendors	
Software		
CardioExcyte Control Software	Nanon Technologies GmbH	
DataControl96 Software	Nanon Technologies GmbH	

Workflow

It is recommended to assess the contractility of co-cultured iCell Cardiomyocytes² (CM2) and iCell Cardiac Fibroblasts (CF) on Day 6 to Day 9 of culture.

- **Day -1:** Coat the 96-well FLX-96 plate with 1:4 Geltrex:DPBS (-/-) overnight in a 37°C incubator.
- **Day 0:** Thaw iCell Cardiomyocytes² and iCell Cardiac Fibroblasts according to their User's Guide or Quick Guide using the online Certificate of Analysis (CoA) viable cell count. The cells are combined in a 10:1 (CM2:CF) ratio and seeded on the FLX-96 plates in iCell Cardiomyocytes Plating Medium (iCPM).
- **Day 2:** After 48hrs of plating in iCell Cardiomyocytes Plating Medium, gently vacuum aspirate media and replace with 200 µl of iCell Cardiomyocytes Maintenance Medium (iCMM).
- **Day 4 and Day 5:** Perform a complete media change with 200 µl of iCell Cardiomyocytes Maintenance Medium. It is important to change the media 24 hours before assay. Do NOT change the media on the day of assay. EX: If you plan to assay on Day 6, perform a complete media change on Day 5. If you plan to assay on Day 7, perform an additional complete media change on Day 6.

- **Day 3 to Day 6:** Read the baseline signal of the wells every day on the FLEXcyte 96 System. (This is optional but recommended for tracking the activity of the plate).
- **Day 6 (day of assay):** Read the baseline of the plate on the FLEXcyte 96 System. Dose with desired compounds at 10X final concentration in 22 μ l while the plate is on the instrument. Read plate on the FLEXcyte 96 after desired amount of time. FCDI recommends assaying the plate on between Day 6-9.

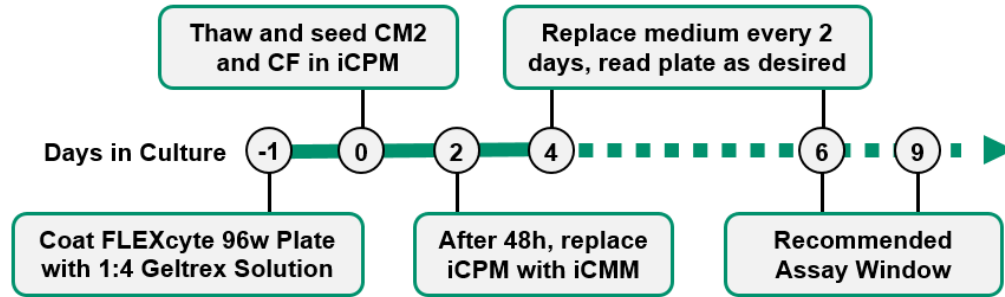


Figure 1: Schematic diagram of the workflow for assaying iCell Cardiomyocytes² (CM2) and iCell Cardiac Fibroblasts (CF) on the FLEXcyte 96 System.

Tips Before Starting

1. Refer to the iCell Cardiomyocytes² and iCell Cardiac Fibroblasts User's Guide and Quick Guide for information on storage and handling of the cells and media.
2. Read this entire Application Protocol before handling the cells to become familiar with the assay workflow.
3. Obtain the Certificate of Analysis (CoA) for each cell type here: www.fujifilmcdi.com/coa-lookup/. Based on the guaranteed number of cells provided per vial ($\geq 5 \times 10^6$ for iCell Cardiomyocytes² and $\geq 0.5 \times 10^6$ for iCell Cardiac Fibroblasts), there are enough cells to seed at least 50 wells of the FLX-96 plate. Two vials of iCell Cardiomyocytes² and iCell Cardiac Fibroblasts are required to seed all 96 wells in a FLX-96 plate.
4. 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.
5. Overnight coating with the Geltrex solution is imperative to assay performance.
6. Cells are thawed and seeded in iCell Cardiomyocytes Plating Medium. Wait 48 hours to perform a media change.
7. The PDMS membrane on a FLX-96 plate is very thin (10 μ m) and is easily ruptured. It is imperative to leave the plate in its protective receiver plate. Do NOT touch the pipette tips to the bottom of the well and carefully rest the tips on the side of the well when dispensing media.
8. A full media change is required 24 hours before assay. Do NOT change the media on day of assay.

Methods

ECM Coating with Geltrex Solution (Day -1)

1. Prepare 1:4 Geltrex:DPBS by diluting 3 ml of Geltrex with 9 ml of DPBS without Ca^{2+} and Mg^{2+} in a solution reagent reservoir.
2. Dispense 100 μl of the Geltrex working solution into each well of a FLX-96 plate with a 200 μl multi-channel pipettor.
3. Incubate the Geltrex-coated FLX-96 plate in a cell culture incubator at 37°C , 5% CO_2 overnight.

Thawing and Plating iCell Cardiomyocytes² and iCell Cardiac Fibroblasts for the FLEXcyte 96 System

These steps outline the requirements for seeding a full FLX-96 plate with iCell Cardiomyocytes² and iCell Cardiac Fibroblasts at a 10:1 ratio.

1. Obtain the number of viable cells per vial from the Certificate of Analysis (CoA) for the specific lot of iCell Cardiomyocytes² and iCell Cardiac Fibroblasts.
2. Calculate the final volume of iCell Cardiomyocytes Plating Medium needed to obtain a viable cell density of 1×10^6 cells/ml, which yields 100,000 viable cells per 100 μl for iCell Cardiomyocytes².
Example: If the number of viable iCell Cardiomyocytes² cells/vial is 5.6×10^6 , the final solution should be 5.6 ml. Multiply the final volume of iCell Cardiomyocytes Plating Medium for the iCell Cardiomyocytes² by 2, because two vials will be thawed.
3. Warm the 30 ml bottle of iCell Cardiomyocytes Plating Medium to 37°C in a water bath prior to use.
4. Thaw the two vials of iCell Cardiomyocytes² into a single sterile 50 ml centrifuge tube according to the iCell Cardiomyocytes² User's Guide. Bring the volume of the cell suspension up to the calculated volume from step 2.

Note: The iCell Cardiomyocytes² User's Guide recommends thawing the vial of cells by transferring 1 ml of cell solution to the 50 ml conical tube, rinsing the vial with 1 ml of iCell Cardiomyocytes Plating Medium and transferring slowly to the cell solution. After this is complete, add additional iCell Cardiomyocytes Plating Medium to the conical tube to reach the calculated volume from step 2. Expect this volume to be greater than or equal to 5 ml for each vial of iCell Cardiomyocytes².

5. Calculate the final volume of iCell Cardiac Fibroblasts Plating Medium needed to obtain a viable cell density of 100,000 cells/ml, which yields 10,000 viable cells per 100 μl for iCell Cardiac Fibroblasts.
Example: If the number of viable iCell Cardiac Fibroblasts cells/vial is 0.7×10^6 , the final solution should be 7 ml. Multiply the final volume of iCell Cardiac Fibroblasts Plating Medium for the iCell Cardiac Fibroblasts by 2, because two vials will be thawed.
6. Thaw the two vials of iCell Cardiac Fibroblasts into a single sterile 50 ml centrifuge tube according to the iCell Cardiac Fibroblasts Quick Guide. Bring the volume of the cell suspension up to the calculated volume from step 5.

Note: The iCell Cardiac Fibroblasts Quick Guide recommends thawing the vial of cells by transferring 0.5 ml of cell solution to the 50 ml conical tube, rinsing the vial with 1 ml of iCell Cardiomyocytes Plating Medium and transferring slowly to the cell solution, then add an additional 0.5 ml of iCell Cardiomyocytes Plating Medium dropwise to the conical tube (the extra 0.5 ml is to make up for cryopreservation solution provided at 0.5 ml per vial). After this is complete, add additional iCell Cardiac Fibroblasts Plating Medium to the conical tube to reach the calculated volume from step 2. Expect this volume to be greater than or equal to 5 ml for each vial of iCell Cardiac Fibroblasts.

7. Transfer 10 ml of each cell suspension to a separate 50 ml conical tube to make a combined master cell suspension. Gently pipette up and down to mix the cells.
8. Transfer the mixed master cell suspension to a reagent reservoir.
9. Remove the Geltrex-coated FLX-96 plate from the cell culture incubator and work in a biological safety cabinet.
10. Aspirate the Geltrex solution from the plate quickly with a glass pipette fitted to a vacuum line from the first four rows. Do NOT touch the bottom of the well with the pipette; it is recommended to have the pipette touching the wall while aspirating.
11. Gently pipette the combined master cell suspension of iCell Cardiomyocytes² and iCell Cardiac Fibroblasts up and down 1-2 times in the reservoir to evenly resuspend cells. Pipette 200 µl of the master cell suspension into each well of the FLX-96 plate using a multichannel pipette. Mix the cells in the reagent reservoir by gently pipetting up and down between each row.
12. Repeat steps 9 and 10 to seed the last four rows.

Culturing of iCell Cardiomyocytes² and iCell Cardiac Fibroblasts in the FLEXcyte 96 System

1. Equilibrate the iCell Cardiomyocytes Maintenance Medium to 37°C in a water bath prior to use.
Note: Aliquots of iCell Cardiomyocytes Maintenance Medium (~20 ml) may be removed from the stock bottle and warmed up separately.
2. On Day 2 (or 48 hours) post-plating, perform a complete media change by vacuum aspirating iCell Cardiomyocytes Plating Medium from the plate 2 rows at a time. Add 200 µl of iCell Cardiomyocytes Maintenance Medium to each well using a multichannel pipette.
3. Incubate the microplate in a cell culture incubator at 37°C, 5% CO₂ after changing the medium.
4. Maintain the co-culture of iCell Cardiomyocytes² and iCell Cardiac Fibroblasts by replacing 100% of the spent medium with fresh iCell Cardiomyocytes Maintenance Medium every 2 days.
5. On Day 5 (24 hours before planned assay), perform a complete media change.

Data Acquisition and Analysis

Data Acquisition

Please refer to the CardioExcyte Control and DataControl96 Software manuals for complete instructions on how to acquire data on the instrument.

Compound Application

1. The day before compound addition, replace 100% of the spent medium with 37°C iCell Cardiomyocytes Maintenance Medium, iCell Cardiomyocytes Serum-Free Medium or iCell CardioTox Assay Medium.

Note: *Changing the medium about 18 hours before compound treatment ensures that the cardiomyocytes have stabilized after medium replacement and that the medium volumes are uniform across the FLX-96 plate.*

2. Prepare stock solutions of test compounds in fresh medium at a concentration of 10X the final concentration in a regular 96-well cell culture plate.

Note: *Final DMSO concentrations above 0.1% should be used with caution. Therefore, if test compounds are dissolved in DMSO, the 10X compound solutions should not exceed 1% DMSO.*

3. Equilibrate the cell culture plate containing the 10X compound solutions (covered with a lid) in a cell culture incubator at 37°C, 5% CO₂.

4. To begin the assay, quickly transfer 20 µl of the 10X compound solutions from the cell culture plate to the FLX-96 plate. Gently mix by pipetting 2-3 times.

Note: *Beating rate and amplitude are temperature dependent. It is recommended to add the compounds while the FLX-96 plate is placed on the FLEXcyte 96 system. If this is not possible, the FLX-96 plate should not be kept outside the incubator for more than 2 minutes while compounds are added.*

Data Acquisition and Analysis

The CardioExcyte Control Software enables automatic beating detection of cardiomyocytes cultured on a FLX-96 plate placed onto the FLEXcyte 96 system. DataControl96 Software allows automated analysis of different contractility parameters before and after compound addition. Furthermore, DataControl96 Software enables the calculation of IC₅₀ and EC₅₀ of test compounds. See the CardioExcyte Control and DataControl96 Software manuals to discover all their features.

Representative Data

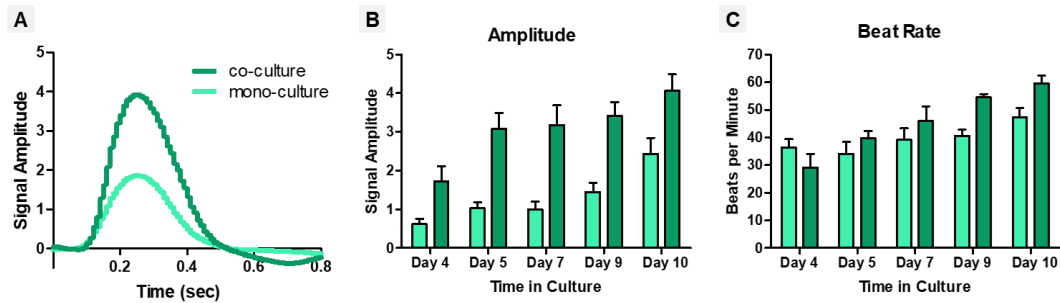


Figure 1. Comparison of iCell Cardiomyocytes² in mono-culture vs. in co-culture with iCell Cardiac Fibroblasts **A)** Co-culture of iCell Cardiomyocytes² (100K) and iCell Cardiac Fibroblasts (10K) results in a higher signal amplitude of contractility on the FLEXcyte 96 (representative data from Day 6 shown here). Contractility can be tracked over time in culture and **B)** the signal amplitude of cells together in co-culture is greater than that of iCell Cardiomyocytes² alone at every time point measured, while **C)** the beat rate of mono- vs. co-culture remains relatively similar over time.

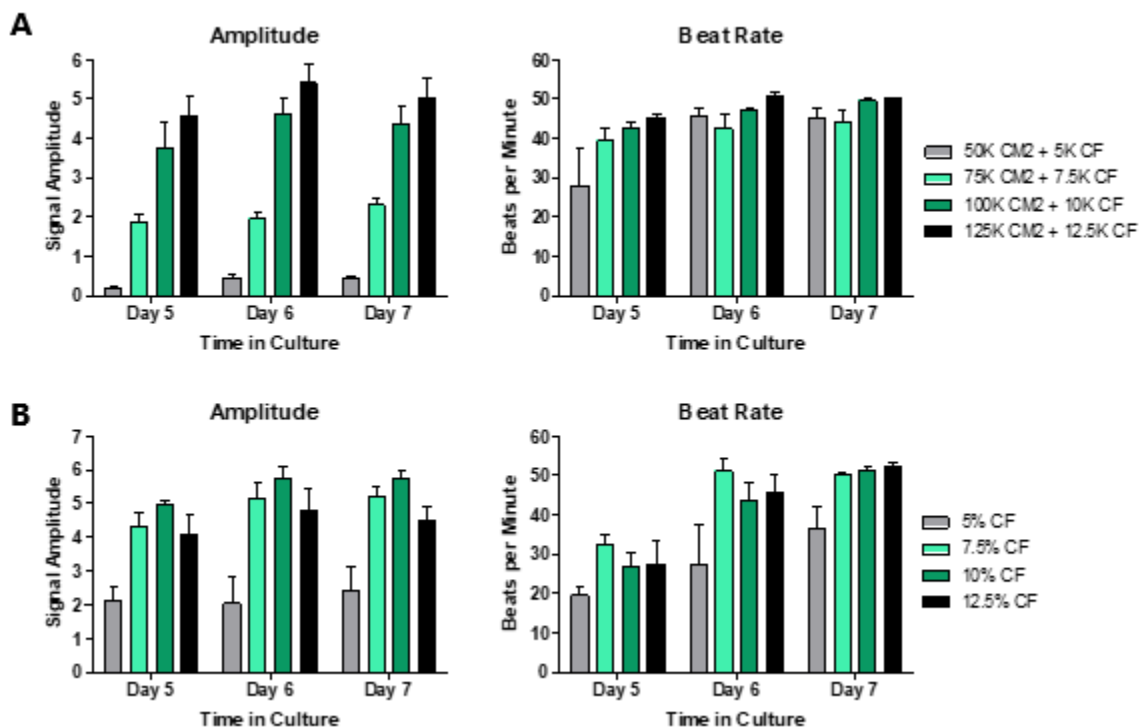


Figure 2. Cell Density Titration **A)** iCell Cardiomyocytes² and **B)** iCell Cardiomyocytes² and iCell Cardiac Fibroblasts in Co-Culture. **A)** iCell Cardiomyocytes² density was varied in co-culture with 10% iCell Cardiac Fibroblasts. Example: 50K iCell Cardiomyocytes² were cultured with 5K iCell Cardiac Fibroblasts, etc. Results showed that the strongest signal amplitude were in cultures containing 100K and 125K iCell Cardiomyocytes². It is recommended to plate 100K Cardiomyocytes² per well in the FLX-96 plate. **B)** 100K Cardiomyocytes² were cultured with varying iCell Cardiac Fibroblasts densities. Results showed the highest amplitude signal in wells containing 10% iCell Cardiac Fibroblasts. It is recommended to culture 100K iCell Cardiomyocytes² and 10K iCell Cardiac Fibroblasts per well of FLX-96 plate.

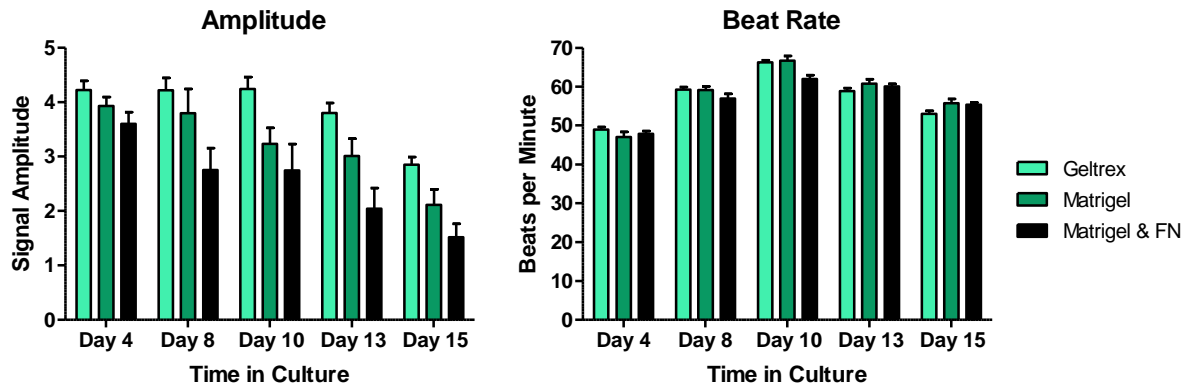


Figure 3. ECM optimization. The type of extracellular matrix coating on the FLX-96 plate had an effect on the cells' contractility signal amplitude over time. Cells seeded on Geltrex (1:4 dilution Geltrex:DPBS(-/-)), 10ug/mL Matrigel, and 10ug/mL Matrigel with 10ug/mL Fibronectin coatings were tested. Results showed that Geltrex supported the highest contractility signal amplitude over time. There was no effect on beat rate from different coatings. It is recommended to plate 100K iCell Cardiomyocytes² and 10K iCell Cardiac Fibroblasts on Geltrex-coated wells in the FLX-96 plate.

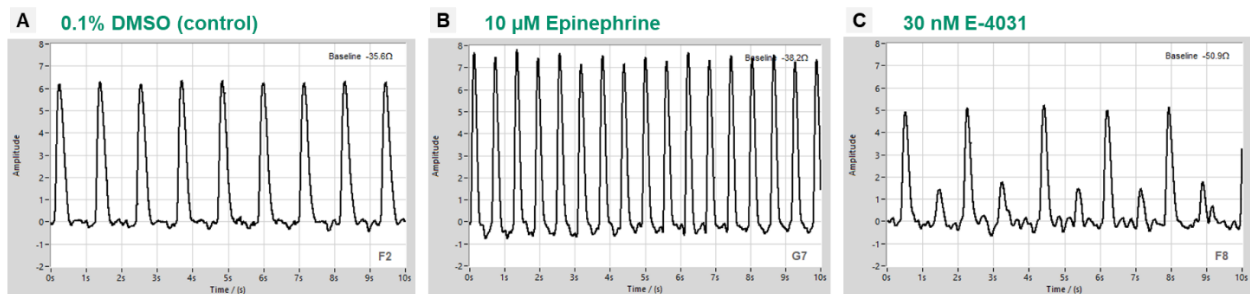


Figure 4. Pharmacology. Co-cultured iCell Cardiomyocytes² and iCell Cardiac Fibroblasts were dosed with compounds on day 7 in culture. Representative traces of the beat pattern when exposed to DMSO control (A), Epinephrine (B), and E-4031 (C). Expectedly, beta-adrenergic receptor antagonist, epinephrine, resulted in an increased beat rate and contraction amplitude. Additionally, hERG channel blocker, E-4031 expectedly decreased the contractility amplitude and disrupted and elongated the beating pattern.

Summary

The details in this application protocol outline the steps to co-culture iCell Cardiomyocytes² with iCell Cardiac Fibroblasts on the FLX-96 plate. The FLEXcyte system measures the contractility force of the co-culture, thereby enabling the user to study the pharmacological effects on contractility force. The data within this protocol is a comprehensive look at representative data for the optimization of culture conditions on the FLX-96 plate with additional pharmacological response to compounds. It has been demonstrated that iCell Cardiomyocytes² co-cultured with isogenic iCell Cardiac Fibroblasts result in a higher contractility force compared to iCell Cardiomyocytes² in mono-culture. The co-culture of cells on Geltrex resulted in the highest contractility force amplitude. Additionally, dosing the co-culture resulted in the expected response to known cardiotoxic compounds.

For questions about culturing our **iCell Cardiomyocytes² and iCell Cardiac Fibroblasts**, please contact FCDI's Technical Support at

- fcdi-support@fujifilm.com
- +1 (877) 310-6688 (US toll-free) or +1 (608) 310-5100

For inquiries about Nanion's **FLEXcyte platform**, please contact Nanion's Technical Support at

- Nanion HQ for general enquiries: info@nanion.de
- Nanion's Technical Support: support.cellular.networks@nanion.de


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