Generation of 3D iCell® CardioSpheres with iCell Products

Introduction

The human heart is a complex tissue. Ventricular cardiomyocytes make up only 50% of all the cells in the left ventricle (1). Therefore, a multi-cellular system containing cardiomyocytes, endothelial cells, and cardiac fibroblasts has the potential to provide greater physiological relevance, predictive power, and mechanistic insight than cardiomyocytes alone (2, 3). These individual cell types are available as iCell® Products through human induced pluripotent stem cell (iPSC) technology as cryopreserved cells that were differentiated from the same donor. Co-culture of iCell Cardiomyocytes, iCell Endothelial Cells, and iCell Cardiac Fibroblasts together in three-dimensional (3D) format offers a simple approach and a complimentary option to generate microtissues *in vitro* with the aim to recapitulate a biologically relevant model of the heart *in vivo*.

This Application Protocol describes a method to generate 3D cardiac tri-culture microtissues, also referred to as “CardioSpheres”. By combining different human iPSC-derived cardiovascular cell types directly from thaw (without any pre-culture) into multi-well ultra-low attachment (ULA) plates, the cells will self-assemble into 3D structures that function as cardiac microtissues. The thawing method, assay plate, cell number, composition, and co-culture media formulation have all been optimized for consistent and robust performance of iCell CardioSpheres. Representative data on spheroid formation, shape, size, and morphology during the course of cell culture is also shown. Furthermore, functional assay performance of cells in 3D has been demonstrated by measuring baseline calcium transient activity and recording the assay response to known modulators of cardiac activity.

1. Litviňuková M, Talavera-López C, et al. (2020). Nature 588(7838):466-472.
2. Ravenscroft SM, Pointon A, et al. (2016). Toxicological Sciences 152:99-112.
3. Giacomelli E, Meraviglia V, et al. (2020). Cell Stem Cell 26(6):862-879.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the respective cell type's User's Guides.

Item	Vendor(s)	Catalog Numbers
Equipment		
12-channel Pipettor, 200 µL	Multiple Vendors	
PrimeSurface® V-bottom 96-well plate ^	FUJIFILM Wako Pure Chemical Corp.	629-01099
PrimeSurface® U-bottom 384-well plate (optional)	FUJIFILM Wako Pure Chemical Corp.	MS-9384UZ
Consumables		
Isogenic iCell CardioSpheres from Donor 01434		
iCell Cardiomyocytes, 01434, 1M †	FUJIFILM Cellular Dynamics, Inc.	R1057
• iCell Cardiomyocytes Plating Medium, 30 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Cardiomyocytes^2, 01434, 1.25M †	FUJIFILM Cellular Dynamics, Inc.	R1059
• iCell Cardiomyocytes Plating Medium, 30 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Cardiac Fibroblasts, 01434, 0.5M	FUJIFILM Cellular Dynamics, Inc.	R1257
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Endothelial Cells, 01434, 0.5M	FUJIFILM Cellular Dynamics, Inc.	C1236
Isogenic iCell CardioSpheres from Donor 11713		
iCell Cardiomyocytes, 11713, 1M †	FUJIFILM Cellular Dynamics, Inc.	R1105
• iCell Cardiomyocytes Plating Medium, 30 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Cardiac Fibroblasts, 11713, 0.5M	FUJIFILM Cellular Dynamics, Inc.	R1256
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Endothelial Cells, 11713, 0.5M	FUJIFILM Cellular Dynamics, Inc.	C1235
Media		
iCell Cardiomyocytes Media Kit	FUJIFILM Cellular Dynamics, Inc.	R1151
• iCell Cardiomyocytes Plating Medium, 30 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
• iCell Cardiomyocytes Maintenance Medium, 100 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Cardiac Co-Culture Supplements Kit ‡	FUJIFILM Cellular Dynamics, Inc.	R1258
• iCell Cardiac Co-Culture Supplement A, 25 ml	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
• iCell Cardiac Co-Culture Supplement B, 250 µl	FUJIFILM Cellular Dynamics, Inc.	(included in kit)
iCell Plating Supplement B (1000X) §	FUJIFILM Cellular Dynamics, Inc.	M1049

† A larger vial size of iCell Cardiomyocytes is available (≥4M size) for donor 11713 (R1106) or 01434 (R1007), if needed. Similarly, a larger vial size of iCell Cardiomyocytes^2 is available (≥5M size) for donor 01434 (R1017).

‡ Two kits of iCell Cardiac Co-Culture Supplements (R1258) are required to make enough Complete iCell Cardiac Co-Culture Medium to carry out the assay for two weeks.

§ iCell Plating Supplement B is optional. For more uniformly "round" and compact spheroids, add iCell Plating Supplement B to the media during cell seeding. Refer to Figure 4 below for additional data on the effects of iCell Plating Supplement B on CardioSpheres formation.

^ Various sources of 3D spheroid-forming ULA plates have been tested with equivalent results (Figure 3), including BIOFLOAT™ 96-well plates (F202003), faCellitate 384-well plates (F224384) and Akura™ 96 Spheroid Microplate (InSphero).

Workflow

Thawing cryopreserved cells into 3D spheroid-forming ultra-low attachment (ULA) plates is a simple approach to create functional cardiac microtissues.

- **Day 0:** Thaw each cell type into iCell Cardiomyocytes Plating Medium (iCPM) with iCell Plating Supplement B (optional).
    - iCell Cardiomyocytes or Cardiomyocytes^2 (C)
    - iCell Cardiac Fibroblasts (F)
    - iCell Endothelial Cells (E)
    - Prepare cell suspensions in Complete iCell Cardiac Co-Culture Medium (with iCell Plating Supplement B (optional)) and combine cells at the recommended ratio of 65:15:20 [C:F:E] to prepare a tri-culture mixture. Dispense mixture of cells into the ULA plate.
- **Day 1:** Add more complete iCell Cardiac Co-Culture Medium (2X the previous plating volume) to each well containing cells.
- **Day 2 or 3:** Replace 50% of the spent medium with Complete iCell Cardiac Co-Culture Medium.
- **Day 4 - 14:** Perform 50% media changes every 2-3 days.
- **Day 14 or later ("Day of Assay"):** Perform a cell viability or calcium oscillation assay as desired.

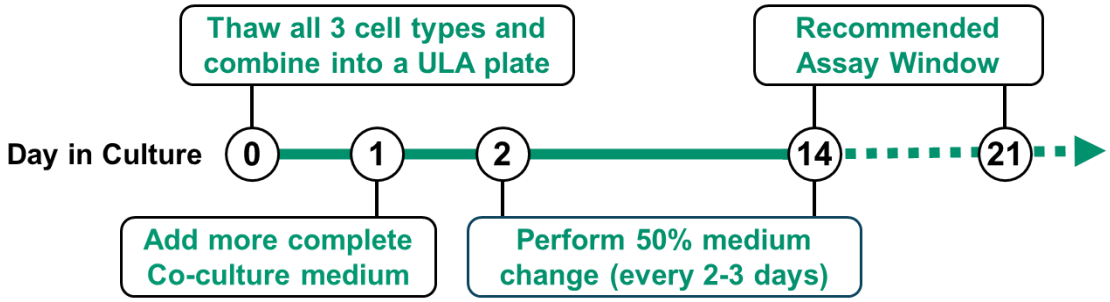

Figure 1. Schematic Workflow for the Generation of iCell CardioSpheres.

Tips Before Starting

1. Refer to the User's Guides for iCell Cardiomyocytes, iCell Cardiomyocytes^2, iCell Cardiac Fibroblasts, and iCell Endothelial Cells for detailed information on storage and handling of the cells and media and supplements.
2. Read the entire Application Protocol to become familiar with the assay workflow.
3. Navigate to www.fujifilmcdi.com/coa-lookup/ to find the Certificate of Analysis (CoA) for each cell type that includes lot-specific information for the number of **Viable Cells/Vial**. It is recommended to use this number instead of counting the cells manually or with an automated cell counter. This Application Protocol was developed using CoA counts for all lots of iCell products tested.
4. iCell Plating Supplement B (1000X) is designed to improve cell survival post-thaw and during the formation of CardioSpheres and should be added only for the first 24-48 hours post thaw.

Methods

Preparing the Medium

1. Thaw iCell Cardiomyocytes Plating Medium, iCell Cardiomyocytes Maintenance Medium, and iCell Cardiac Co-Culture Supplement B overnight at 4°C.
2. Prepare the Compete iCell Cardiac Co-Culture Medium by adding 250 µl of iCell Cardiac Co-Culture Supplement B to 25 ml of iCell Cardiac Co-Culture Supplement A first, then mixing by inversion 2-3 times, and finally transferring the entire 25 ml mixture into the 100 ml bottle of iCell Cardiomyocytes Maintenance Medium. It is recommended, but optional, to add 1.25 ml of penicillin/streptomycin to the complete media (Co-Culture Medium).
3. Store the Co-Culture Medium at 4°C for up to 3 weeks.

iCell Cardiac Co-Culture Medium – 125 ml

Component	Volume	Final Concentration
iCell Cardiomyocytes Maintenance Medium	100 ml	N/A
iCell Cardiac Co-Culture Supplement A (5X)	25 ml	1X
iCell Cardiac Co-Culture Supplement B (500X)	0.25 ml	1X
Penicillin-streptomycin (100X) **<optional>**	1 ml	1X

Thawing the Cells and Forming CardioSpheres

The following procedure outlines the steps for forming iCell CardioSpheres in a 96-well ULA plate containing 10,000 cells in 100 µl of media per well. For 384-well format, adjust total cell counts and volumes to 5,000 cells in 50 µl of media per well.

1. Equilibrate iCell Cardiomyocytes Plating Medium (iCPM) and Co-Culture Medium to room temperature.

   Note: *For workflow with iCell Plating Supplement B, create separate aliquots of iCPM (20 ml) and the Co-Culture Medium (10-15 ml) with 1X iCell Plating Supplement B.*

2. Thaw one vial of iCell Cardiomyocytes into iCPM (with iCell Plating Supplement B, if desired), according to the iCell Cardiomyocytes User's Guide.

   Note: *Smaller sizes of cells (EX: 1M size) typically have ≤1 ml of cryo solution in the vial.*

3. After thawing and pelleting the cells, determine the total number of viable cells from the reported value on the Certificate of Analysis (CoA). Use Co-Culture Medium (with iCell Plating Supplement B, if desired) to gently resuspend the cell pellet to a concentration of 100,000 cells/ml.

   Note: *The Certificate of Analysis (CoA) for the iCell products can be found online here:* *www.fujifilmcdi.com/coa-lookup/*

4. Repeat steps 2 – 4 for each additional cell type.
5. Prepare a mixed cell suspension for all wells in a 50 ml conical tube according to Table 1. The total volume needed to fill a 96-well plate is ~10 ml.

Table 1. Cell Suspension Preparation for iCell CardioSpheres.

	%	Cells per microtissue	Amount for 1x 96-well plate (ml)
iCell Cardiomyocytes or iCell Cardiomyocytes[2]	65	6,500	6.5
iCell Cardiac Fibroblasts	15	1,500	1.5
iCell Endothelial cells	20	2,000	2
Total cells	100	10,000	10

6. Transfer cell suspension to a reagent reservoir with a multi-channel pipette.
7. Using a multi-channel pipette, dispense 100 µl of cell suspension (10,000 cells/well) to each well of a 96-well ULA plate.

   Note: *Gently pipette up and down once between each transfer step to mix the cells in the reagent reservoir, which helps to maintain a uniform seeding density.*
8. Centrifuge the 96-well ULA plate at 1000 x g for 5 minutes to ensure the cells are collected at the bottom of the wells.

   Note: *A centrifuge with a microplate adaptor is required for this step. The high spin speed is recommended for efficient and uniform spheroid formation.*
9. Place the ULA plate in a cell culture incubator at 37°C, 5% CO_2 and culture overnight.
10. After 24 hours, add an equal volume (100 ul) of warmed up Complete Co-Culture Medium to all wells containing cells. Return the ULA plate back to the incubator for continued culture. 3D spheroids will form within the first 24-48 hours.

Maintaining the CardioSpheres

For a weekend-free workflow, perform 50% media changes every Mon, Wed, and Fri.

1. Warm an aliquot (~10 ml per 96-well plate) of Co-Culture Medium to room temperature prior to every media change step.
2. Carefully remove 100 µl of spent media with a multi-channel pipette from each well and replace with 100 µl of fresh Co-Culture Medium.

   Note: *Visually inspect pipette tips to ensure that all spheroids remain in well following media removal.*
3. Maintain the ULA plate containing 3D spheroids in a cell culture incubator at 37°C, 5% CO_2 until ready for assay. For most assays, it is recommended to culture iCell CardioSpheres until Day 14.

Representative Data

The modular nature of this protocol enables flexibility for multiple assay endpoints. The data presented in this section highlights how iCell CardioSpheres can be tracked over time and their morphology analyzed. Additionally, it shows how they can be imaged via microscopy and/or tested in functional calcium oscillation assays.

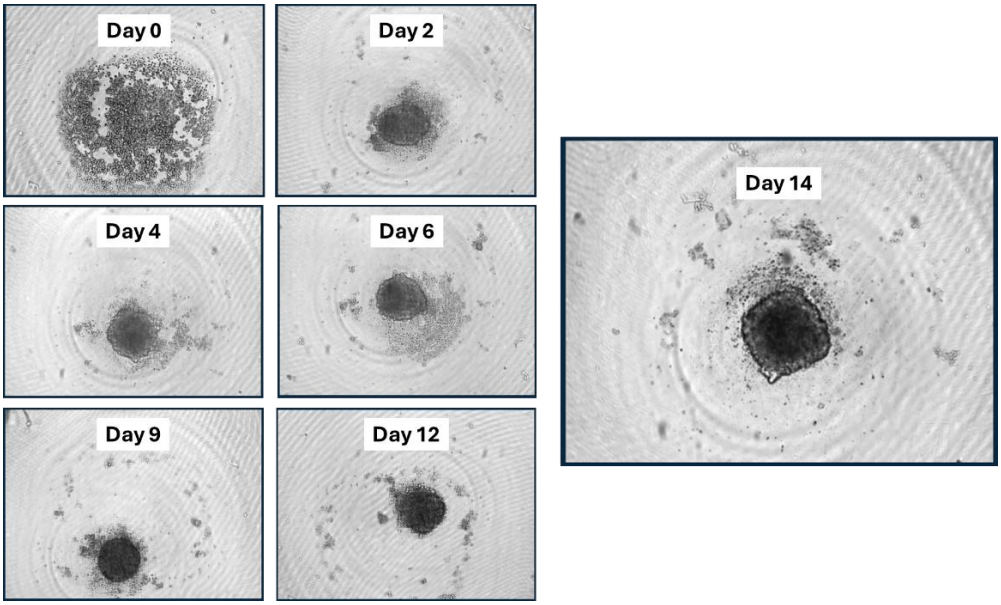

Figure 2. Structure of iCell CardioSpheres Over Time.
iCell Cardiomyocytes, iCell Cardiac Fibroblasts, and iCell Endothelial Cells (all from donor 11713) were used to form iCell CardioSpheres in a 384-well ULA plate (Sbio). Each well contains 5,000 total cells that were seeded on Day 0. Spheroid formation happens within the first 24-48 hours (see Day 2 image) and is maintained over time (through Day 14 and beyond; data not shown). An Incucyte® SX5 Live-Cell Analysis System (Sartorius) equipped with the Incucyte® Spheroid Analysis Software Module was used for kinetic acquisition of spheroids in the ULA plate.

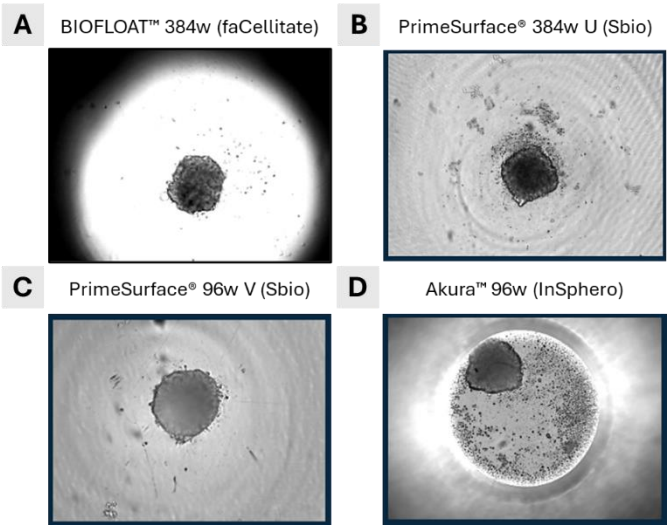

Figure 3. iCell CardioSpheres in Various ULA Spheroid-Forming Plates.
5,000 total cells were seeded in (**A**) BIOFLOAT™ 384-well plate or (**B**) PrimeSurface® 384-well U-bottom plates and representative images of 3D spheroids were captured on Day 14. Similarly, iCell CardioSpheres from 10,000 total cells were formed in a (C) PrimeSurface® 96-well V-bottom plate or a (D) Akura™ 96-well spheroid microplate (InSphero). All 3D spheroid images were acquired on an Incucyte® SX5 Live-Cell Analysis System (Sartorius). iCell Cardiomyocytes^2, iCell Cardiac Fibroblasts and iCell Endothelial cells from donor 01434 were used to form these CardioSpheres. The cells were seeded in Co-Culture Medium containing iCell Plating Supplement B (M1049).

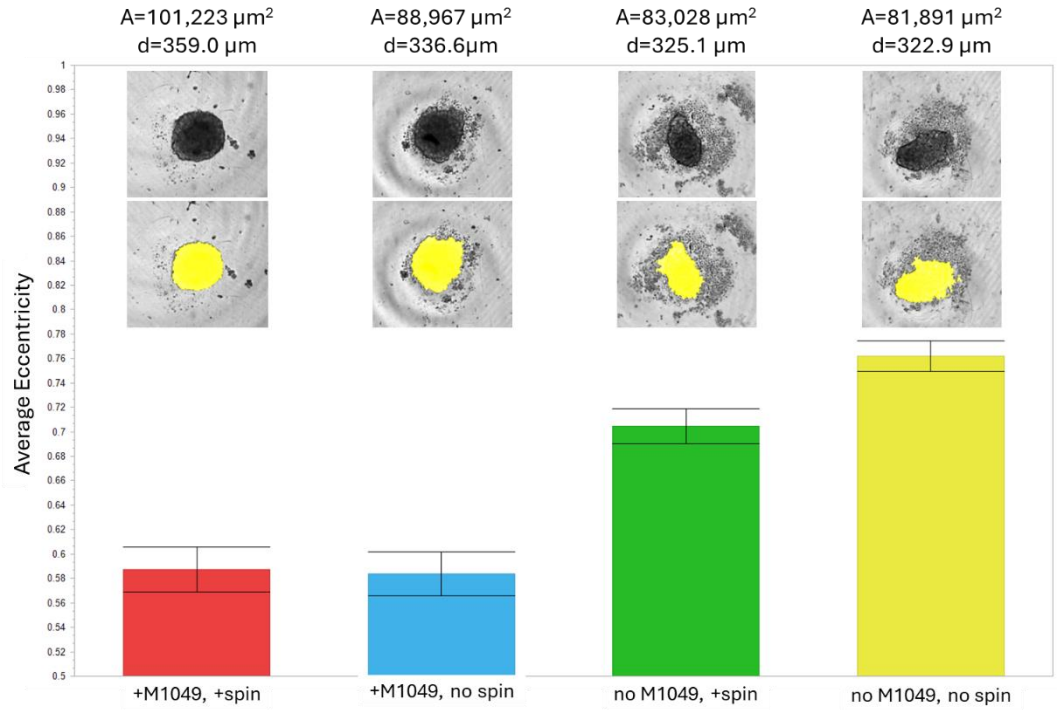

Figure 4. Impact of iCell Plating Supplement B and Centrifugation on the Size and Eccentricity of iCell CardioSpheres at Day 14.

Tri-culture cell suspensions (5000 total cells) were prepared in the Co-Culture Medium in the presence or absence of iCell Plating Supplement B (M1049) during initial seeding in a PrimeSurface® 384-well U-bottom ULA plate and with or without centrifugation of the ULA plate after seeding. After 14 days in culture, the average area (A), diameter (d), and eccentricity of iCell CardioSpheres formed under the different conditions (n=77 wells for each) were analyzed on the Incucyte® SX5. A perfect circle has an eccentricity of 0, and an ellipse has an eccentricity between 0.5 and 1. iCell Cardiospheres formed with M1049 are typically rounder and more uniform, with area (A) =90-100,000 µm^2, diameter (d) = 336-359 µm, and eccentricity around 0.55. Tri-culture microtissues generated in the absence of M1049 are a little smaller (A = 81-83,000 µm^2 and d = 325 µm), display more heterogeneity and adopt a slightly more elliptical shape with eccentricities ranging from 0.7-0.75.

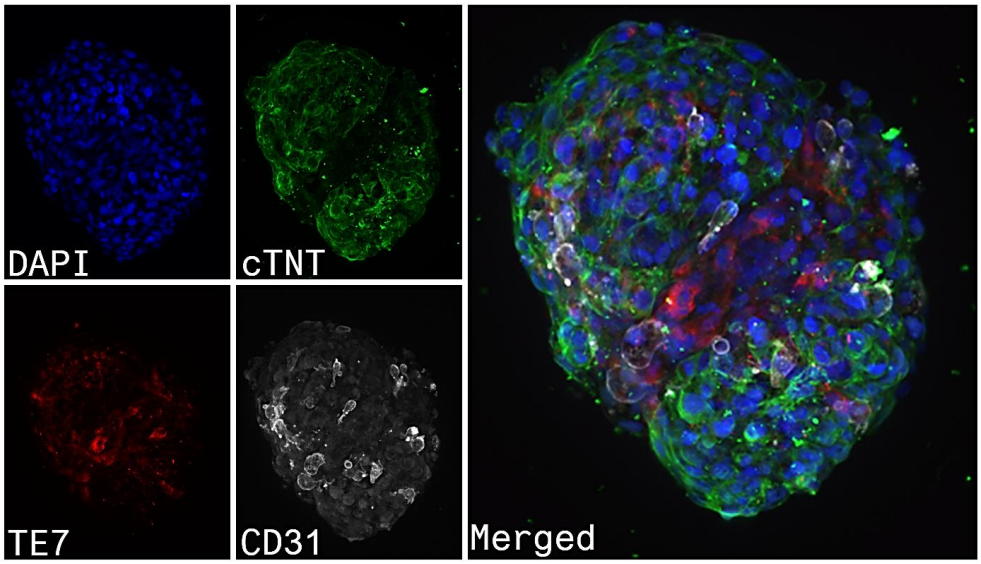

Figure 5. Immunostaining of iCell CardioSpheres Enables Identification of the Three Cell Types.

Photo micrographs of iCell CardioSpheres fixed and immunostained on DIV 14 identifies iCell Cardiomyocytes (green, anti-cTNT), iCell Cardiac Fibroblasts (red, anti-TE7), and iCell Endothelial Cells (gray, anti-CD31) with nuclei (blue, Hoechst) in 3D tri-culture spheroids (merged image of iCell CardioSpheres).

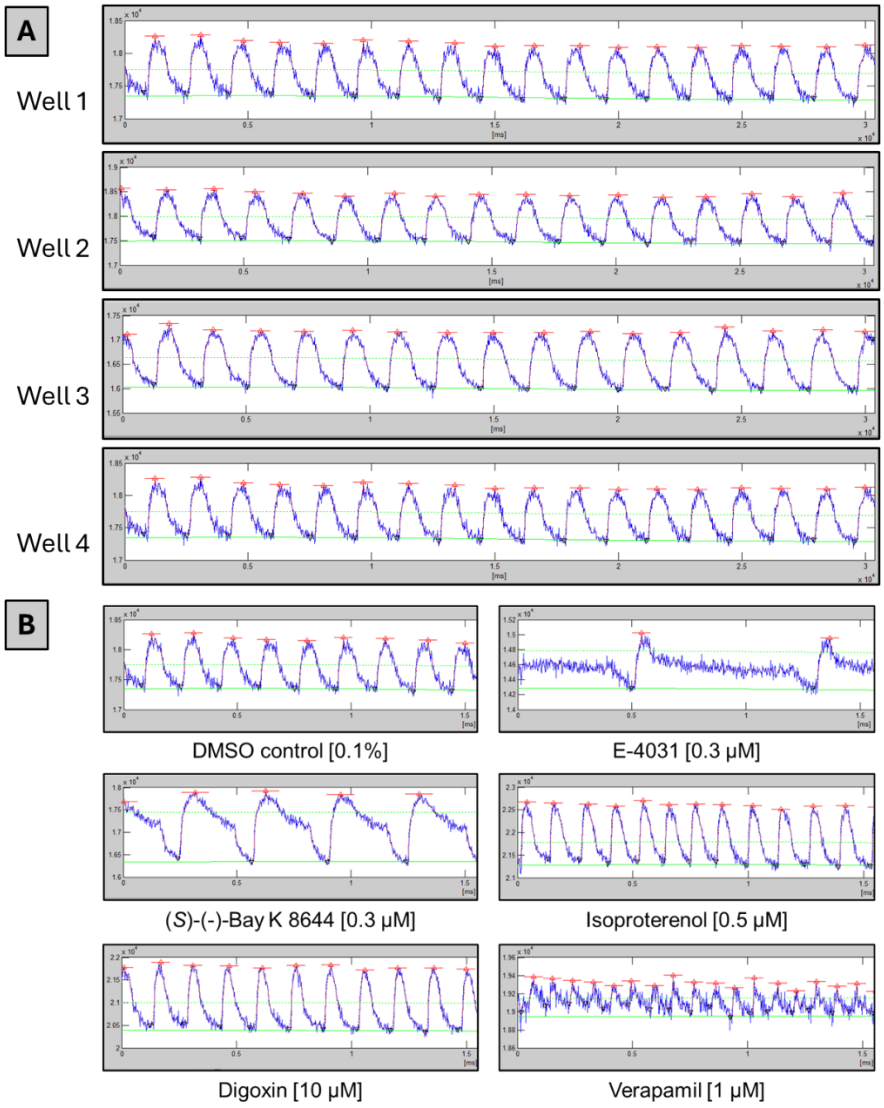

Figure 6. Calcium Transient Recordings from iCell CardioSpheres.
CardioSpheres (5000 total cells per well) were formed from iCell products from donor 11713 in a 384-well ULA plate (Sbio) and assayed on Day 14 using the FDSS/µCell (Hamamatsu). CardioSpheres were loaded with 1X Calcium 6 dye (Molecular Devices) for 2 hours prior to testing. (**A**) Representative 30 second baseline recordings from 4 different wells illustrate the consistent calcium waveform shape in terms of peak height (amplitude), peak width (calcium transient duration; CTD), and peak spacing (peak rate or beat rate). (**B**) iCell CardioSpheres respond to compounds known to modulate cardiac activity, including E-4031 (hERG channel blocker), Bay K 8644 (calcium channel activator), Isoproterenol (beta-adrenergic receptor agonist and positive inotrope), Digoxin (Na-K ATPase enzyme inhibitor and positive inotrope), and Verapamil (calcium channel blocker). Calcium waveform traces indicate that each drug showed the expected response compared to DMSO control. The timescale is ~15 seconds.

Summary

Co-culturing isogenic iCell Cardiomyocytes, iCell Endothelial Cells, and iCell Cardiac Fibroblasts together right out of thaw (no pre-plating needed) into ULA plates offers a simple and direct approach to generate consistent 3D CardioSpheres *in vitro*. iCell CardioSpheres offer a physiologically relevant model system that can be used for high-throughput screening for drug discovery, and safety pharmacology studies. This model system will aid in understanding cardiac liability or drug effects on not only the cardiomyocytes but also the non-myocyte (fibroblasts and endothelial) population that are the other major cell types in the heart.

Revision History

Version 2.0: April 2024

AP-CMC_CardioSpheres_30APR2024