FUJIFILM Cellular Dynamics

iCell® Cardiomyocytes
Application Protocol

Culturing and Assaying Calcium Transients of 3D Cardiac Tri-Culture Microtissues

Introduction

The human heart is a complex tissue. Ventricular cardiomyocytes make up only 50% of all the cells in the left ventricle (1). Therefore, there is potential for a three-dimensional (3D) multi-cellular system containing cardiomyocytes, endothelial cells and cardiac fibroblasts to have greater physiological relevance, predictive power, and mechanistic insight than cardiomyocytes alone (2,3). Culturing iPSC-derived cardiomyocytes in a 3D microtissue offers a simple and physiologically relevant approach to recapitulate in vivo heart biology while providing an approach for stem cell-derived cardiomyocyte maturation in vitro.

This Application Protocol describes a method for combining three cardiovascular cell types (iCell® Cardiomyocytes, iCell Endothelial Cells, and primary cardiac fibroblasts) directly into 3D cardiac tri-culture microtissues in a 96 well plate without pre-culture. The thawing method, assay plate, microtissue cell number, composition, and co-culture media formulation have all been optimized for robust performance in calcium transient assay. Representative data on microtissue size, structure and composition during the assay window is also shown. Using the EarlyTox calcium indicator dye and Clariostar for measuring calcium transients, baseline cardiomyocyte function in tri-culture microtissues was measured. Furthermore, 3D cardiac tri-culture microtissues demonstrated positive response to the inotropic compound isoproterenol, which is characteristic of a mature cardiomyocyte.

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, Consumables, and Software

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

Item	Vendor(s)	Catalog Numbers
Equipment		
12-channel Pipettor, 200 µL	Multiple Vendors	
Consumables		
iCell Cardiomyocytes, 01434 or 11713	FUJIFILM Cellular Dynamics, Inc. (FCDI)	R1057 or R1105
• 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 Cardiomyocytes^2, 01434, 1.25M	FUJIFILM Cellular Dynamics, Inc. (FCDI)	C1058 or R1059
• 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 Endothelial Cells, 11713	FUJIFILM Cellular Dynamics, Inc. (FCDI)	C1114
Primary Human Cardiac Fibroblasts	Promocell	C-12375
Endothelial Cell Growth Medium Kit	Promocell	C-22110
PrimeSurface® V-bottom 96-well plate	Wako	629-01099
Corning 384-well plates (optional)	Millipore Sigma	CLS4516
EarlyTox Cardiotoxicity Kit	Molecular Devices	R8210

Item	Vendor(s)	Catalog Numbers
Software		
Clariostar	BMG Labtech	

Workflow

The recommended timepoint for measuring calcium transients is 14 to 21 days after microtissue formation.

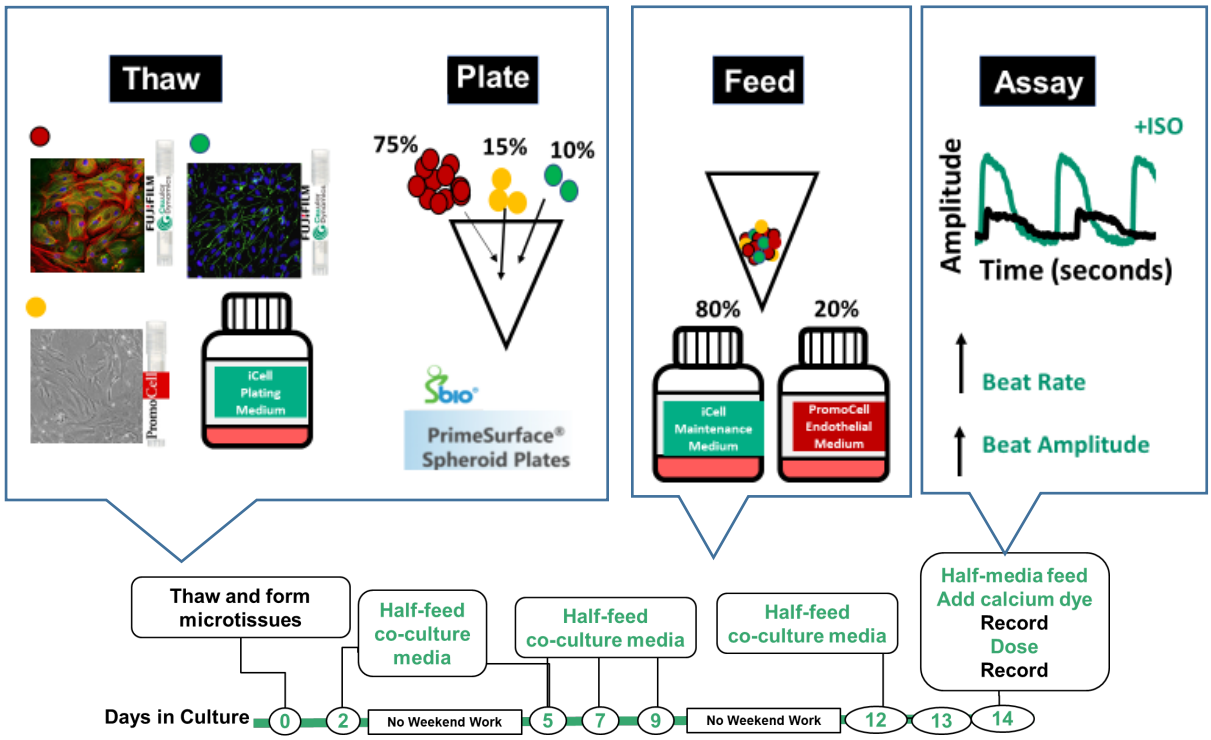

Figure 1: A Schematic Showing Different Steps Involved in Assembling the Triculture Microtissue and Timeline of the Workflow From Day 0 Until Ready for Assay.

- **Day 0:** Thaw the iCell Cardiomyocytes, iCell Endothelial Cells and cardiac fibroblasts into iCell Cardiomyocytes Plating Medium. Count cells and distribute 5,000 viable cells in 200 µl media per well of a 96-well plate.
- **Day 2 - Day 14 or Day 21**. Half feed every other day. In 96-well plate, replace 100 µl media with 100 µl co-culture media.
- **Assay Day:** Half feed as described above. Then, replace 100 µl media with calcium dye. Incubate two hours. Record baseline calcium transients. Dose with compounds. Record post-dose calcium transients after 5-30 minutes.

Methods

Preparing the Medium

1. Thaw iCell Cardiomyocytes Plating Medium, iCell Cardiomyocytes Maintenance Medium, and Endothelial Cell Growth Medium supplement overnight at 4°C.
2. Prepare complete Endothelial Cell Growth Medium by adding supplement to the according to manufacturer's instructions.
   - Aseptically, mix the supplement solution by carefully pipetting up and down and transfer the entire contents to the Basal Medium.
   - Close bottle and swirl gently until homogenously mixed.
   - Complete Endothelial Growth Medium is good for 6 weeks at 4°C.
   - Do not freeze the complete medium.
3. Prepare the co-culture medium by adding complete Endothelial Cell Growth Medium to iCell Cardiomyocytes Maintenance Medium in a suitable container according to Table 1.

Table 1. Co-culture Medium Preparation – *Stable for 2 weeks at 4°C*

	Vendor	Catalog #	%
iCell Cardiomyocytes Maintenance Medium	FCDI	M1003	80
Endothelial Cell Growth Medium	Promocell	C-22010	20
Total			100

Thawing the Cells and Forming Microtissues

1. Pre-warm the iCell Cardiomyocyte Plating Medium and the prepared co-culture medium in 37ºC water bath for 10 minutes.
2. Thaw all cells into iCell Cardiomyocyte Plating Medium and confirm cell viability and count using a hemocytometer with trypan blue or an automated cell counter.
   - Thaw cell vials in 37°C water bath for 2-3 minutes per User's Guide.
   - Transfer contents of cell vial into separate 50 ml conical tubes. Slowly add 2 ml of iCell Cardiomyocyte Plating Medium drop-wise into each conical tube while swirling gently to mix.
   - Transfer the contents of each 50 ml conical tube to individual 15 ml conical tubes.
3. Centrifuge cells at 300 x g for 5 minutes and adjust each cell type to 100,000 viable cells/ml in co-culture medium.

   Note: *The Certificate of Analysis (COA) for the iCell products can be found online here:* fujifilmcdi.com/resources/coa-lookup/
4. Prepare a mixed cell suspension for all wells in a 50 ml conical tube according to Table 2.

Table 2. Cell Suspension Preparation for 5,000 Cells per Microtissue.

	%	Cells per microtissue	Viable cells per ml	Amount for 1x 96-well plate (ml)
iCell Cardiomyocytes or iCell Cardiomyocytes[2]	75	3,750	$1x10^5$	3.75
Cardiac fibroblasts	15 (*)	750	$1x10^5$	0.75
iCell Endothelial cells	10	500	$1x10^5$	0.5
Total cells	100	5,000		
Co-culture media				15
Final volume				20

**The optimal concentration of cardiac fibroblasts depends on the source and passage number of the cardiac fibroblasts. We recommend using 15% PromoCell human cardiac fibroblasts at passage 2.*

5. Transfer cell suspension to a trough compatible with a multi-channel pipette.
6. Using a multi-channel pipette, dispense 200 µl of cell suspension (5,000 cells/well) to the center of each well of a 96-well S-Bio V bottom well plate.

   Note: *Gently pipette up and down once to mix cell suspension in trough prior to dispensing each row to maintain an even seeding density.*
7. Culture the cells in a cell culture incubator at 37°C, 5% CO_2 overnight. Microtissues will form in 24 hours.

Maintaining the Microtissues

1. Warm an aliquot of 10-12 ml of prepared co-culture medium per 96-well plate.
2. Carefully remove 100 µl from the top of each well with multi-channel pipette and replace with 100 µl of co-culture media.
3. Visually inspect pipette tips to ensure that all spheroids remain in well following media removal.
4. Culture the spheroids in a cell culture incubator at 37°C, 5% CO_2.

Assaying for Calcium Flux

1. On the day of the assay, replace the medium as described above.
2. Reconstitute EarlyTox Cardiotoxicity Dye (Component A) from the kit according to the manufacturer's instructions.
   - Briefly, remove one vial of Component A from the freezer and equilibrate to 37°C.
   - Dissolve the contents of Component A by adding 10 ml of dilution buffer (Component B).
   - Mix by vortexing for 1-2 minutes.
3. Replace 100 µl medium with 100 µl of reconstituted Component A and incubate at 37ºC, 5% CO_2 for 2 hours.

   Note: *An assay volume of 200 µl lowers the risk of aspirating the microtissues when removing the medium.*

4. Pre-warm the plate reader to 37°C at least 30 minutes before assay.
5. Perform a baseline recording of calcium flux activity on the CLARIOstar using the parameters listed in Table 3.

Table 3A. and 3B. CLARIOstar Settings

3A.

Instrument Settings	Parameter
Measurement Method	Fluorescence Intensity, Well Mode
Monochromator	Ex: 483-14 and Em: 530-30.
Reading Mode	Bottom Reading
Gain	1000
Focal Height	2.4 mm
Gain Adjustment	40%

3B.

Kinetic Settings	Parameter
No. of Intervals	1000
No. of Flashes per Interval	1
Interval Time	0.02
Gain	1000
Total Measurement Time per Well	20 sec

6. Prepare stock solutions of dosing compounds in co-culture medium at a concentration of 5X higher than the final concentration in a separate 96-well cell culture plate.
7. Equilibrate the plate with the dosing compounds in the incubator at 37°C, 5% CO_2 for at least 10 minutes.
8. Remove the plate with the dosing compounds from the instrument and dispense 50 µl of the 5X dosing compound solution per well containing the spheroids with the reconstituted Component A.
9. Gently mix the dosing compound solution in with the medium of each well containing a microtissue using a P200 pipette set to 100 µl.

   Note: *Mixing the solutions improves the assay performance. The microtissues will settle very quickly following the mixing step.*
10. Return the plate containing the microtissues to the incubator and incubate at 37°C, 5% CO_2.
11. Perform a post-dose recording of calcium flux activity on the CLARIOstar using the parameters listed in Table 3 after 5-30 minutes.

Representative Data

Table 4. Success Rate of Creating Microtissue.

Contracting tri-culture microtissues per plate	**96/96**
Independent experiments	**7**
Total MT assayed	**692**

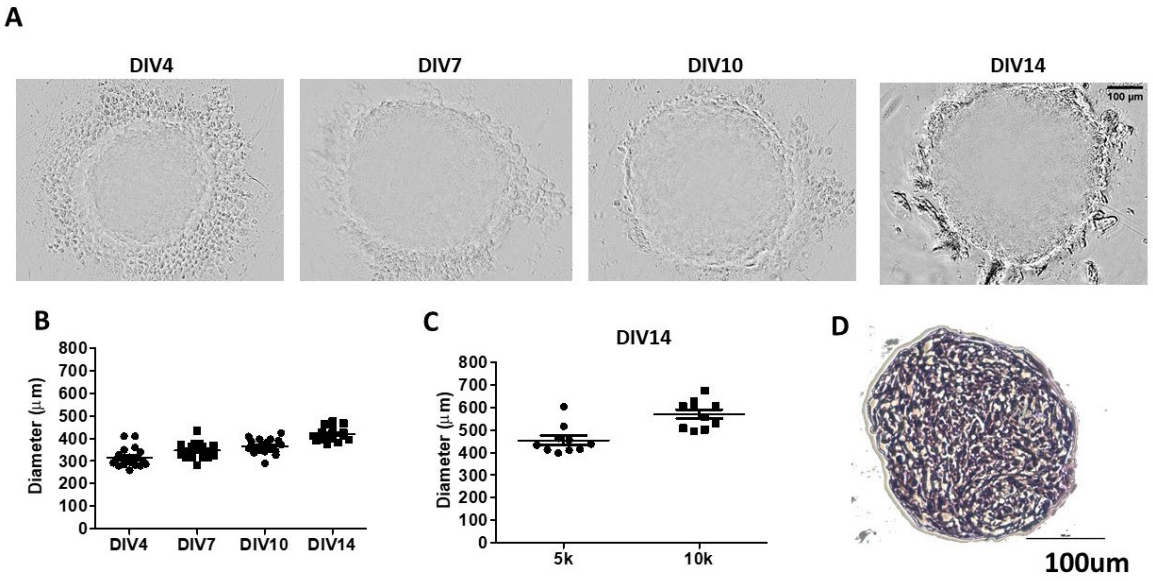

Figure 2. Structure of 3D Cardiac Tri-culture Microtissues Over Time.
Microtissues were formed containing 5,000 or 10,000 total cells in S-bio 96-well plates. Compact, contracting microtissues were obtained by Day 4. (**A**) Panel of phase contrast 10X images of 5,000 cell tri-culture spheroids over time using the Incucyte S3. (**B**) Quantification of 5,000 cell tri-culture microtissue diameter. Each dot represents a microtissue. Mean and SEM are indicated. (**C**) Comparison of Day 14 microtissue diameter of 5,000 cell tri-culture microtissues with 10,000 cell tri-culture microtissues. Each dot represents a microtissue. Mean and SEM are indicated. (**D**) H&E staining of Day 14 3D cardiac tri-culture microtissue formed with 10,000 total cells. Staining shows the absence of a necrotic core.

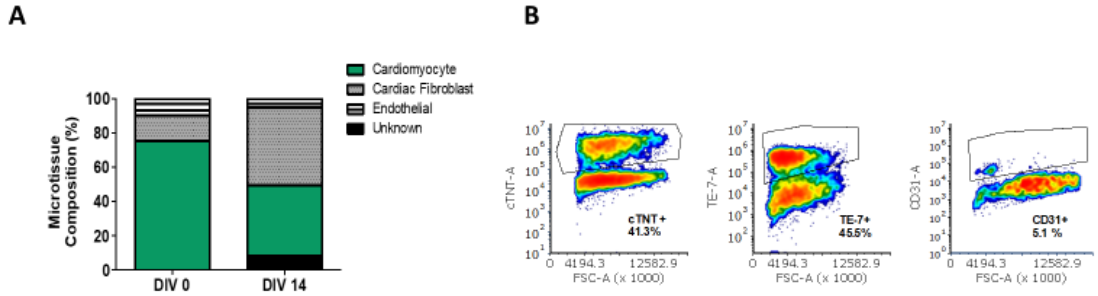

Figure 3. Composition of 3D Cardiac Tri-culture Microtissues Over Time.
Microtissues were formed according to this protocol (75% iCell Cardiomyocytes, 15% Human Cardiac Fibroblasts, 10% iCell Endothelial Cells) and assayed by flow cytometry for cell type specific markers at Day 14. (**A**) Summary of composition at Day 0 and Day 14. (**B**) Representative flow plots cardiomyocyte (cTNT+), cardiac fibroblast (TE-7+), and endothelial cells (CD31+) from microtissues dissociated at Day 14. The composition of cardiac fibroblasts tripled over the course 14 days while the composition of endothelial cells decreased slightly.

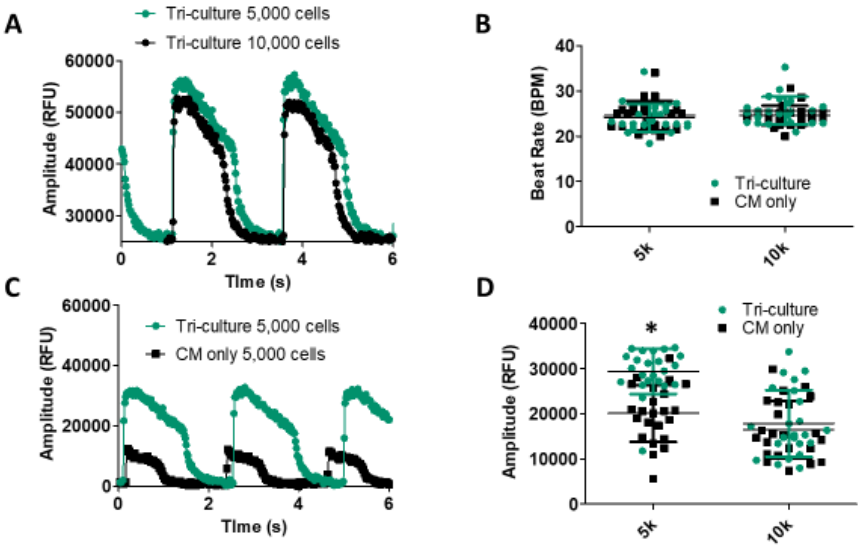

Figure 4. Baseline Calcium Transients in Cardiomyocyte and Tri-culture Microtissues at Day 14.
Calcium transients were measured using EarlyTox calcium dye. (A) Representative calcium traces for 5,000 and 10,000 total cell 3D Tri-culture cardiac microtissues. (B) Beat rate is not different between 5,000 or 10,000 cell tri-culture microtissues and control cardiomyocyte only microtissues. (C) Representative calcium traces for 5,000 cell tri-culture and 5,000 cell cardiomyocytes only microtissues (CM only). (D) Amplitude is significantly higher in tri-culture microtissue compared to cardiomyocyte only microtissues (CM only) at 5,000 cells, but similar at 10,000 cells.

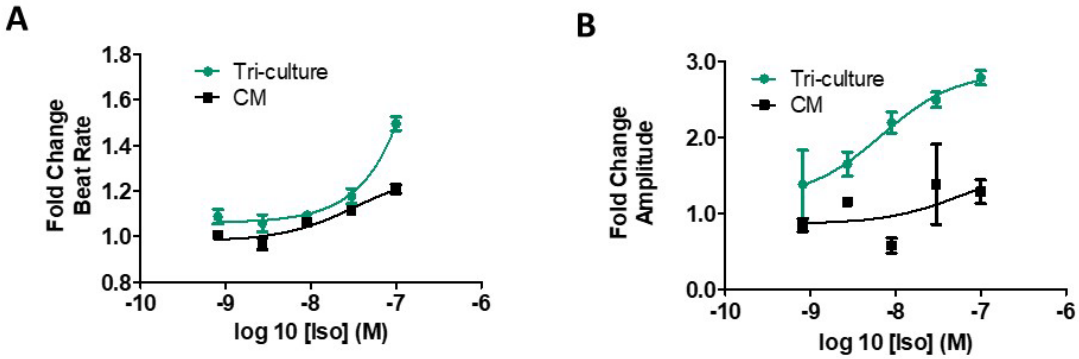

Figure 5. 3D Cardiac Tri-culture Microtissues Response to Beta Adrenergic Agonist Isoproterenol.
(A) Chronotropic response: Control iCell Cardiomyocytes, 11713 only microtissues (CM) and tri-culture microtissues exhibit an increase in beat rate with increasing concentrations of isoproterenol. (B) Inotropic response: Control iCell Cardiomyocytes, 11713 only microtissues (CM) do not increase amplitude with increasing concentrations of isoproterenol. Tri-culture microtissues demonstrate a twofold increase in beat amplitude with increasing concentrations of isoproterenol.

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Revision History

Version 1.0: May 2021

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