Structural Organization and Functional Analysis of Compound Response in 3D Human iPSC-derived Cardiac Tri-Culture Microtissues

Simon Lydford, Zhisong Tong, Carole Crittenden, Angeline Lim, Oksana Sirenko | Molecular Devices LLC
Sarah Himmerich, Cara Rieger, Ravi Vaidyanathan, Coby Calson | FUJIFILM Cellular Dynamics, Inc.

Introduction

The human heart is a complex organ providing highly regulated processes of moving blood through the body. The adult human ventricle is comprised of cardiomyocytes, endothelial cells, fibroblasts and other supporting cell types. Though cardiomyocytes make up 75% of the total volume of human ventricle, it only constitutes 50% of the total cell number. Recent publications show that tri-cellular co-culture microtissues of cardiomyocytes, endothelial cells and cardiac fibroblasts that are all derived from human iPSC enhance the maturation and functional activity of cells compared to 2D cardiomyocytes and thus more closely mimics actual heart physiology.

In this study, we used a tri-culture model created by mixing iPSC-derived cardiac cells with primary adult fibroblasts and iPSC-derived endothelial cells at 75:15:10 ratio in ultra-low attachment (ULA) plates directly from thaw. We used a Biomek i7 liquid handling system for cell plating and subsequent media exchange (every 2 days). 3D microtissues were formed within 48 hours and started to contract spontaneously and regularly on day 5. We investigated the functional activity of microtissues by recording calcium oscillations after addition of calcium dye using a fast kinetic fluorescence recording instrument (FLIPR® Penta High-Throughput Cellular Screening System—Figure 1). We tested the response of the microtissues to a number of known modulators of cardiac activity. Importantly, we found that molecules like isoproterenol significantly accelerated the oscillation rate and also increased the peak amplitude (inotropic response). Other compounds, including hERG inhibitors, ion channel blockers, or beta-blockers, demonstrated changes in the Ca^{2+} oscillation patterns consistent with expected mode of action. Waveform analysis was performed using the ScreenWorks® Peak Pro 2™ software module.

Additionally, we used high content imaging to characterize the structure and morphology of our 3D microtissues with the ImageXpress® Micro Confocal High-Content Imaging System—Figure 1. Different cell types were immunostained using antibodies specific for Troponin T (for cardiomyocytes), VE-Cadherin (for endothelial cells), and COL1A1 (for fibroblasts). The 3D structure of microtissues was reconstructed and analyzed using MetaXpress® High-Content Image Acquisition and Analysis Software. The data presented here highlights the utility and biological relevance of using iPSC-derived cell types in 3D micro-tissues as promising model for measuring compound effects on human cardiac tissues in high throughput format.

Instruments

- We used a high speed EMCCD camera on the FLIPR Penta cellular screening system to measure the patterns and frequencies of the Ca^{2+} oscillations of cardiac tri-culture microtissues as monitored by changes in intracellular Ca^{2+} levels with the EarlyTox™ Cardiotoxicity Kit (Molecular Devices). The instrument was equipped with ScreenWorks PeakPro2 peak analysis software allowing analysis and characterization of the primary and secondary peaks and complex oscillation patterns.
- We used the ImageXpress Micro Confocal High-Content Imaging System equipped with spinning disk confocal and sCMOS camera to capture the 3D structures of the whole microtissues, stained with antibodies in different fluorescent channels. The custom module editor which can be customized for adequate image analysis was used to reconstruct the 3D structure of the microtissues.

Figure 1. FLIPR Penta High-Throughput Cellular Screening System and ImageXpress Micro Confocal High-Content Imaging System.

Methods

Procedures

Cryopreserved human iPSC-derived iCell Cardiomyocytes[2], iCell Endothelial Cells from FUJIFILM Cellular Dynamics, Inc. and Primary Human Cardiac Fibroblasts Cells from Promocell were seeded at 75%:15%:10%. The timepoint of workflow is shown above. The presence of strong synchronous contractions in the 3D cultures was confirmed visually prior to running experiments. To assess phenotypic changes, cells were stained live using a mixture of two dyes: the viability dye Calcein AM (1 µM) and the Hoechst nuclear dye (2 µM) (all from Life Technologies). After the compound treatment, cells were measured with FLIPR, then fixed and stained with either a combination of anti Troponin T A647 and anti VE-Cadherin A568 or anti Troponin T A647 and anti COL1A1 A568.

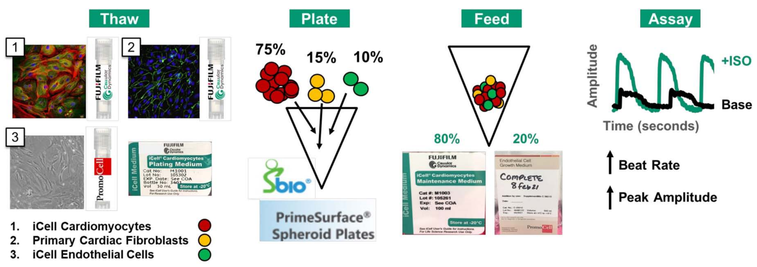

Figure 2. The workflow of the iPSC-derived Cardiac Tri-Culture Model.

Results

Recording and Analysis of Kinetic Patterns

On the day of assay, cardiac tri-culture microtissues were loaded with the EarlyTox Cardiotoxicity Kit and treated with compounds for 60 minutes. Spontaneous calcium oscillations were recorded using 30–50 frames per second to allow proper resolution of the complex oscillation patterns. Advanced analysis methods were implemented to provide multi-parametric characterization of the Ca^{2+} flux oscillation patterns. This phenotypic assay allows for the characterization of readouts such as oscillation frequency, amplitude, peak width, peak raise and decay times, and irregularity. In addition, appearance of EAD-like (early-depolarization-like event) patterns, peak prolongation, and peak irregularity were evaluated. Representative tracing curves of the fluorescence signals are shown in Figure 3.

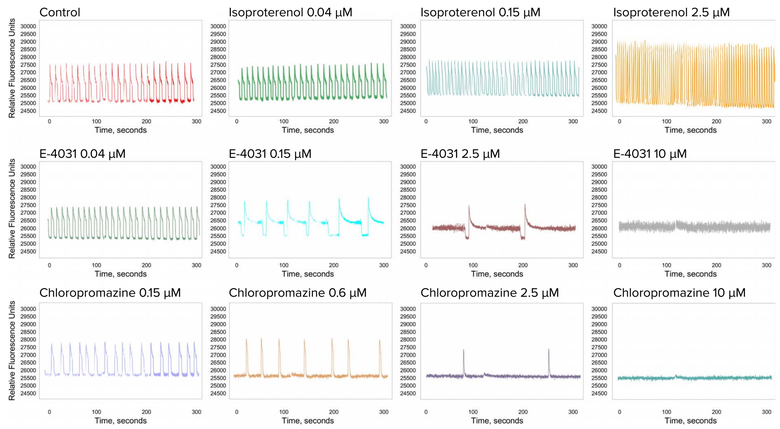

Figure 3. The tracing curve of fluorescence signals of Isoproterenol at 0.04, 0.15, and 2.5 µM; E-4031 at 0.04, 0.15, 2.5, and 10 µM; and Chloropromazine at 0.15, 0.6, 2.5, and 10 µM.

Summary of Parametric Effects of Compounds

The trend of effects of 8 modulators on cardiac activity of microtissues are summarized in Figure 4, where parameters like peak counts/300 sec, CTD 90 (sec) and amplitude (RFU) are shown. The well-known agonists, like isoproterenol, increase the beat rate and reduce CTD90 as expected. While other antagonists, like cisapride and chlorpromazine, decrease the beat rate and amplitude after a certain threshold of concentration. The EC_{50} values of the compound concentration are summarized in Table 1.

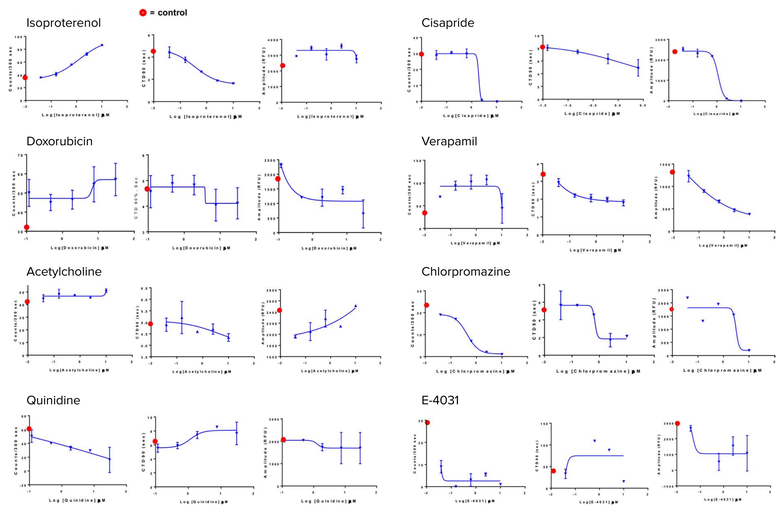

Figure 4. The trend of the change of Peak Counts/300 sec, CTD90 and Amplitude with the increasing concentration of compounds. Red dots indicated control values for appropriate measurements.

Compound	Peak Count per 300 sec	CTD90 (width of peak/sec at 90% of height)	Amplitude	MOA
Isoproterenol	1.25	0.35	12.76*	B2 adrenoreceptor agonist
Cisapride	1.9*	1.4	1.1	hERG blocker, antacid
Chlorpromazine	.43	0.69*	0.31*	Inhibits calmodulin stimulation of CNP
Quinidine	0.2	0.57	0.46	Class IA antiarrhythmic; reduces both Na^+ and K^+ channel currents, Blocks hERG
E-4031	0.14*	0.04*	0.15	hERG Blocker
Doxorubicin	0.03	3.8*	.004*	Anti tumor, inhibits DN topoisomerase II
Verapamil	6.7*	0.16*	0.34*	Cav1.x channel blocker
Acetylcholine	20.1	3.5	0.028	Acts on voltage gated ion channels

*denotes an ambiguous value in Prism.

Table 1. Summary chart of EC_{50} values (µM) calculated with Prism.

Results

Investigation of 3D Cardiac Microtissue Structure by High Content Imaging

High-content Imaging of the microtissues were taken with Cy5, TRITC and DAPI channels with multiple z-planes captured to cover the whole microtissue. The captured images were then analyzed with the MetaXpress built-in custom module editor to analyze the 3D coordinates of each cell. Figure 5 shows the representative overlay images of the combination of cardiomyocytes and fibroblast cells staining and the combination of cardiomyocytes and endothelial cells staining. The images suggest that the cardiac fibroblast cells tend to form clustering structures compared to that of endothelial cells. We thus performed a silhouette analysis to further investigate this phenomenon.

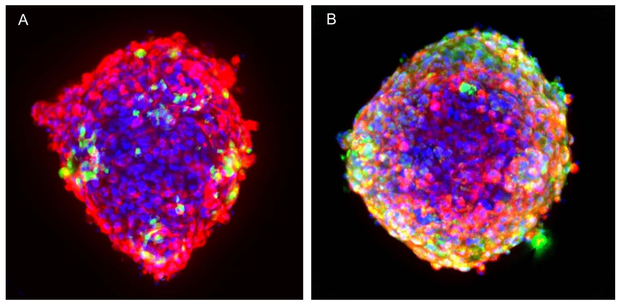

Figure 5. Composite images of cardiac tri-culture microtissues. After fixation and permeabilization, cells were stained either with a combination of anti-Troponin T AF647 + anti-VE-Cadherin AF568 or anti-Troponin T AF647 + anti-COL1A1 AF568. Cells were imaged with the DAPI, TRITC and Cy5 channels, 10X Plan Fluor objective. (A) Red: Cardiomyocytes, Green: Cardiac Fibroblast Cells, Blue: Hoechst; (B) Red: Cardiomyocytes, Green: Endothelial Cells, Blue: Hoechst.

3D Reconstruction of Cell Distribution and Silhouette Analysis of Clustering

To study the clustering of the cardiac fibroblast cells and endothelial cells, we used a silhouette score ranging from -1 to +1 to measure how close each point in one cluster is to points in the neighboring clusters, where +1 indicates the point is far away from the neighboring clusters. The 3D reconstruction of the fibroblast cells in Figure 5A is shown in Figure 6A with silhouette analysis shown in Figure 6B, where 4 clusters are obvious and gains the highest silhouette score. The overall silhouette analysis of fibroblast cells and endothelial cells in Figure 6C and Figure 6D shows that the fibroblast cells have a higher tendency to form clusters compared to endothelial cells, though whether this tendency helps the maturation of cardiac microtissues or not needs more research.

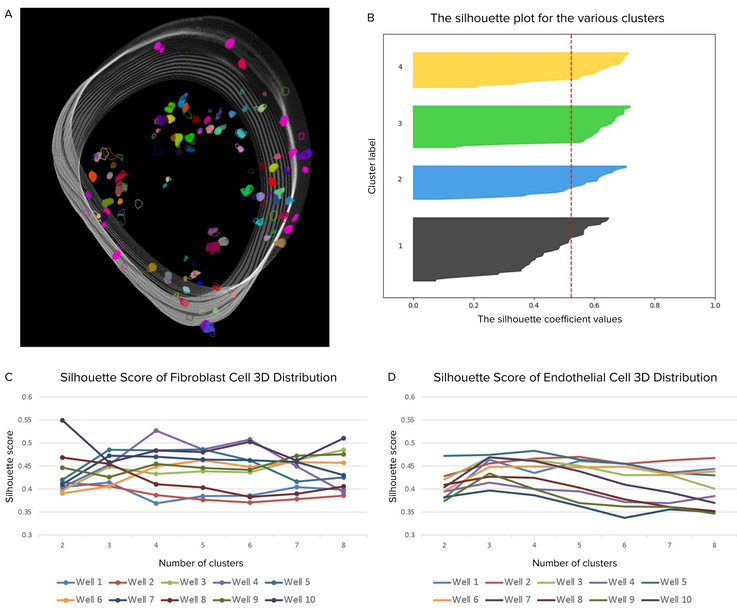

Figure 6. (A) 3D reconstruction of fibroblast cells from the microtissues shown in Figure 5A. (B) Silhouette analysis of the 3D coordinates from Figure 6A which shows that the number of 4 clusters is a good pick due to the presence of clusters with above average silhouette scores. (C) The silhouette score trend of different number of clusters for fibroblast cells from different microtissues. (D) The silhouette score trend of different number of clusters for endothelial cells from different microtissues.

Summary

- Tri-culture model created by mixing iPSC-derived cardiac cells with primary adult fibroblasts and iPSC-derived endothelial cells provides a novel tool for evaluation of functional and morphological effects on cardiac cells.
- The FLIPR Penta System equipped with new high-speed camera allows better resolution calcium oscillation patterns in cardiomyocytes.
- The ImageXpress Micro Confocal High-content Imaging System allowed fast acquisition of complex biological samples with high resolution.
- The MetaXpress custom module editor is a powerful analysis tool to locate the individual cells and reconstruct the 3D structure of complex cell models.
- A variety of parametric responses were demonstrated using a set of 8 known modulators of cardiac activities.
- The assay can be used to test developing drugs and screen chemicals for potential cardiotoxic hazard.