Full workflow automation for cardiac differentiation and functional analysis of compound responses in human iPSC-derived 3D cardiac organoids

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Introduction

Modeling human tissues using induced pluripotent stem cell (iPSC)-derived three-dimensional (3D) organoids is a highly promising technology to facilitate drug development and toxicity assessment. However, time and process complexity prevents wider adoption of this method for compound screening. harvested, study, we developed a method to automate the full process for cell differentiation from iPSC, formation and maintenance of 3D cardiac organoids. The CellXpress.ai™ Automated Cell Culture System contains four essential components for automated organoid culture: liquid handler, automated incubator and automated imager, plus integrated software that provides machine learning-enabled processing of complex protocols, scheduling, and image-based analysis.

Cardiac cells were derived from iPSC then expanded and triggered to differentiation using the CellXpress.ai system. After 36 hours of differentiation, cells were harvested, and cardiac micro-tissues were created in low attachment U-bottom plates. The CellXpress.ai system automated cell plating and subsequent media exchanges every day. During culture, cardiac spheroids were automatically monitored every 24 hours using automated imaging in transmitted light (TL) and recorded size and morphology of microtissues during 3 weeks in culture. 3D microtissues were formed within 48 hours and started to contract spontaneously on day 7. After additional maturation for 2 weeks in culture, we tested the functional activity of cardiac organoids by recording calcium oscillations after the addition of calcium dye using the FLIPR® Penta High-Throughput Cellular Screening System.

We tested the responses of the microtissues against 16 compounds, including modulators of cardiac activity, blockers of ion channels, and a panel of known cardiotoxic compounds (CIPA). Tested compounds, including hERG inhibitors, ion channel blockers and other compounds, demonstrated changes in the Ca^{2+} oscillation patterns consistent with the expected mode of action or toxicity effect. Waveform analysis of patterns was performed with Peak Pro 2 software and provided multiple read-outs, including peak frequency, amplitude, peak prolongation, irregularity, appearance of secondary peaks, and other measurements characterizing modulations of oscillation patterns.

The data presented here highlights the utility and biological relevance of using iPSC-derived cell types in 3D microtissues as a promising model for measuring potential cardiotoxic effects on human cardiac tissues. The cell model is suitable for automation and compound screening in high-throughput formats.

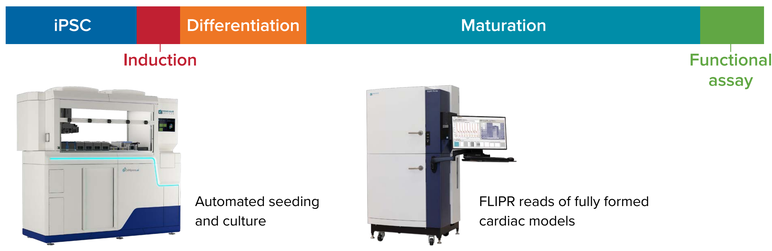


Figure 1. Schematic diagram of the process workflow. Organoids were formed and matured using a method and reagents provided by HeartBeat.bio as described below. Differentiation of iPSC to cardiac cells, formation of organoids, and maintenance of the microtissues and imaging were done using the CellXpress.ai automated cell culture system (Molecular Devices).

Methods

Cells: iPSCs Clone Gm25256 cell line from Coriell Institute for Medical Research was used for this experiment. Cells were grown with E8 media. Single cell iPSC passaging was performed manually when cells reached 70% confluency. IPSC cells were then seeded into 12-well plates and the protocol was continued with full automation. First, cells were induced to differentiation using induction media. After induction, cells were actively differentiating into fibroblast-like phenotypes (Figure 1) and reached confluency within 36 hours. Then, cells were harvested with Tryple E reagent and re-seeded into 96-well U-bottom ultra-low attachment plates (Corning 7007) 11,000 cells/well. Cells formed tide spheroidd after 48h. Media exchanges and imaging were done automatically with the CellXpress.ai system every 24 hours. After which, media was switched to differentiation media, and then to maintenance media. After 5–6 days, spontaneous contractions were observed and cardioid morphology changed showing the cavity inside. After continuous culture for 21 days, cardiac organoids were used to record calcium oscillation activity.

Calcium oscillation analysis: The presence of strong synchronous contractions in 3D cultures was confirmed visually prior to running experiments. On the day of assay, cell spheroids were loaded with 2X conc. of FLIPR Calcium 6 dye indicator (Molecular Devices) and incubated for 2h. We used a high-speed EMCCD camera on the FLIPR Penta instrument (Molecular Devices) to measure the patterns and frequencies of spontaneous calcium waveforms from 3D neural organoids. Baseline recordings were acquired for 2 min then plates were dosed with drugs for 15–90 min. Peak analysis was accomplished with ScreenWorks Peak Pro 2 software (Molecular Devices) which allowed the characterization of both primary and secondary peaks and complex calcium oscillation patterns.

High-content imaging was done using the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices) which captured the 3D structures of the spheroids and evaluated viability. To assess phenotypic changes, cells were stained live using a mixture of three dyes: the viability dye Calcein AM (1 µM), EtHD (2 µM), and Hoechst nuclear dye (2 µM) (all from Life Technologies).

Compound treatment: Cardioids were treated with 12 compounds that previously were shown to have cardiotoxic effects. Short-term (up to 90 min) effects were evaluated.

Instruments

IPSC cells were automatically plated and cultured with periodic media exchanges and monitoring using the CellXpress.ai system. The CellXpress.ai system contains four essential components for automated cell culture: liquid handler, automated incubator, and automated imager, plus integrated AI-powered software to automate complex protocols, scheduling, and image analysis.

For recording of calcium oscillations and analysis of the oscillation patterns, we used a high-speed EMCCD camera on the FLIPR Penta 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 Peak Pro 2 peak analysis software which allows analysis and characterization of the primary and secondary peaks and complex oscillation patterns.

Results

Formation of cardiac organoids and automated culture using the CellXpress.ai system

Since the manual process of developing iPSC-derived cardiac organoids is labor–intensive, we developed protocols for automated media exchanges and imaging of cardiac organoids using the CellXpress.ai Automated Cell Culture System. IPSC were differentiated and then automatically seeded into 96-low attachment plates and cultured for 21 days with imaging and media exchanges every 24h. Images were taken with transmitted light (10X, see below) and media exchanges were done by automatically replacing ½ of media volumes.

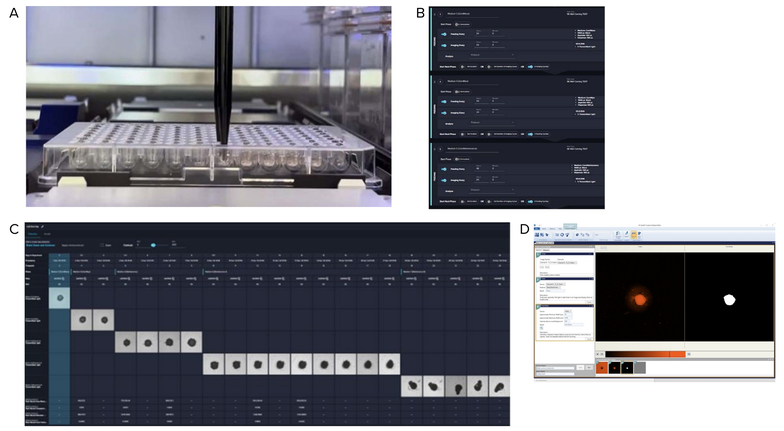


Figure 2. A. Image of the CellXpress.ai system performing media exchanges in 96-well plates. B. Steps of the fragment of automated workflow. C. iPSCs were expanded, harvested, and seeded into 96-well U bottom plates. Various cardioid differentiation media were added and contracting cardioids were successfully generated over a period of 4 weeks. D. Segmentation and analysis of TL images of cardiac microtissues during the culture.

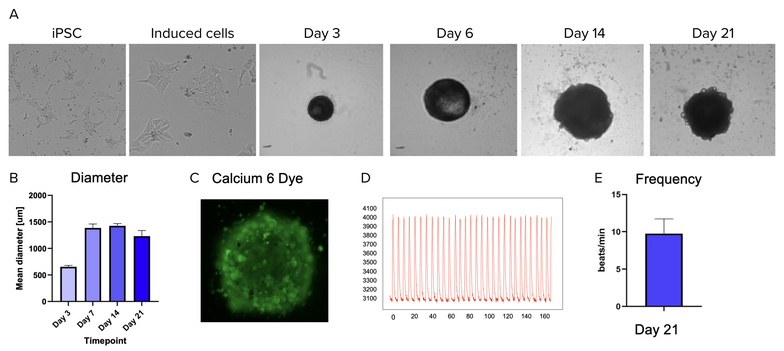
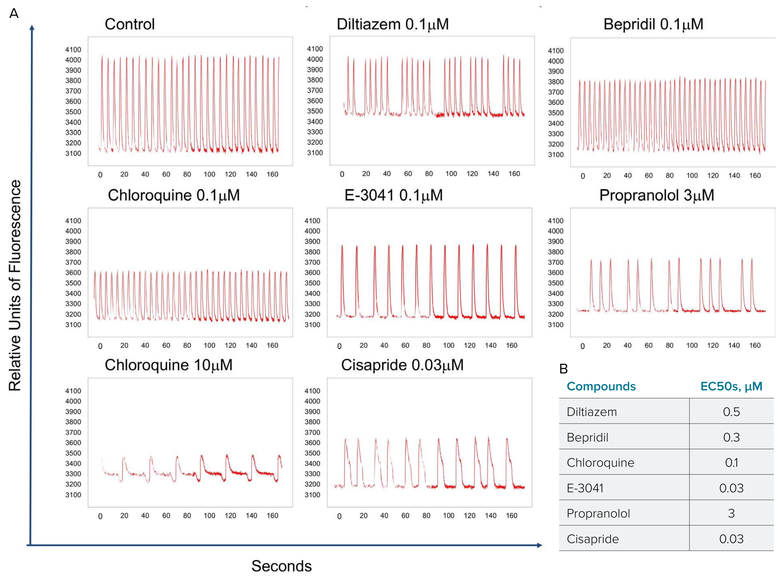


Figure 3. A. Transmitted light images of cells and spheroids during development. B. Calculated diameters of spheroids. C. Image of microtissue stained with Calcium 6 dye. Images taken–using the integrated imager on the CellXpress.ai system—for Calcium 6 dye in FITC 10X, best focus projection of 8 images taken 10 µm apart. D. Calcium oscillations recorded by the FLIPR Penta system. E. Bar graph showing average peak frequency from control wells.

Compound efficacy evaluation by modulation of calcium oscillation patterns

3D cardiac spheroids generated spontaneous contraction activity and corresponding calcium oscillations that can be visualized with calcium-sensitive dyes. Spontaneous oscillations were recorded after loading microtissues with Calcium 6 dye with the FLIPR Penta system. Microtissues were treated with several cardio-active compounds in concentration-depended manner.



B

Compounds	EC50s, µM
Diltiazem	0.5
Bepridil	0.3
Chloroquine	0.1
E-3041	0.03
Propranolol	3
Cisapride	0.03

Figure 4. A. Calcium oscillations recorded by the FLIPR Penta system and analyzed by Peak Pro 2 software. Changes in frequency and patterns observed 45 min after treatment with indicated compounds. B. Table showing estimated effective concentrations of compounds.

Results

Compound testing and evaluation of toxicity effects measuring changes in calcium oscillation patterns

Using a commercially available, curated library of reference compounds and controls (CIPA), we assessed the functional responses of 3D Cardioids and 2D cardiomyocytes side by side. Spontaneous oscillations were recorded with FLIPR instrument. The cardioids were treated with variety of cardio-active and cardiotoxic compounds in triplicates or quadruplicates with 6-point concentration response.

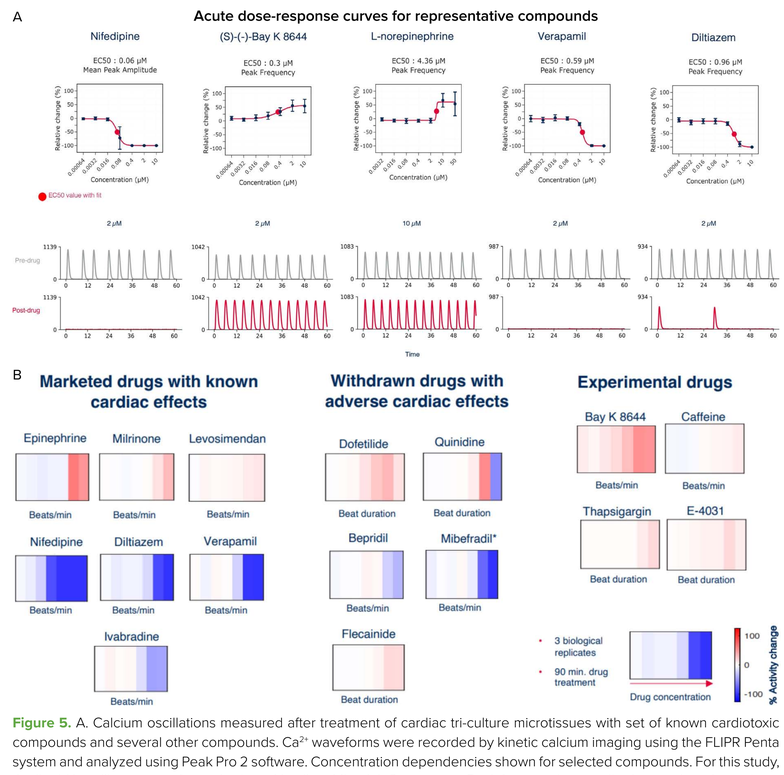


Figure 5. A. Calcium oscillations measured after treatment of cardiac tri-culture microtissues with set of known cardiotoxic compounds and several other compounds. Ca^{2+} waveforms were recorded by kinetic calcium imaging using the FLIPR Penta system and analyzed using Peak Pro 2 software. Concentration dependencies shown for selected compounds. For this study, the liquid handling was performed using a Hamilton Microlab Star system. Peak frequencies, amplitudes, peak prolongations, and other measurements were evaluated for different compounds and concentrations. B. Color coded modulations of patterns with increasing compound concentrations.

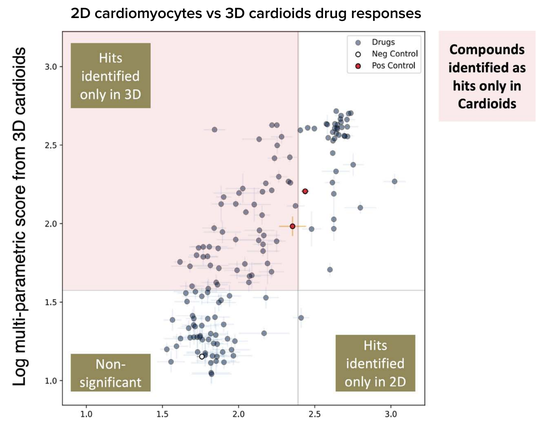


Figure 6. An additional study was performed by HBB using a sub-set of known cardiotoxic compounds from the Enzo library. To quantify cardiotoxic effects, we developed a custom multi-parametric scoring system based on several functional key features — each measured at the 30-, 45-, and 90-minute timepoints. Each compound was scored based on its Euclidean distance from the control baseline, with a higher score indicating a greater deviation and stronger toxic effect. Compounds were classified as cardiotoxic "hits" if their score exceeded two standard deviations above the control mean at any timepoint — a threshold selected to balance sensitivity and specificity, and adjustable for future analyses. Our results show that 3D Cardioids are significantly more effective in detecting functional changes upon compound administration. Out of 130 compounds tested, 39 compounds were identified as cardiotoxic exclusively in the Cardioid model whereas 35 compounds were identified as hits in both the 2D cardiomyocyte system and 3D Cardioid model.

Summary

- Analysis of kinetic calcium oscillations demonstrates functional activity of cardiac microtissues and provides readouts for drug-induced changes in oscillation patterns and for toxicity evaluation.
- We developed a fully automated method for the formation and culture of organoids that included automated media exchanges and imaging. Automation of organoid culture facilitates scalability and increases assay throughput.
- This benchmarking study confirms the added value of 3D Cardioids as a highly sensitive and physiologically relevant platform for cardiotoxicity screening. Compared to traditional 2D models, Cardioids consistently demonstrated improved fidelity in detecting cardiotoxic effects, supporting their role as a powerful tool for early-stage safety evaluation in both preclinical research and drug development pipelines. These findings also underscore the potential for expanding assay capabilities in cardiotox applications beyond calcium dynamics to capture a broader spectrum of cardiac responses.