Automated Functional Cellular Analyses of Human iPS-derived Cardiomyocytes For Cardiovascular Research, Toxicity, and Drug Discovery

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Introduction

Induced pluripotent stem cell (iPSC)-derived human cardiomyocytes are emerging as a biorelevant model system for testing toxicity and cardioprotective effects of drug candidates and developing drugs. Human iPSC-derived iCell® Cardiomyocytes (Cellular Dynamics International) were dosed with generally toxic and cardiotoxic compounds in the presence and absence of agents known to have cardioprotective effects. Molecular Devices' ImageXpress® Velos and ImageXpress® Micro High Content Screening Systems were used to obtain and analyze cell images for overall viability and early damage indicated by disruption of the mitochondrial membrane.

Cells were evaluated for phenotype and function by staining with cardiac-specific markers as well as observing spontaneous beating of live cells using time-lapse imaging under environmental control in the ImageXpress Micro System. Appropriate and functional ion channels were detected via automated patch clamp using IonWorks Barracuda™ System.

Materials & Methods

- iCell® iCell® Cardiomyocytes (Cellular Dynamics Intl.)
- ImageXpress® Micro High Content Screening System
- ImageXpress® Velos Laser Scanning Cytometer
- IonWorks Barracuda™ Automated Patch Clamp System
- Detection Reagents
  - Anti-α Actinin (Novus Biologicals)
  - Anti-Troponin T (Cell Signaling Technology)
  - AlexaFluor labeled anti-mouse and anti-rabbit IgG (Cell Signaling Technology or Invitrogen)
  - Cell Meter™ JC-10 (AAT Bioquest)
  - ToxCount™ Cell Viability Assay/Calcein AM + Ethidium Homodimer (Active Motif)
  - MitoTracker® Red CMXRos (Invitrogen)
- Compounds tested (from Sigma or Biomol):
  - Doxazocin mesylate, Verapamil, Antimycin A, Valinomycin, Nifedipine, Terfenadine, Mitomycin C, Imatinib mesylate, Calcium Ionophore
- Cardioprotective agent (from Sigma):
  - Sphingosine 1-Phosphate

Cardiomyocytes were thawed and plated at 40,000 cells/well in 96 well plates or 15,000 cells/well in 384 well plates. Compounds were serially diluted and added at 2-3 days post-plating. 96 or 384 well microplates were imaged at various time points (from 90 min. to 72 hours post-treatment) for different toxicity assays. Some plates were fixed with 4% methanol-free formaldehyde at the conclusion of toxicity studies and were assayed for cardiac-specific markers.

Confirming Phenotype

Live Cells were stained with Calcein AM to provide fluorescent output and time-lapse video was acquired while cells were maintained in the environmentally controlled (37ºC, 5% CO_2) chamber of ImageXpress Micro System.

Videos of the beating cardiomyocytes were analyzed with MetaXpress® Imaging Software and beat rates were counted by measuring the change in cell perimeter as they expanded and contracted. This not only demonstrates cell functionality but can also be used to measure drug-induced changes on cardiomyocyte beat rate and contraction magnitude (Figures 1 & 8).

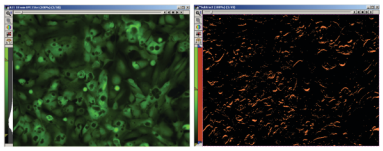

Figure 1. Time-lapse images of beating cardiomyocytes can be analyzed using MetaXpress software to track changes in cell perimeter.

Cells were stained for cardiac-specific markers to confirm they were fully differentiated (Figure 2).

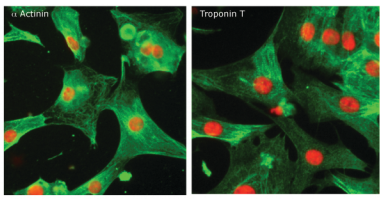

Figure 2. iCell Cardiomyocytes fixed and stained on Day 6 post-plating and acquired on ImageXpress Micro Widefield system with 40X objective. Red = Hoechst stained nuclei, Green = AlexaFluor 488 labeled alpha Actinin & Troponin T.

An automated patch clamp experiment showed that functional endogenous channels are present in the cells.

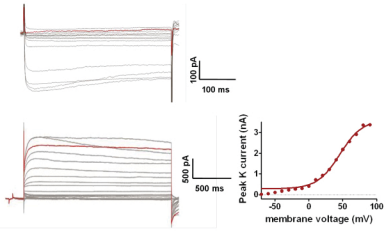

Figure 3. Endogenous inward currents (top) and outward currents (bottom) are detected using IonWorks Barracuda Automated Patch Clamp System. The current-voltage relationship for the outward current matches that of potassium channels.

Toxicity Testing

Cardiomyocytes were treated in a dose-wise manner and returned to the incubator. They were assayed after 24 or 48 hours for viability. The assay was performed on multiple days with both ImageXpress Velos and ImageXpress Micro Systems. Calcein AM staining of only live cells allowed measurements of total fluorescence of cells either in the entire well (Figure 4) or in 1-4 fields of view using 20X objective (Figure 5).

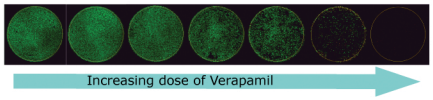

Figure 4. Whole well imaging using ImageXpress Velos illustrates the effect of increasing doses of Verapamil after 48 hours of treatment in a 96 well microplate. Calcein AM fluorescence indicates live cells.

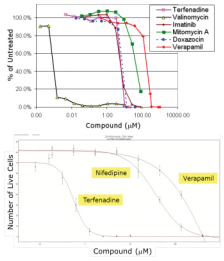

Figure 5. General cytotoxicity results from a dilution series of various compounds in a 96 well microplate. Top: ImageXpress Velos Scanning Cytometer data normalized to red/green ratio of untreated cells in the entire well. Bottom: ImageXpress Micro Automated Microscope reports number of live cells identified in the fields of view plus other information on subcellular staining. AcuityXpress™ software was used to analyze results.

Gross toxicity assays are typically read 24-72 hours after treatment. Some compounds interfere with maintenance of the mitochondrial membrane potential which leads to cell death. This disruption of the mitochondria occurs quickly when treating cells with Antimycin A or Valinomycin and can be measured in live cells using CellMeter JC-10 reagent. If the mitochondrial membranes are intact, the cationic dye enters the mitochondria and forms red "J-aggregates" but if the membrane potential is disrupted the dye turns to green in the cytoplasm. The impending cell death may be apparent within minutes. The ratio of red to green can be visualized with an imaging system and analyzed with MetaXpress® image analysis software using a pre-optimized module for finding granules within cells (Figure 6).

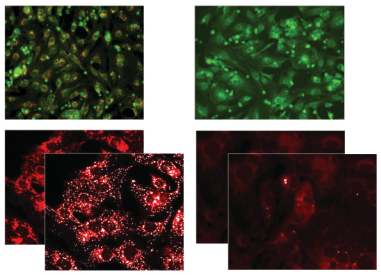

Figure 6 . Top: Representative images taken at 20X magnification using ImageXpress Micro system showing the results of a JC-10 assay. Healthy cells on left show red dye aggregates in mitochondria while cells treated with Antimycin A on right show increase in green dye and loss of aggregates in mitochondria. Bottom: Granules (white masked areas) are identified and counted in the images shown at 40X magnification and pseudo colored red (without the green overlay). The untreated well on the left has many intact mitochondria while the treated cell on the right shows failure to retain the red dye inside the mitochondria. Cells were grown in a 384 well microplate and tested 90 minutes after compound addition.

Cardioprotection

Plated cardiomyocytes were preincubated for 8 hours with 3 concentrations of sphingosine 1-phosphate for cardioprotection then dosed with 5 uM Antimycin A + 1 uM Calcium ionophore for 30 minutes in the presence of mitochondrial stain which is only visible inside the organelle if the membrane potential is maintained.

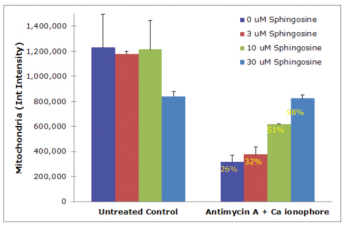

Figure 7. Mitochondrial integrity assay indicates that increasing doses of Sphingosine 1-Phosphate can protect cardiomyocytes from toxic effects of Antimycin A and Calcimycin.

Effect on Beat Rate

Spontaneously beating iPS-derived cardiomyocytes were stained with Calcein AM then media was replaced and cells were treated with varying concentrations of compounds that affect heart rate: isoproterenol, epinephrine, caffeine or acetylcholine. Time-lapse images were acquired under temperature and CO_2 control using ImageXpress® Micro System. Video from each well was analyzed as described in Figure 1.

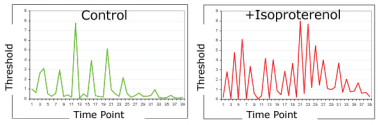

Figure 8: Automatic analysis of the fluorescent images leads to a plot of thresholded intensities vs. time. Beat frequency was determined 10 min. after compound addition and generally the beat rates remained stable between 5-60 min after compound addition (not shown).

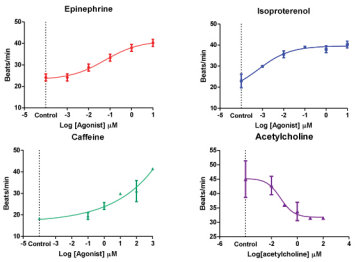

Figure 9: Dose dependent beat rate modulation of iPSC-derived cardiomyocytes was as expected for these four compounds.

Conclusion

Many drug products have been removed from the market or failed late clinical trials due to cardiovascular toxicity. These experiments illustrate the use of high content imaging for powerful and automated assessment of toxicity and to determine the functional effects (beating or loss of mitochondrial membrane potential) of compounds on living human cardiac cells.

Summary

- Dose response curves and IC50 results from biologically relevant cells are obtainable using a combination of iPSC-derived cardiomyocytes, cellular imaging platforms, image analysis, and data analysis software.
- When a compound interferes with the mitochondrial membrane potential, toxic effects can be measured using CellMeter JC-10 reagent as early as 90 minutes post-treatment, a full day before general cell death is easily measurable.
- Many drugs affect heart rate and that effect can be measured in cardiac cells in a microplate by utilizing ImageXpress High Content Imaging Systems in conjunction with sophisticated MetaXpress image analysis software.

Together through life sciences.

Cellular Dynamics International

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