mES Cell-derived Cardiomyocytes Characterization using the xCELLigence RTCA HT System

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ABSTRACT (#TP51)

Cardiac toxicity, a drug-induced adverse effect, accounts for most drug recalls and delays in gaining regulatory approvals, and is critical to uncover in the early stage of the drug discovery process. Primary cultures of cardiomyocytes are tedious to establish for screening purposes, and embryonic stem (ES) cell-derived cardiomyocytes provide a novel approach as an *in vitro* model for studying drug-induced cardiac toxicity.

In this study, we will characterize cardiomyocytes derived from mES cells, and examine the effects of various drugs and compounds on the ES cell-derived cardiomyocytes by monitoring the cellular events in a real-time manner with the xCELLigence* RTCA HT system from Roche Applied Science. Cells and compounds were dispensed into the sensor-coated 384-well E-Plates using a Biomek® FX^P workstation.

*Note: Biomek® FX^P workstation is For Laboratory Use Only; not for use in diagnostic procedures. *All trademarks are property of their respective owners.*

INTRODUCTION

Functional assay on cells could be very complicated due to the variation of compound concentrations and combinations, and it requires strict control of timing before and after compound addition. To automate the characterization assay of ES cell-derived cardiomyocytes, we integrated the xCELLigence RTCA HT system on a Biomek FX^P workstation.

Biomek® FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor

The Biomek FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor (Figure 1) is a dual-pod automated liquid handling system with a Span-8 configuration featuring independent well access as well as tube-based operations, and a 96- or 384-multichannel pipetting head which are easily swapped out for the maximum flexibility and system reconfiguration. The gripper built into the multichannel pod provides fast and reliable labware movement around the deck, as well as the capability to de-lid and re-lid plates, create vacuum manifold assemblies and access integrated devices.

Figure 1. Biomek FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor.

RTCA HT System, integrated on Biomek FX^P

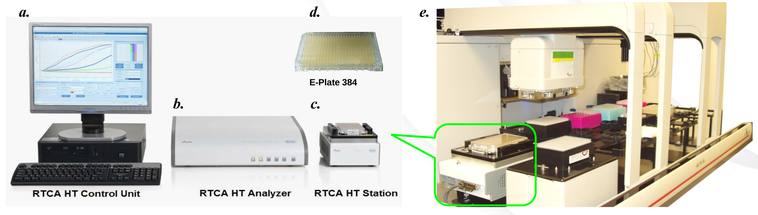

Figure 2. RTCA HT system, including a controller (*a*), analyzer (*b*), station (*c*) with E-Plate 384 (*d*), is integrated on Biomek FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor (*e*).

The RTCA HT system (Figure 2, *a - c*) provides label-free and real-time monitoring of cellular behavior in a 384-well format. With microelectrodes attached at the bottom, the E-Plate 384 (*d*) can sense changes in electrical impedance, reflected as cell index (CI) corresponding to the alteration of cell number, cell morphology and adhesion degree (quality of attachment to the plate). The RTCA HT Instrument is integrated on Biomek FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor (*e*). Up to four stations can be integrated on the deck and all the stations are connected to the RTCA HT Analyzer, which records and analyzes the electronic signals obtained from the E-Plate 384 then converts and transfers the signals to the controller. The RTCA HT Software on the controller allows the user to schedule steps with individual reading intervals and frequencies, as wells as perform data analysis on-line or off-line. Biomek software can communicate with the RTCA software and send commands during an automation method to initialize, run or resume a pre-defined schedule.

ASSAY PROTOCOL & BIOMEK FX^P WORKSTATION SETUP

Cardiomyocytes preparation

Mouse embryonic stem cells (from Invitrogen) were maintained on growth media containing LIF before differentiation. For detailed cardiomyocyte differentiation protocols please refer to Dr. Michael Kowalski, et al's poster (#MP75). Briefly, cells were cultured in media containing 15% FBS without LIF and plated into a 384-well round-bottom polypropylene plate in 40 µL at a density of 500 cells per well for two days before being treated with or without (controls) 100 µM of ascorbic acid. The embryo bodies that formed five days after plating were gently harvested and re-seeded to a gelatin-coated 96-well plates. Two days later, a portion of the adhered cells showed visible contractions. The differentiated cells were then harvested and plated to a 384-well E-Plate using a Biomek workstation at a density of 15,000 cells and a volume of 60 µL per well. After overnight incubation at 37°C, cells were treated with various compound doses at a 1:3 serial dilution and signals were recorded and analyzed on a RTCA HT analyzer.

Clonal mouse cardiomyocytes (from Celprogen) were primary cultured cells derived from mouse neonatal heart. Cells were maintained and sub-cultured every 24 to 48 hours on mouse cardiomyocytes extracellular matrix before analysis. Cells were harvested and plated in a 384-well E-Plate at 40,000 cells per 60 µL per well the day before compound treatment and results were recorded and analyzed using RTCA HT system. Data were compared with mouse ES cells derived cardiomyocytes.

Biomek FX^P Workstation deck setup and assay protocol

Cell seeding and the addition of serially diluted compound were automated on a Biomek FX^P automation workstation with dual-pod with a 384 multi-channel pipetting head and a span-8 with 1 mL syringes. It is a two-day procedure with the 1st day of cell seeding and overnight incubation, and the 2nd day of compound additions and assay (Figure 3). The method provides options to run the two days' procedure separately or continuously with the option to start from any column on the plates with the desired number of compounds and replicates in the User Interface step (Figure 3, *left*).

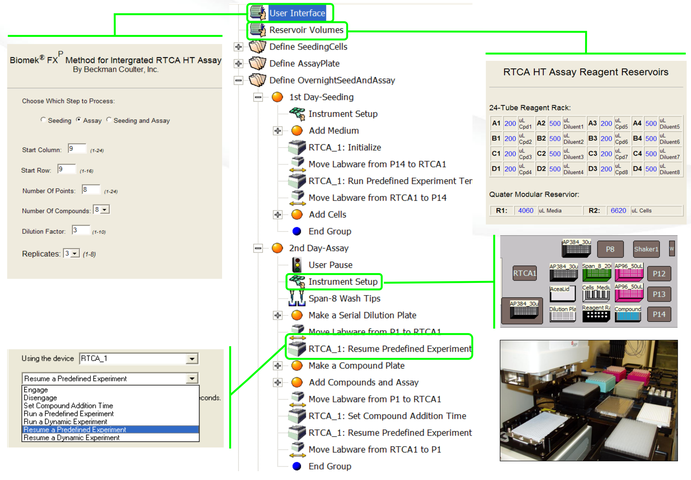

Figure 3. Biomek FX^P deck set up and assay protocol.

Prior to seeding of 40 µL of cells per well into the 384w E-Plate, 20 µL of media was dispensed, centrifuged and read on RTCA as background. The cells were allowed to attach and proliferate overnight. On the 2nd day, a compound plate was generated with a serial dilution step. A customized script step allows the total volumes of reagents to be automatically calculated based on the number of compounds and replicates, and updated in the Reagent Volume step to simplify reagent preparations (Figure 3, *right*). The serially diluted compounds were then simultaneously transferred using a 384 multi-channel head to the 384w E-Plate where cells were attached after overnight incubation, and the remaining scheduled steps on RTCA software were then resumed for real-time recording of the cell responses to various concentrations of compounds.

RTCA HT setup

The background readings (media only) were set to 3 sweeps at 1 min intervals for 3 min before adding cells. The values at the time points right before adding the compounds were set as 1 and used to normalize the following dose responses which were read at with 40 sweeps at 15 seconds intervals. Dose response curves were plotted using DRC function (CI at a time point vs. concentration) and Sigmoidal Dose-Response function, and the EC50/IC50 were calculated using the RTCA software as well.

*Note: [1]Method cited Not for Clinical purposes. *All trademarks are property of their respective owners.*

RESULTS

Data from clonal mCardiomyocytes

As shown in Figure 4, mouse cardiomyocytes were density titrated from 5,000 to 80,000 cells per well and stimulated with 10 µM of dobutamine (ryanodine receptor agonist) after overnight seeding. The wells containing 40,000 cells gave the highest resting CI reading (***a***) and activation (delta CI reading) after dobutamine stimulation (***b***). From this figure, 40,000 cells per well were used for the following assays for mouse cardiomyocytes.

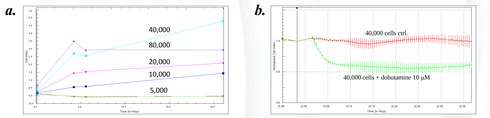

Figure 4. Results of mCardiomyocytes density titration with 10 µM of dobutamine. ***a.*** Density titration from 5,000 to 80,000 cells per well. ***b.*** Zoom in of dobutamine response vs. control curve (***red***, control wells with 5 µL of PBS; ***green***, treated wells with 5 µL of dobutamine, error bars are standard deviations of 3 replicates).

To characterize cardiomyocytes, compounds that were reported as having functional effects on cardiomyocytes—ouabain (NKA α1 and α2 inhibitor, ***a***), esmolol (β_1AR inhibitor, ***b***), staurosporine (Ca^{2+}-dependent protein kinase inhibitor, ***c***), amoidarone (non-selective ion channel inhibitor, ***d***) and nifedipine (L-type Ca^{2+} channel blocker, ***e***) were applied on clonal mouse cardiomyocytes, and the cell index was read on the RTCA HT system, as shown in Figure 5. All compounds induced dose-dependent responses in clonal mouse cardiomyocytes with different IC50 values (***f***).

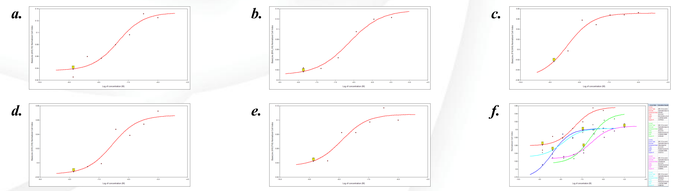

Figure 5. Dose response curves of: ***a.*** ouabain (1.37 nM to 1 µM; IC50 = 45.4 nM); ***b.*** esmolol (13.7 nM to 10 µM; IC50 = 0.2 µM); ***c.*** staurosporine (1.37 nM to 1 µM; IC50 = 3.9 nM); ***d.*** amiodarone (13.7 nM to 1 µM; IC50 = 0.3 µM) and nifedipine (1.37 nM to 1 µM; IC50 = 12.7 nM) on mCardiomyocytes. ***e.*** Overlay of dose response curves. Data were normalized to the time point before the addition of compounds and controls (vehicles without compounds) were subtracted as baselines.

Data from mEScells-derived cardiomyocytes

As shown in Figure 6, dobutamine induced dose-dependent responses in both ESc-derived cardiomyocytes differentiated in the presence of ascorbic acid (Figure 6***a***) and the differentiated cells in control group (without ascorbic acid treatment, Figure 6***b***). Dobutamine-induced responses at 30 min were superimposed in Figure 6***c***. The responses of ESc-derived cardiomyocytes differentiated in the presence of ascorbic acid were 2-fold higher than the differentiated cells in the control group. These results (~2-fold increasing in the number of cardiomyocytes in the ascorbic acid conditions) are consistent with flow cytometry data of similarly treated cells (refer to Michael Kowalski's poster #MP75); Undifferentiated ES cells (Figure 6***c. blue***) showed minor response to dobutamine.

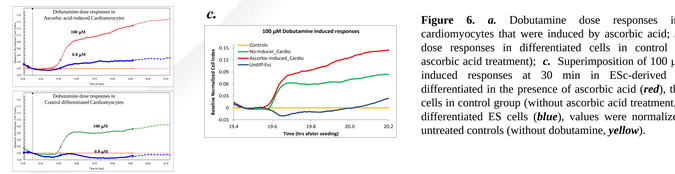

Figure 6. ***a.*** Dobutamine dose responses in ESc-derived cardiomyocytes that were induced by ascorbic acid; ***b.*** Dobutamine dose responses in differentiated cells in control group (without ascorbic acid treatment); ***c.*** Superimposition of 100 µM dobutamine-induced responses at 30 min in ESc-derived cardiomyocytes differentiated in the presence of ascorbic acid (***red***), the differentiated cells in control group (without ascorbic acid treatment, ***green***) and un-differentiated ES cells (***blue***), values were normalized to their own untreated controls (without dobutamine, ***yellow***).

Acknowledgments: *Thanks to ACEA Biosciences and Roche Applied Science for providing the RTCA instrument and technical support.*

SUMMARY

- The RTCA HT system was successfully integrated on Biomek FX^P Dual Arm System with Span-8 and Multi-Channel Pipettor, and functional assays on mouse cardiomyocytes were automated for cell treatments with various compounds at serially diluted concentrations.
- Cardiomyocytes characterization for both clonal primary cultured mouse cardiomyocytes and mouse ES cell-derived cardiomyocytes were successfully performed on the integrated system. Comparison data were obtained and dose response curves were generated with RTCA HT software.