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A phenotypic in vitro model for the main determinants of human whole heart function

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Abstract

This article details the construction and testing of a phenotypic assay system that models in vivo cardiac function in a parallel in vitro environment with human stem cell derived cardiomyocytes. The major determinants of human whole-heart function were experimentally modeled by integrating separate 2D cellular systems with BioMicroelectromechanical Systems (BioMEMS) constructs. The model featured a serum-free defined medium to enable both acute and chronic evaluation of drugs and toxins. The integration of data from both systems produced biologically relevant predictions of cardiac function in response to varying concentrations of selected drugs. Sotalol, norepinephrine and verapamil were shown to affect the measured parameters according to their specific mechanism of action, in agreement with clinical data. This system is applicable for cardiac side effect assessment, general toxicology, efficacy studies, and evaluation of in vitro cellular disease models in body-on-a-chip systems.

Keywords

cardiomyocyte; human; functional assay; force generation; conduction; serum-free

INTRODUCTION

The current drug development process is costly (ca. 1.2 billion dollars) and time consuming (10 to 15 yr.). To reduce cost and time, as well as increase success rates, improvement in pre-clinical testing must be achieved, especially to identify drug side effects. Since animal

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research has not translated well to the human condition in many cases, human organ mimics may be more germane for this type of testing. A human-based *in vitro* cardiac system could provide a key technology necessary to speed up the drug discovery process by demonstrating that function-based human cell models can accurately capture and predict complex organ responses. Development of a simple, low cost system would facilitate widespread usage and maximize the benefit of this approach in pharmacological screening for predicting cardiac side effects, general toxicity and efficacy.

Currently, there is no high-information content, phenotypic *in vitro* test system capable of predicting both acute and, more importantly, chronic cardiac side effects from drug treatments. The most relevant assays, such as *in vivo* dog EKG measurement, the *ex vivo* rabbit Langendorff heart, or the left ventricular wedge assay[1–4] are slow, expensive and suffer from interspecies differences. The *in vitro* hERG channel inhibition test[5], which became mandatory[3] for all new drugs, has a higher throughput, but it has limited information content and its predictive value is still debated[6]. QT interval prolongation was one of the leading causes for the recent retraction of several, already commercialized drugs[3], thus, measurement of QT interval has also become a focus of preclinical cardiac side effect testing. Unfortunately, prolongation of the QT interval can result in either arrhythmogenic or anti-arrhythmogenic effects. According to Antzelevitch and coworkers, dispersion of the repolarization of the cardiac action potential (AP), measured in a ventricular wedge preparation model, has a better predictive value for arrhythmogenic effect than the QT interval[7–10]. Hondeghem and coworkers have developed a more complex system (SCREENIT model) for the assessment of arrhythmogenic effect of drug candidates: the measurement of the TRIad parameters (Triangulation, Reverse use dependence, Instability) on the Langendorff-perfused isolated rabbit heart[4, 11–15].

Recently, attempts have been made to develop an integrated, automated system for the measurement of electrical and contractile properties of 2D and 3D cardiac tissues. Electrical properties of cultured rat and human cardiomyocytes have been successfully measured using substrate embedded microelectrode arrays[16–19], but these methods do not give any information concerning the contractile ability of the cardiac tissue. Calcium imaging has been shown to provide physiological and pharmacological data from human cardiomyocytes[20], but intracellular calcium is only one of the determinants of force generation, and there are other mechanisms and pathways that could be evaluated with a direct force measurement system that cannot be detected through intracellular calcium imaging. Contraction force of cardiomyocytes has been measured by biopolymer microcantilevers[21], microfabricated pillars[22], videomicroscopy of flexible substrates [23, 24], calcium imaging [25] and with magnetic beads[26]. However, the fabrication of these devices and/or the difficulty involved in analyzing the data obtained using these devices as well as the use of animal cells or serum-containing medium makes them inappropriate for high-content screening. Other attempts using 3D cardiac tissue analogues and macroscopic force measurements suffer from being relatively low throughput[27–29]. In summary, none of the described methods fulfill all of the requirements of a functional high content human cardiomyocyte-based screen for cardiac force generation.

Electrical activity in the heart as well as the force of muscle contraction are the primary focus of both pre-clinical toxicity and efficacy evaluation. Previously, recapitulation of the physiology of the heart has proven to be extremely difficult in an *in vitro* system because it was assumed 3D structure is necessary to reproduce function. However, the main functions of interest in pre-clinical drug discovery, rhythm generation, electrical conduction[17] and cardiac muscle force measurements[30–32], are readily measurable in individual 2D *in vitro* systems. Using patterned cardiomyocytes, *in vitro* parameters such as QT interval dispersion, reverse use dependence, and relative refractory period after APs can be measured and have their analog parameters in the validated SCREENIT cardiotoxicity test. The major factors for adapting Multi-Electrode array (MEA) and Atomic Force Microscopy (AFM) technologies to cardiac screening are: 1) The application of scalable photolithographic patterning technology on MEAs to cardiomyocytes[17], which enables the high-fidelity measurement of conduction velocity and stimulation; 2) The development of defined surfaces and a serum-free culture medium and for cardiac cells[33]; 3) The development of AFM cantilever technology for muscle force measurement[31]; 4) The incorporation of a commercial human embryonic stem-cell derived cardiac cell line in the screen[34]. Combining all these technological advances facilitated the establishment of a defined system for functional cardiac analysis of acute and chronic drug responses. In this work, the combination of two *in vitro* test systems with human embryonic stem cell derived cardiomyocytes allows for the measurement of defined parameters which have a high predictive value for cardiac side effects: spontaneous beating rate, conduction velocity, Action Potential (AP) length or QT interval, minimal inter-spike interval (mISI) (upon high frequency stimulation) and peak contractile force. In addition, the speed of contraction and the time to relaxation can also be monitored (not reported here).

MATERIALS AND METHODS

Cell Source Description

The cells were commercially available human cardiomyocytes derived from the NIH approved stem cell line hESC-10-0061, Cytiva (product code 28976398) from GE Healthcare, Cardiff, UK. GE cardiomyocytes possess an adult phenotype, comprising ventricular, atrial and nodal subtypes and express Gata4, Nkx2.5, MYH6/7, troponin I, α MHC and α -actinin, and are negative for Oct4, TRA-1-81 and TRA-1-60. The cells were supplied cryopreserved in 10% DMSO and 90% fetal bovine serum (FBS) and stored in liquid nitrogen until use. Two separate lots were used in this study, Lot 39 and 43, which corresponds to the cardiomyocyte percentage of each batch of cells.

In a typical experiment, one cryovial was removed from cryostorage and placed into a –80°C freezer for 2 minutes. The cell suspension was first thawed in a 37°C water bath with gentle agitation, then transferred into a sterile 50 ml centrifuge tube, and diluted with 9 ml of warm plating medium added dropwise while slowly mixing the vial. The diluted cell suspension was centrifuged at 300 g for 5 minutes at room temperature, supernatant removed, and the remaining pellet re-suspended in 1 ml of plating medium. The serum-free plating and maintenance media were the same and consisted of: 100 ml Ultraculture medium (BioWhittaker, cat# 12-7257F), 10% B27 (Gibco, cat#17504044), 1% glutamax (Gibco, cat#

35050–061), 1% Antibiotic/Antimitotic (Gibco, cat# 15240–062), 1% MEM non-essential amino acids (Gibco, cat# 11140–050), 1% HEPES buffer (Gibco, cat# 15630–080). To improve cell survival in the serum free medium, it was supplemented with 0.1 µg/ml of L-Thyroxine (Sigma, cat# T1775), 10 ng/ml Epidermal Growth Factor (Sigma, cat# E9644), 0.5 µg/ml Hydrocortizone (Sigma, cat# H088) and 20 mg/ml dextrose (Fisher Scientific, BP 350–500).

Surface Modification and Patterning of MEAs

Multielectrode arrays (MEAs, item# MCSMEA-S2-WO, Multichannel-Systems, Germany) containing both 60 and 30 µm diameter electrodes, were used for extracellular recording experiments. A cell-adherent foreground (Fibronectin) surrounded by a cell-repulsive background (PEG-silane) was created on the MEA surface by using surface modification and photolithography techniques[17]. Surfaces were cleaned by soaking in 1% Tergazyme solution for 2 h, followed by sonication in DI water for 2 min. Prior to chemical modification, MEAs were O₂ plasma treated at high frequency for 15 min at 600 mtorr to enhance surface hydrophilicity. Chemical modification of the substrates was achieved by incubation of MEAs in 3 mM PEG-silane (2-[Methoxypoly(ethyleneoxy)propyl]trimethoxysilane, Gelest, cat# SIM6492.7) in acidified (0.08% v/v HCl) toluene solution for 45 minutes at room temperature. Non-covalently bonded PEG moieties were removed by rinsing the MEAs once in freshly distilled toluene (VWR, cat# BDH1151), twice in ethanol (VWR, cat# BDH1160) followed by sonication in DI water for 2 min[35]. The MEAs were then dried with nitrogen and stored until patterning in a dessicator. Patterning of the PEG-modified substrates was done by exposing the arrays to a deep UV (193 nm) excimer laser (Lambda Physik) beam with a pulse power of 200 mJ and a frequency of 10 Hz for 45 seconds through a fused-quartz chromium photomask (Bandwidth Foundry, Eveleigh, Australia). The patterns on the photomask were precisely aligned with the electrodes on the MEAs via microscope, translation stage and a vacuum controller.

MEAs are commercially available with built-in cell culture chambers (glass rings permanently glued on the MEAs). As these are not suitable for contact photolithography and surface modification MEAs were used without cell culture chambers and fabricated removable rings out of Polydimethylsiloxane (PDMS). A mold was created using two concentric quartz rings (12 mm height, 15 mm and 24 mm outside diameter, respectively) placed on a microscope slide. A 10% mixture (v:v) of Sylgard 184 curing (Dow Corning, cat# 3097358–1004) to base polymer (Dow Corning, cat# 3097366–1004) was slowly injected between the two rings, and let cure at RT for 10 min and further at 40°C for 20 min. Following the curing process, the PDMS chamber was carefully removed from the mold using Teflon pestle and plastic tweezers.

Cantilever Fabrication and Cleaning

The cantilevers were fabricated onto a silicon wafer using standard photolithographic and deep reactive ion etching techniques at the Cornell's Nanoscale Science & Technology Facility (CNF). Cantilevers with dimensions of 737 µm × 150 µm × 4 µm were fabricated from silicon-on-insulator (SOI) wafers following a slightly modified protocol from

previously published work[30, 31]. The front side of the SOI wafer was primed with a 10 nm layer of hexamethyldisilazane (HMDS) and coated with 2 μm S1818 photoresist (Shipley Co, Marlborough, Massachusetts) layer, followed by softbake (110°C, 1 min on a hot plate), alignment, exposure, and development (2 min in 726 MIF developer - AZ Electronic Materials Co, USA). The device layer was etched using a deep reactive ion etch (DRIE) process. Prior to backside processing of the wafer, the front side was protected by spinning a thick layer of SPR 220 photoresist (Shipley Co, Marlborough, MA) which was baked in an oven at 90°C for 4 hours. The wafer was then flipped over and a 1.5 μm silicon oxide layer was deposited on the backside via Plasma Enhanced Chemical Vapor Deposition (PECVD) to act as a mask during subsequent processing steps. The backside was primed with a 10 nm layer of HMDS and spincoated with a 5 μm layer of SPR220 photoresist. Similar to the front side, coating of the backside was followed by softbake, exposure and photoresist development. The protective oxide mask was partially detached first by plasma etching, followed by DRIE etch for 6 h (4 $\mu\text{m}/\text{minute}$), leaving behind the native buried oxide layer, which was removed with buffered hydrofluoric acid (HF, dilution 6:1) for 45 minutes. The cantilevers were cleaned by soaking in 1% Tergazyme solution for 2 h, and then rinsed in DI water. Prior to cell plating, the chips were O₂ plasma treated for 15 min such that to enhance surface hydrophilicity and fibronectin adsorption.

Human Cardiomyocyte Culture

Patterned MEAs and freshly O₂ plasma cleaned cantilevers were sterilized by soaking in 100% ethanol (Sigma Aldrich, cat # 459844) for 10 minutes. At the same time, the PDMS cell culture chambers were sterilized by soaking in 70% isopropanol (VWR, cat#BDH1131) for 20 min. Once dried, the PDMS chambers were pressed onto the MEA surfaces to encircle the electrode arrays. Cantilevers and MEAs were incubated with 50 $\mu\text{g}/\text{ml}$ fibronectin (Millipore, FC010) in 1X Phosphate buffered saline (PBS) for 30 minutes in an incubator. The solution was aspirated, and the surfaces rinsed twice with 1X PBS before the cells were plated. Cells were manually plated with a cell density of 750 cells/ mm^2 on the MEAs and 2,500 cells/ mm^2 on the cantilevers. A higher cell density was required for the cantilevers, since many cells missed the cantilevers and adhered to the bottom of the dish. The medium was changed 24 h after plating, and every third day thereafter.

Baseline recordings were taken for each lot of hESC derived cardiomyocytes on both MEA and AFM systems before drug evaluation in order to verify that there were no significant differences in the physiological properties. MEA recordings were taken after 15 minutes equilibration in the lab atmosphere at 37°C. There was only a small, non-significant ($p = 0.3$) difference in spontaneous beating frequencies between the 2 lots utilized: 0.69 ± 0.15 Hz ($n = 14$) for lot 38, and 0.78 ± 0.24 Hz ($n = 9$) for lot 43. However, the traces recorded with the AFM system did show a significant difference in beating frequency ($p < 0.01$): 1.15 ± 0.13 Hz ($n = 10$) for lot 38 and 1.31 ± 0.03 Hz ($n = 5$) for lot 43. The repeated measures statistical analysis blocked for differences among MEAs and among cantilevers, including any differences between the two lots. The difference in spontaneous beating frequency between the MEA and cantilever systems could be the result of the different plating density or differentiation stage of the cardiomyocytes, but did not affect the results.

Experimental Setup

MEA Recordings and Drug Experiments—Electrical activity of the human cardiomyocytes plated on patterned MEAs was recorded following 4–10 days *in vitro* (DIV) using a Multichannel Systems 60 channel amplifier (MEA 1040, Multichannel Systems). Prior to recording, the cells were allowed to equilibrate for 15 min in the lab atmosphere at 37°C. Temperature was maintained with a TC02 temperature controller (Multichannel Systems). The cells were stimulated using a STG 1002 stimulator (Multichannel Systems) by applying 1 ms wide bipolar square pulses of 500–700 mV amplitude at increasing frequencies ranging from 0.5 Hz to 2.5 Hz in 0.5 Hz increments. The recording medium was the same as the plating medium. For drug experiments, sotalol, (Sigma, cat#S0278), norepinephrine (Sigma, cat#A7257) or verapamil (Sigma, cat#381195) were added to the bathing medium in increasing concentrations of 10, 30, 100 and 300 μ M – sotalol, 0.1, 0.3, 1 and 3 μ M – norepinephrine, and 0.3, 1, 3 and 10 μ M – verapamil. In all drug experiments, for control and each new drug concentration a total of 5 minutes of recordings were performed, the first 150 seconds with no stimulation, followed by 30 seconds of stimulation, and ended with no stimulation. The data was converted to pClamp (Axon Instruments) format using MC-Data Tool (Multichannel Systems) and analyzed with Clampfit (Axon Instruments) software and software written in Matlab.

Cantilever Recordings and Drug Experiments—Contractile stress of the human cardiomyocytes plated on the cantilevers chips was recorded following 10–14 DIV utilizing a detection system similar to that used in atomic force microscopy (AFM) described previously[31]. Electrical functionality was not found to change over 4–10 DIV, hence it was decided to use the widest range of sampling times possible in order to maximize the data obtained from each culture, and so sampling was initiated on day 4. Contractile activity required higher degrees of maturation, so could not be performed until later in the culture period starting at day 10.

The cantilever system consisted of a Class 2 red photodiode laser (Newport, Irvine, CA), a heated chamber (Delta T Dish, Biopetechs Butler, PA), a 4-quadrant photodetector (Noah Industries, Melbourne, FL) and a computer running Clampex software. The chip was placed in the middle of the chamber, maintained at $37 \pm 1^\circ\text{C}$, between two stimulation electrodes. The laser photodetector was aligned so that the beam was directed onto the tip of the cantilever, and subsequently reflected into the center of the photodetector's 4 quadrants. The deflection of the cantilever in response to contracting cells was measured and recorded in real time. For stimulation experiments, square electrical pulses, 40 ms in duration and 4–5 V in amplitude, were applied every 0.5–1 seconds via an isolated pulse stimulator (A–M Systems, Sequim, WA). Prior to recording, the cells were allowed to equilibrate for 15 min in the lab atmosphere at a temperature of $37 \pm 1^\circ\text{C}$ maintained by a Delta T4 culture dish temperature controller (Biopetechs, Butler, PA). To minimize the light-induced toxicity of cells, the system was fitted with an automatically controlled laser shutter (Auto Mate Scientific, Berkeley, California). Verapamil (1 μ M, 3 μ M) was administered cumulatively every 10 minutes. Sotalol (100 μ M) was administered once. Washout was performed by rinsing the cells 5 times with 1 ml of cell culture medium. Short recordings (10–15 seconds) were taken every 5 minutes throughout the drug experiment. Calibration of the system was

done by recording the spontaneous beating traces throughout a 30 minutes time interval every 5 minutes for 10–15 seconds. During this timeframe the amplitude change was $6.19\% \pm 4.41$ ($n = 5$), and the beating frequency change was $9.86\% \pm 1.28$ ($n = 5$).

The positive inotropic effect of norepinephrine on the contraction force was captured only when cells were exposed to an extracellular solution containing 1 mM Ca^{2+} . In a typical experiment, the cells were washed out carefully with 6 ml of calcium free Tyrode solution[36] in portions of 1 ml per treatment for 5 minutes. The cells were equilibrated to the new conditions until a stable baseline was obtained prior to adding 1 mM Ca^{2+} and the norepinephrine treatment. The cell response was continuously monitored during the experiment, recordings were taken at 0 mM Ca^{2+} , 1 mM Ca^{2+} and after adding 1 μM norepinephrine.

Cantilever displacement, recorded as laser deflection by the photodetector, was converted to force exerted by the cardiomyocyte layer using a modified version of Stoney's equation for thin films using methods described previously for the cantilever system[31]. Using this approach, physical dimensions and parameters relative to the silicon cantilevers, photodetector, and arrangement of the system were used to determine the deflection of the cantilever tip. The modified version of the Stoney's equation was applied to convert deflection to the force in the cardiomyocyte layer by incorporating the dimensions of the cantilever, the physical properties of the single crystal silicon, and the thickness of the cardiomyocyte layer (measured to be $21 \pm 1 \mu\text{m}$ via confocal laser microscopy).

Equations 1 and 2 are restated versions previously published[31] for cantilever tip deflection (δ) and stress produced by the cardiomyocytes layer (σ_c). The system parameters used in these equations are the system-specific coefficient relating voltage to laser position on the photo-detector (C_{detector}), the angle of the laser and detector relative to the plane of the cantilever (θ), the path length of the laser from the cantilever tip to the detector (P), the elastic modulus of silicon (E_{Si}), the thicknesses of the cantilever (t_{Si}) and cardiomyocyte layer (t_c), poison's ratio of silicon (v_{Si}), cantilever length (L), and the width of the cantilever (w_{Si}).

$$\delta = \frac{2L}{3} \tan \left[\frac{\theta}{2} - \frac{1}{2} \arctan \left(\tan \theta - \frac{\text{Voltage}}{C_{\text{detector}} \times P \times \cos \theta} \right) \right] \quad 1$$

The force in the cardiomyocyte layer is equal to the stress times the cross sectional area (width x thickness), leading to Equation 3.

$$\sigma_c = \frac{E_{\text{Si}} t_{\text{Si}}^3}{6 t_f (1 - v_{\text{Si}}) (t_f + t_{\text{Si}})} \frac{3\delta}{2L^2} \times \frac{1}{1 + \frac{t_f}{t_{\text{Si}}}} \quad 2$$

$$F_{\text{myotube}} = \sigma_c \times t_f \times w_{\text{Si}} \quad 3$$

Immunostaining—Cardiomyocyte cultures were fixed for 10 minutes with fresh 4% paraformaldehyde in PBS, followed by three washes in PBS for 30 minutes. Cells were permeabilized with a solution of 0.1% Triton X-100 in PBS for 15 minutes then blocked with 5% donkey serum in PBS for 20 minutes. Cell cultures were then incubated overnight at 4°C with a primary antibody against Troponin T (Millipore, MAB 1693, 1:300). Following three washes with PBS, cells were incubated for 2 hours at room temperature in the dark with a secondary antibody conjugated to Alexa-488 fluorophore (Invitrogen Molecular Probes, A21206, 1:300). Cells on cantilevers and MEAs were rinsed three times in PBS, with the second rinse containing phalloidin-594 (Invitrogen A12381, 1:40) and DAPI (Sigma, D9564, 1:10,000). Cells on control coverslips were mounted on glass slides using Vectashield mounting medium with DAPI (Vector Labs), and affixed using clear nail polish. Images were collected with a Hamamatsu spinning disk confocal microscope and the accompanying software.

Statistical Analysis

For each drug, a repeated measures ANOVA was applied to the data for each response variable ($\alpha = 0.05$), followed by the Holms-Sidak multiple comparisons test ($\alpha = 0.05$) for datasets in which the repeated measures ANOVA was significant. Nonlinear regression was performed using a power equation on the percent change data for each drug and response variable, following the form $\%Change_{output} = A \times Concentration^B$. A significant trend was found if the regression was significant and both regression coefficients were significant ($\alpha = 0.05$). Further nonlinear regression was performed using the power equation on the percent change data for each drug by combining the data from all output variables for which a significant regression was found. The percent change data from all response variables were first normalized to the value obtained for the highest concentration, and then combined into a single dataset for each drug. Nonlinear regression was performed on the combined dataset for each drug following the form $\%Change_{output} = A \times Concentration^B$ to find the coefficients describing the shape of the drug-response curve.

RESULTS

The system demonstrated here was developed to model the major determinants of cardiac output (CO) in a 2D, high-content analysis format for both acute (< 24 hours) and chronic evaluation of compounds. CO is determined by the electrical rhythm generation and conduction system of the heart, and by the force generation ability of the cardiac muscle. To measure those parameters, two BioMicroelectromechanical Systems (BioMEMS) subsystems were employed. Extracellular surface-embedded microelectrodes were used for the measurement of the electrical properties of cultured 2D cardiac tissue analogues derived from human stem cells, while AFM cantilever technology was used for the measurement of human cardiomyocyte contractile force (Figure 1). For cardiac muscle, the parameters evaluated were spontaneous beating rate, conduction velocity, Action Potential (AP) length or QT interval, minimum interspike interval (mISI) (upon high frequency stimulation) and peak contractile force. In the demonstrated parallel system the GE cells formed monolayers of spontaneously contracting cardiomyocytes within days of thawing and replating with stable activity for greater than 3 weeks. Both spontaneous and stimulated electrical activities

were successfully measured utilizing this system, but the stimulated activity allowed for more control and better reproducibility.

To demonstrate equivalence in our system to this previously collected data, patterned human cardiomyocytes on MEAs were used to measure AP length and, by using electrical stimulation at different frequencies, conduction velocity (Figure 2A–B). Conduction velocity was calculated as distance between electrodes divided by the time-difference between the recorded peaks and was statistically the same independent of the path length. The stimulated conduction velocities between short paths (average length 1200 μm) and long paths (average length 7400 μm) on average were 0.173 and 0.177 m/s, respectively ($p > 0.9$). Additionally, regression analysis of conduction velocities along a wide range of distances among multiple cultures indicated that distance is not a significant predictor of conduction velocity ($p > 0.4$), accounting for less than 7% of the variation in the velocities.

This indicated that using patterned surfaces eliminated the dispersion found with all unpatterned MEA publications to date. This improvement in methodology allows the recording of AP length, by using electrical stimulation at different frequencies, to determine the length of the refractory period following a cardiac AP, or the minimum interspike interval (mISI) (Figure 2C). Regression analysis indicates that mISI and QT interval are highly linearly interrelated regardless of the drug and concentration applied ($p < 0.001$), with the mISI equal to 3.0 times the length of the QT interval. The QT interval was measured as the time difference between the positive and the negative peak of the field potential. Even in cases in which the QT interval cannot be accurately measured due to system constraints that obscure the shape of the T wave, such as low signal-to-noise ratio, the mISI can still be easily measured to provide a robust measurement of deviations in heart function. According to recent publications the shape of the depolarization phase of the AP (Triangulation[5]) determines the possibility of reentry and fibrillation in the heart. Thus, the refractory period after AP, measured in our system, may have higher predictive value towards arrhythmogenic side effects than QT interval.

Three drugs were tested that have a proven effect on electrical activity in the heart: norepinephrine, sotalol and verapamil. The parasympathetic nervous system regulates heart rate and contraction force through the neurotransmitter norepinephrine. In our system norepinephrine significantly increased spontaneous beating rate by up to 85% ($p < 0.001$), increased amplitude by up to 33% ($p < 0.001$), decreased QT interval by up to 20% ($p = 0.004$), and the QT interval's analogue mISI by up to 21% ($p = 0.002$) (Figure 2D). These results are in good agreement with previous published data[37]. Sotalol is an arrhythmogenic agent, with potent hERG channel blocking properties and significantly increased QT intervals by up to 84% ($p = 0.003$). It also increased mISI by up to 58% ($p < 0.001$) and decreased conduction velocity by up to 43% ($p = 0.002$) (Figure 2e) and these results are in agreement with published data[38]. The major effect of verapamil is the blockade of voltage dependent calcium channels in the cell membrane. In this system it significantly, and in a concentration dependent manner, increased the spontaneous beat rate of the cardiomyocytes by up to 77% ($p < 0.001$)[39]. Also, regression analysis indicates a decrease in amplitude of up to 12% ($p < 0.001$), a decrease in QT interval by up to 38% ($p < 0.001$) and a decrease in mISI by up to 26% ($p = 0.003$).

All investigated drugs, except sotalol, had an effect on cardiac muscle force generation. Figure 3 illustrates the effect of each compound on the force produced either by stimulation of the cardiac monolayer cultured on a BioMEMs cantilever or by spontaneous beating. Cantilever deflection was converted to peak force utilizing cell thickness as determined by immunocytochemistry[31]. Figure 3A indicates representative immunocytochemical analysis of the beating cardiomyocytes on an unpatterned coverslip control as well as on a cantilever, which indicates that narrow cantilever geometry produces the same alignment effect as seen with the patterning of the cardiomyocytes. Cellular monolayer thickness was determined from the immunocytochemical analysis using z-stack reconstruction from confocal images. Since contraction force generation in cardiomyocytes primarily depends on intracellular calcium concentrations, verapamil was expected to decrease contraction peak force given its function as a calcium channel blocker. After exposure to 3 μ M verapamil the peak force for spontaneous beating cardiomyocytes was reduced by 43% ($p = 0.01$) of the control, and the change was concentration dependent (Figure 3B), in agreement with previously published data[39]. The frequency of contraction also increased by 30%, in good agreement with the frequency change measured on the MEAs at 3 μ M verapamil. Sotalol's effect on contractile force was expected to be minimal since its role is in modulating QT interval and has little effect on the cell's contractile apparatus. This assumption was confirmed as no change in contraction force frequency was observed from a spontaneous beating cardiomyocyte layer after drug treatment (Figure 3C). Equivalent results for force changes after treatment with verapamil and solatol were seen with stimulated experiments on the cantilevers.

Norepinephrine slightly increased the stimulated peak contraction force of the human cardiomyocytes in the concentration range of 0.1 – 3 μ M, using our base medium formulation. However, to capture the positive inotropic effect of norepinephrine, experiments were done in an extracellular solution containing 1 mM Ca^{2+} (CaCl_2 solution) to amplify the drug's effect. The initial medium, containing roughly 2 mM Ca^{2+} , was stepwise removed and replaced with the calcium-free medium. Cells were allowed to equilibrate to the new conditions before a 1 mM Ca^{2+} addition and norepinephrine treatment were made; cell response was continuously monitored throughout this procedure. Figure 3D indicates the force traces at 0 mM Ca^{2+} (a), 1 mM Ca^{2+} (b) and after addition of 1 μ M norepinephrine to the medium containing 1 mM Ca^{2+} (c). In 0 mM Ca^{2+} conditions, the cardiomyocytes exhibited no beating activity on cantilevers even when electrically stimulated, while in 1 mM Ca^{2+} , the cardiomyocytes beat in synchronization with electrical stimulation. The addition of 1 μ M norepinephrine caused an increase in contraction force by 84% ($p < 0.01$) compared to the same conditions without norepinephrine, thereby demonstrating the inotropic effect of norepinephrine in this system. It should be noted that recording from MEA systems utilizing the reduced calcium medium indicated no significant changes in the cardiac electrical parameters.

The measurement of the drug effects with either set of BioMEMs systems in simple 2D configurations separately would be powerful predictors of drug function, however, their combination now allows for prediction of some of the most critical *in vivo* effects seen with intact human heart. The pharmacological data is summed up in Figure 4A for verapamil,

norepinephrine and sotalol, and indicates dose dependent responses that are highly correlative to known *in vivo* clinical data for toxicity and efficacy. This approach is different than analysis of molecular biomarkers in that *functional* characteristics are combined for this predictive capability much like evaluation of a human in a doctor's office. Figure 4B shows the effect of reference compounds on rhythm generation, conduction velocity, field potential amplitude, QT interval, mISI, and peak contraction force in a concentration dependent manner. Data are represented as percentage changes compared to the control and were expressed as mean $\pm$ SEM, n = 4–8. Each drug indicated a different combination of effects. A: Sotalol significantly increased QT interval and mISI and decreased conduction velocity. It had no effect on force generation. Norepinephrine increased spontaneous beating frequency and decreased QT-interval. It increased contraction force. Verapamil decreased peak force, increased beating frequency and decreased QT interval. Combined, the data gives a fingerprint of a drugs action that should be unique to a given compound.

This screening system facilitates an estimation or even prediction of drug effects on whole heart function through measurement of the two main cellular functions, electrical conduction changes and contractile force. This data can be displayed in a continuous stream, much like an ECG monitor, and also allows for non-invasive *chronic* monitoring of drug effects as this system is routinely maintained for 3 weeks in culture, and we have previously shown data can be collected for over 60 days when using a comparable rat based system[17]. Combined, these systems supply comparable information content to the gold standard for *in vitro* testing, the Langendorff whole heart model. Instead of dissected rabbit hearts however, this model is composed entirely of human cardiac cells in a simple, 2D, defined, serum-free culture system.

DISCUSSION

The development of a parallel, high information-content, phenotypic screening system to measure the major determinants (rhythm generation, conduction, AP length and muscle force) of human cardiac function using a 2D cell-culture based format, integrated with BioMEMs devices, that reproduces the main functional components of 3D cardiac tissue was detailed in this article. The parallel system adapted a silicon cantilever myotube system[40] to measure contractile force, and MEA technology to monitor the two main parameters of interest for the human heart in drug discovery (electrical conduction, force generation), yet as independent measurements. Such independent measurements are not possible in whole heart or whole animal/human systems. This separation, and the use of 2D systems, greatly simplifies the construction and operation of the model. The use of human cardiomyocytes also eliminates extrapolation problems due to interspecies differences.

The demonstrated *in vitro* parallel system accurately reproduced and confirmed well characterized *in vivo* effects of pharmaceutical compounds on the heart. Norepinephrine showed a significant increase in frequency and force which correlated with *in vivo* inotropic action. In humans, intravenous administration of norepinephrine led to an increase of 32% in the ventricular contractile force in 15 patients[41] and an increase of 32 beats/min in the rate of the donor atrium in patients who had received a transplanted human heart[42]. Sotalol, a Class III antiarrhythmic, prolongs the atrial and ventricular monophasic action potential.

Clinical observations confirmed a dose dependent increase in the QT interval in healthy volunteers (between 4–20%)[43, 44] and in patients with supraventricular tachyarrhythmias (of up to 27%)[45] after treatment with sotalol. In this model system, sotalol increased both the QT and the mISI in a dose response manner similar to that observed with the clinical data. Verapamil, a voltage-dependent Ca^{2+} channel blocker decreased cardiomyocyte force in our model. Data in canine ventricular myocytes, as well as in humans, supports a negative inotropic effect (force reduction) upon verapamil administration[39]. Besides affecting the calcium ion influx, verapamil also inhibits the hERG protein, similar to sotalol, but it does not increase the QT interval because the inhibition of both channels compensates[44]. Unlike the *in vitro* hERG channel inhibition test, this functional cardiac system did not give a false positive when exposed to Verapamil. The tested reference compounds affected the measured parameters in a unique and characteristic way ('fingerprints') in this system, according to their mechanism of action and their known physiological effects obtained from clinical data.

There is a widely held belief that, if one seeks to recapitulate *in vivo* organ function, tissue-engineered constructs need to be in three dimensions. This point is definitely true for applications of tissue engineering and regenerative medicine for *in vivo* applications, but not necessarily so for *in vitro* applications, as the main goal should be demonstration of function, irrespective of system geometry. While certain *in vitro* systems probably require 3D architecture, these results clearly demonstrate that 2D cellular models, integrated with the proper microfabricated systems, can reproduce critical aspects of 3D *in vivo* function. In addition, since microfluidic and BioMEMs technology are being employed, if necessary, biomarkers can also be evaluated through fluid draws or with continuous monitoring of specific test compound parameters. It is important to note, however, that by utilizing functional measures in this system, the number of biomarkers necessary for complete analysis is drastically reduced, if not eliminated altogether.

Another major tenet is that, for organ systems other than CNS, serum is a necessary component for proper organ development and function. The argument generally being made is that various growth factors and biomolecules are necessary for proper development of tissues. However, little attention is paid to the fact that other signaling molecules, whose role is to repress different stages of development, are also present in serum. It may be one of the main drivers for why many stem cell-based systems fail to achieve functional maturity and de-differentiate after short periods of time in culture. Rumsey et al. 2009 showed complete node of Ranvier formation on motoneurons by Schwann cells, where the only variable changed was removal of serum[46]. Thus, the absence of serum, in addition to providing a totally defined medium for drug candidate evaluation, also may be a driver for the differentiated human stem cells in the demonstrated parallel system to maintain their state of maturity.

Concerning the predictive value of the system: the measured *in vitro* electrophysiological parameters are analogous to the parameters used in the SCREENIT scoring system introduced by Hondeghem and coworkers in 1994[47]. In this system beating frequency, conduction velocity, QT interval and peak force, as well as reverse use dependence, variability in QT intervals and relative refractory period (which is related to triangulation)

can be measured. There is still controversy concerning the predictive value of QT interval measurement towards arrhythmogenic side effects of drugs, so the ability to measure both the QT interval and the mISI difference is advantageous. The simplification of the device is further enhanced by using the repeated measures approach for data analysis, as the variation among cantilevers is accounted for and eliminated from the analysis, thus abolishing the need to determine differences among cardiomyocyte layers on specific cantilevers. However, determination of absolute force values can still be made with high accuracy if cardiomyocyte thickness is obtained from immunocytochemical imaging[31].

The use of repeated measures analysis also enables us to account for the system differences among samples including any variation among cultures and cell source manufacturer lots. An analysis of the variation among samples demonstrated that the use of repeated measures analysis decreased the ratio of standard deviation to difference between means by an average of 70% versus a standard multiple comparisons analysis that does not take into account variation among samples using the data collected for this work. In situations with higher variability between samples, a system such as the one presented becomes increasingly important. Overall, the advantage of using repeated measures statistics is that it enables the use of cultures with significant variation while maintaining the ability to distinguish differences among treatments, making the system extremely robust.

CONCLUSIONS

A drug screening device must identify key cellular responses that report physiological states, either by functional outcomes or from biomarker analysis. Moreover, it must produce physiologically relevant predictive modeling, based on known clinical responses to drugs, to ensure the device accurately reports drug toxicology and efficacy. Besides the novel predictive capability, reduced cost will be the main driver to realize widespread acceptance of this technology. In addition, low-cost disposables and replacement chips, and rapid assembly to minimize personnel hours are required. This system offers a potentially low cost solution in terms of technology and operation, which are essential for the success of this microphysiological system. Application of a phenotypic, high information content human-based cardiac screen earlier in the preclinical drug development process would save significant time and money, and reduce drug failures in the clinical phase resulting in safer and cheaper drugs on the market. Application of patient-derived human cardiomyocytes (from induced pluripotent stem cell sources) in the system would open new avenues in drug testing, targeting disease state modeling or individual genetic variability in the patient population. Integration of this system with other constructs, such as physiologically correct functional systems for skeletal muscle[31], neuromuscular junctions[48], myelination[46] and neuronal networks[49], modeling different tissue functions would be straightforward.

This system demonstrates microscale structures of iPSC-cardiomyocytes used for both electrophysiological and force measurements, with the active area on the order of tens of mm^2 . The cantilevers used for force measurements are produced in an array, which is itself a demonstration of feasibility of high-throughput measurements. Likewise, the electrodes used for electrophysiological measurements are also arrayed and demonstrate feasibility for expansion for high-throughput screens. The technology described here is fully capable of

being extended to higher throughput, with such an extension simply requiring larger arrays of electrodes and cantilevers and an expanded data acquisition system. The benefits of this approach are the simplicity and relevance of the readouts and the ease of reconfiguration of the modular systems into body-on-a-chip devices[50].

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Figure 1.

Key elements for the platform for the determination of cardiac function with human cells. Cardiac function was extrapolated from measurement of rhythm generation (frequency and amplitude), conduction velocity, action potential length (QT interval) and force generation of the heart. A) Schematic of the system used to pattern SAMs on MEAs (top). Phase contrast micrograph of patterned human-derived cardiomyocytes on top of substrate embedded extracellular electrodes. Immunostaining verified that human derived cells differentiated to cardiomyocytes (middle) and exhibited cardiac rhythm generation as measured by the embedded electrodes (bottom). B) Diagram of the cantilever-based force measurement system (top) cardiomyocytes integrated into the BioMEMs device and immunocytochemistry indicating cardiac alignment along the cantilever (middle). Example traces of deflection and torsional force with the device after myocyte contractions (bottom). The repeated measures statistical analysis blocked for differences among MEAs and cantilevers. Scale: The interelectrode distance on the MEA is 200 μm .

Representative Recordings with MEAs

Figure 2.

Measurement of rhythm generation, conduction velocity, AP length or QT interval and minimum interspike interval with patterned multielectrode arrays. A: Phase contrast image of human cardiomyocytes patterned to produce defined conduction pathways of variable lengths. B: Representative recordings of electrically stimulated APs propagated between two electrodes located at the opposite ends of a short loop and long loop pattern. C: Consistent conduction velocity over the entire pattern enabled measurement of mISI. Electrical stimulation was applied to the patterned cardiomyocytes at increasing frequency and the inability of the cells to beat in synchrony at a given frequency was used to determine the minimum relaxation time or mISI. D: Effect of norepinephrine on spontaneous beating rate of human cardiomyocytes. Representative recordings of control and 3 μM norepinephrine treated cultures, and histogram indicating the change in beating frequency with increasing norepinephrine concentration. E: Effect of sotalol on measured AP length or QT interval (an analogue to the QT interval on the ECG recordings). The histogram indicates the QT interval change with increasing sotalol concentration. Data in both histograms (figure D and E) are presented as mean $\pm$ SEM, $n = 5$. Scale: The interelectrode distance on the MEA is 200 μm .

Representative recordings with AFM-based cantilever system

Figure 3.

Measurement of force generation. A: Representative confocal images showing Troponin T (green), Actin (red) and DAPI (blue) immunostaining of human cardiomyocytes plated on coverslip controls (left) as well as on cantilevers (right). B: Cantilever force measurements indicating the effect of 3 μM verapamil on the cardiomyocytes (red) compared to control (black). C: Cantilever force values indicating no effect from 100 μM sotalolol (red) compared to control (black). D: Modification of the medium to enhance the visualization of the effect of calcium on contraction force. From top to bottom: medium containing 0 mM Ca^{2+} , no contraction, after adding 1 mM Ca^{2+} , contraction restored, and after adding 1 μM norepinephrine, which produced enhancement of force. No frequency changes could be measured for norepinephrine as they were stimulated measurements.

Drug (μM)	Frequency (Hz)	CV (m/s)	Amplitude (Relative)	QT Interval (ms)	mISI (ms)	n	Force (Relative)	n
Sotalolol	0.62 ± 0.04	0.27 ± 0.07	1	0.19 ± 0.02	0.71 ± 0.06	4	1	6
10	0.63 ± 0.10	0.25 ± 0.09	1.01 ± 0.03	0.19 ± 0.03	0.74 ± 0.07	4		
30	0.64 ± 0.07	0.22 ± 0.11	1.04 ± 0.03	0.24 ± 0.06	0.80 ± 0.17	4		
100	0.70 ± 0.12	0.21 ± 0.11	1.10 ± 0.17	$0.29 \pm 0.06^*$	0.91 ± 0.11	4	1.04 ± 0.42	6
300	0.69 ± 0.11	$0.17 \pm 0.11^*$	0.95 ± 0.19	$0.34 \pm 0.08^*$	$1.13 \pm 0.25^*$	4		
Norepinephrine	0.74 ± 0.21	0.16 ± 0.06	1	0.21 ± 0.03	0.72 ± 0.42	5	1	4
0.1	$1.01 \pm 0.30^{**}$	0.18 ± 0.06	1.19 ± 0.24	$0.20 \pm 0.03^{**}$	0.63 ± 0.33	5		
0.3	$1.17 \pm 0.24^{**}$	0.16 ± 0.06	1.23 ± 0.38	$0.18 \pm 0.03^{**}$	0.57 ± 0.27	5		
1	$1.24 \pm 0.23^{**}$	0.17 ± 0.06	1.26 ± 0.37	$0.17 \pm 0.03^{**}$	0.54 ± 0.24	5	$1.75 \pm 0.50^*$	4
3	$1.33 \pm 0.22^{**}$	0.17 ± 0.06	1.35 ± 0.52	$0.17 \pm 0.03^{**}$	0.51 ± 0.20	5		
Verapamil	0.92 ± 0.19	0.21 ± 0.08	1	0.28 ± 0.06	0.68 ± 0.23	8	1	4
0.3	$0.97 \pm 0.23^*$	0.21 ± 0.09	0.98 ± 0.04	0.26 ± 0.05	0.65 ± 0.27	8		
1	$1.09 \pm 0.23^*$	0.21 ± 0.09	0.98 ± 0.04	0.25 ± 0.05	0.61 ± 0.22	8	0.77 ± 0.20	4
3	$1.20 \pm 0.29^*$	0.22 ± 0.10	0.96 ± 0.06	$0.21 \pm 0.05^*$	0.60 ± 0.22	8	$0.58 \pm 0.26^*$	4
10	$1.56 \pm 0.49^*$	0.21 ± 0.09	0.88 ± 0.22	$0.17 \pm 0.06^*$	$0.53 \pm 0.19^*$	8		

* Significant at $p < 0.05$ level. Holms-Sidak multiple comparisons test

** Significant $p < 0.01$ level. Holms-Sidak multiple comparisons test

Figure 4.

Effect of reference compounds on frequency (rhythm generation), conduction velocity (CV), field potential amplitude, AP length or QT interval, mISI and peak contraction force as noted in A) Table and B) graphs. Data are represented as percentage changes compared to

the control and are expressed as mean $\pm$ SEM, n = 4–6. * marks significant difference ($p < 0.05$).

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