High-Throughput Phenotyping of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes and Neurons Using Electric Field Stimulation and High-Speed Fluorescence Imaging

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ABSTRACT

Electrophysiology of excitable cells, including muscle cells and neurons, has been measured by making direct contact with a single cell using a micropipette electrode. To increase the assay throughput, optical devices such as microscopes and microplate readers have been used to analyze electrophysiology of multiple cells. We have established a high-throughput (HTP) analysis of action potentials (APs) in highly enriched motor neurons and cardiomyocytes (CMs) that are differentiated from human induced pluripotent stem cells (iPSCs). A multichannel electric field stimulation (EFS) device enabled the ability to electrically stimulate cells and measure dynamic changes in APs of excitable cells ultra-rapidly (>100 data points per second) by imaging entire 96-well plates. We found that the activities of both neurons and CMs and their response to EFS and chemicals are readily discerned by our fluorescence imaging-based HTP phenotyping assay. The latest generation of calcium (Ca²⁺) indicator dyes, FLIPR Calcium 6 and Cal-520, with the HTP device enables physiological analysis of human iPSC-derived samples highlighting its potential application for understanding disease mechanisms and discovering new therapeutic treatments.

Keywords: Cal-520, calcium, cardiomyocyte, electric field stimulation, FLIPR Calcium 6, induced pluripotent stem cell

INTRODUCTION

Measurement of electrical activity in live biological samples has been challenging and multiple approaches have been developed. The technique for measuring action potentials (APs) using elec-

trodes was established more than 50 years ago.¹ This approach, that is, patch clamp of a single cell, requires significant technical expertise to analyze rapidly changing APs from biological samples. Optically measuring AP is an alternative to techniques using electrodes and has been successful in analyzing electrophysiology of various sample types, including whole hearts.² The recent development of optical probes that are much more sensitive for detecting APs lowers the technical barrier for measuring APs in cardiomyocytes (CMs) and neurons. In addition, optical imaging enables simultaneous recording of many neurons at the same time without damaging cells and measurement of multiple forms of activities, including AP firing, synaptic activity, and other subcellular signals.

APs in neurons allow for the communication of biological signals between neurons.³ Deciphering how the brain generates cognitive, emotional, and motor function from patterns of these electrical signals is one of the ultimate challenges in neuroscience.^{4,5} Advancing toward this goal is facilitated by the development of technologies for monitoring the electrical signals of large neuronal populations with fine spatiotemporal resolution. The APs in cardiac pacemaker cells propagate through cardiac tissues and regulate cardiac contractions through the excitation-contraction coupling mechanism. Nearly 45% of drugs withdrawn from the market between 1975 and 2007 were eliminated due to cardiac safety concerns.⁶ Drug-induced interference of APs is one of the primary concerns of cardiac safety issues. Human induced pluripotent stem cell (iPSC)-derived CMs respond to well-known proarrhythmic compounds⁷ and will be used to improve the guidelines for cardiac safety testing.⁸

Since the physiological activity of neurons and CMs is represented by changes in membrane voltage, voltage sensors are a logical choice for high-throughput (HTP) assays. Although there are many new voltage-sensitive dyes (VSD),^{9,10} imaging voltage is exceptionally difficult in HTP. Voltage signals in

neurons last around 1–5 ms (10–100 times faster than even the fastest calcium $[Ca^{2+}]$ signals) and are confined to the plasma membrane, which leads to low signal-to-noise ratio (SNR). Duration of membrane potentials lasts at least a few hundred milliseconds in CMs. However, optical signals for APs still have low SNR. Changes in membrane potential lead to calcium (Ca^{2+}) influx through voltage-gated calcium channels located throughout the cell, and calcium transients are significantly longer in duration compared with voltage fluctuations. Therefore, quantification of free Ca^{2+} changes can be used as a reliable proxy for neural and cardiac activity. Small molecule Ca^{2+} dyes have achieved widespread use in the last three decades, and state-of-the-art indicators such as Oregon Green BAPTA-1¹¹ and fluo-4¹² have been the workhorses of HTP imaging *in vitro*.

Cellular activities regulated by the electrophysiology of cells, such as membrane potential and intracellular calcium levels, by nature are very rapid. It requires technology to acquire signals in millisecond time frames. The HTP parallel acquisition of ultra-rapid data brings additional challenges. For instance, the effort to identify subtype-selective Na^+ channel blockers is still underway to develop highly effective and better-tolerated treatments for cardiac arrhythmias, epilepsy, cancer, neurodegenerative diseases, spasticity, and chronic and neuropathic pain.¹³ To perform such phenotypic assays in electrophysiology in HTP, a stable electrical stimulator that can trigger APs in a multiwell format is critical for reproducible compound screening. In this study, we describe a HTP phenotypic compound screening approach for analyzing the electrophysiology of many neurons or CMs growing in 96-well plates excited by an electrical field potential. Rapidly changing electrophysiology of neurons and cardiomyocytes is recorded optically using an ultra-rapid CCD camera. The various applications of electrical pacing on electrically excitable cells are introduced.

MATERIALS AND METHODS

Neuronal Culture

Human iPSC-derived motor neurons (BrainXell, Inc., Madison, WI) were plated at a density of 20,000 cells per well on poly-D-lysine-coated CellCarrier™ 96-well microplates (PerkinElmer, Waltham, MA). Motor neurons were cultured in 100 μ L Neurobasal® Medium plus B27 supplement (Thermo Fisher Scientific, Waltham, MA) and motor neuron maturation supplement (BrainXell, Inc.) for 7–10 days.

Neuronal Electric Field Stimulation

The neurons were loaded with artificial cerebrospinal fluid (ACSF) containing 5 μ M calcium-sensitive fluorescent dye, Cal-520/AM (AAT Bioquest, Sunnyvale, CA). ACSF was added to neuronal cultures (ACSF containing 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4 , 1 mM $MgSO_4$, 1.2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose) to improve the neuronal activity. After incubating for 30 min at 37°C in 5% CO_2 , neuronal cultures were treated with compounds and incubated for 15 min at 37°C in 5% CO_2 . Stock solutions of compounds were first diluted (1:200) in ACSF, then added 25 μ L/well. Neuronal cultures were imaged on a functional drug screening system (FDSS)/ μ CELL (Hamamatsu Photonics) at 480 nm excitation and 540 nm emission with electric field stimulation (EFS). EFS was performed using an EFS device developed by Hamamatsu Photonics for use with the FDSS/ μ CELL. Neuron cultures were stimulated by EFS at 20 V, pulse width at 2.0 ms, number of pulses at 50 times, and frequency at 30 Hz (Table 1).

Table 1. Neuronal Culture Protocol

Step	Parameter	Value	Description
1	Coat plate	100 μ L	Poly-D-lysine
2	Plate cells	100 μ L	20,000 cells/well (0.63×10^5 cells/cm ²)
3	Feed cells	100 μ L	Neurobasal medium with B27 supplement (Thermo Fisher Scientific, Waltham, MA) and motor neuron maturation supplement (BrainXell Inc., Madison, WI)
4	Culture time	7–10 days	37°C, 5% CO_2
5	Dye loading	100 μ L	Cal-520 AM (5 μ M) in ACSF
6	Incubation time	30 min	37°C, 5% CO_2
7	Drug addition	25 μ L	TTX, glycine, GABA, THIP, CGP 54626, DMSO
8	Incubation time	15 min	37°C, 5% CO_2
9	Assay readout	540 nm	FDSS/ μ Cell fluorescence imager, 480 nm excitation, 540 nm emission; EFS stimulation

Step Notes

1. CellCarrier™ 96-well microplate (black, clear-bottom).
2. Human iPSC-derived motor neurons (BrainXell, Inc.).
3. Change half media every 3 days.
4. Cells cultured to reach maturation.
5. Dye mix is warmed to 37°C.
6. Green fluorescence observed under microscope.
7. Compounds are diluted (1:200) to 4 $\times$ of final concentration in ACSF, DMSO (control) is 0.1% in ACSF.
8. Incubation time may vary depending on the compounds used.
9. EFS Stimulation voltage: 20 V, frequency: 30 Hz, pulse-width: 2 ms, number of pulses: 50 times.

ACSF, artificial cerebrospinal fluid; DMSO, dimethyl sulfoxide; EFS, electric field stimulation; FDSS, functional drug screening system; GABA, gamma-aminobutyric acid; iPSC, induced pluripotent stem cell; THIP, 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride; TTX, tetrodotoxin.

CM Culture

Human CMs were derived from human iPSCs (Applied Stem Cell, Milpitas, CA) similar to previously published methods using small molecule modulators of Wnt signaling pathways. The RPMI 1640 Medium (Thermo Fisher Scientific) supplemented with B27 minus insulin (Thermo Fisher Scientific) was used during CM differentiation and the Advanced RPMI 1640 Medium was used to maintain beating CMs. Differentiated CMs were harvested from the plates by treatment with 0.25% Trypsin (Thermo Fisher Scientific) and Accutase (Innovative Cell Technologies, San Diego, CA). CMs were harvested 20–30 days after the start of differentiation and replated onto Matrigel-coated wells of a 96-well μ -Plate (ibidi, Madison, WI) at 100,000–200,000 cells per well ($1.8\text{--}3.6 \times 10^5$ cells/cm²). CMs were cultured for 7–10 days in Advanced RPMI 1640 (Thermo Fisher Scientific).

Cardiomyocyte EFS

CMs were stained with the Ca²⁺ transient dye FLIPR Calcium 6 (Molecular Devices, Sunnyvale, CA) at a 1:1 media to dye ratio for 40–60 min before fluorescence recordings were performed. CMs were treated with dofetilide or E-4031 and incubated for 30 min at 37°C in 5% CO₂. Time-matched controls (<0.1% dimethyl sulfoxide [DMSO]) were included in all assay plates for comparison to account for changes in signal intensity with time. CM cultures were recorded on an FDSS/ μ CELL (Hamamatsu Photonics) at 480 nm excitation and 540 nm emission with EFS. CM cultures were recorded without EFS for 0–30 s and with EFS for 30–60 s at 5 V, pulse width at 10 ms, number of pulses at 100 times, and frequency at 0.5 Hz (Table 2).

Calcium Transient Analysis

Custom software developed at InvivoSciences using MATLAB (MathWorks) was used to detect and assess the average calcium transient amplitude and calcium transient duration.

Calcium transient duration-90 (CaTD-90) is defined as the duration at 90% from the peak of the transient.

Assessment of Z-Factor

Z-factor was assessed for each assay using the baseline and drug-treated measurements from the same wells.

Z-factor was assessed according to the following equation:

$$Z' = 1 - \frac{3(SD\ p + SD\ n)}{|\text{Mean } p - \text{Mean } n|}$$

Values for negative control (*n*) were time-matched DMSO control measurements and values for positive controls (*p*) were the drug-treated measurements for the indicated dose.

RESULTS

Optimizing 96-Electrode Array for Stable Electrical Stimulation

A 96-electrode array (patent pending) (Fig. 1) is designed to stably stimulate cells growing in a monolayer on the bottom of 96-well plates. Applying electrical pulse to each central pin-electrode surrounded by a grounded cylindrical electrode enables distribution of an intense electric field near the center of wells. While biphasic stimulation has been shown to improve organoid culture,^{14,15} monophasic stimulation was applied to deliver

Table 2. Cardiomyocyte Culture Protocol

Step	Parameter	Value	Description
1	Coat plate	100 μ L	Matrigel, >2 hrs at 37°C
2	Plate cells	100 μ L	100,000–200,000 cells/well ($1.8\text{--}3.6 \times 10^5$ cells/cm ²)
3	Feed cells	100 μ L	Advanced RPMI 1640 (Thermo Fisher Scientific)
4	Culture time	7–11 days	37°C, 5% CO ₂
5	Dye loading	100 μ L	FLIPR Calcium 6 (1:1 media to dye) or Cal-520 AM (2 μ M)
6	Incubation time	60 min	37°C, 5% CO ₂
7	Wash	100 μ L	Cells are washed 1 $\times$ with warmed media
8	Replace media	90 μ L	Conditioned cell media
9	Drug addition	10 μ L	E-4031, Dofetilide, DMSO (control)
10	Incubation time	30 min	37°C, 5% CO ₂
11	Assay readout	540 nm	FDSS/ μ Cell fluorescence imager, 480 nm excitation, 540 nm emission; EFS stimulation: 30 s unpaced, 30–60 s paced

Step Notes

1. ibidi μ -Plate 96 well or Corning 96 well (black, clear bottom).
2. Cardiomyocytes harvested 20–30 days after differentiation with 0.25% trypsin and accutase.
3. Change media every 2 days.
4. Cardiomyocytes cultured until stable beating is observed.
5. Dye mixture is warmed to 37°C.
6. Flashing green fluorescence observed under microscope.
7. Dye media are aspirated and washed with Advanced RPMI 1640 media (37°C).
8. Replaced with conditioned media (stored at 37°C, 5% CO₂).
9. Drug stocks are 10 $\times$ of final concentration in media, DMSO (control) is 0.1% in media.
10. Incubation time may vary depending on the drug used.
11. EFS Stimulation voltage: 5V, frequency: 0.5–2.0 Hz, pulse-width: 10 ms, number of pulses: 100 times.

Fig. 1. EFS head, illuminator optics, and sensor camera for FDSS/μCELL. Image of EFS head unit (right) that descends into a 96-well plate (middle) for electrical stimulation at various frequencies. Below the 96-well plate are optics for fluorescence/luminescence excitation and the fluorescence/ luminescence sensor, which is capable of up to 120 Hz image capture. The EFS unit can be combined with an assay plate heating unit and fast data acquisition software as a part of the FDSS/ μCELL system (left). (Images and diagrams were provided by Hamamatsu Photonics.) EFS, electric field stimulation; FDSS, functional drug screening system. Color images available online at www.liebertpub.com/adt

a minimum, but sufficient electrical stimulation to depolarize cells that are electrically polarized at their resting state. A finite element analysis was employed to simulate electric field distribution to optimize electrode positions and identify the distance between the tips of the central electrode to the cell surface, which is a critical factor that determines the strength of the electric field (Fig. 2A). The cylindrical electrode is reproducibly placed close to the bottom of the plate. The distance from the tip of the central electrode to the bottom of the cylindrical electrode determines the maximum voltage applied near the center of the wells (Fig. 2B).

Ultra-Rapid Image Acquisition

Achieving a stable sampling rate for rapid image acquisition is also critical to generate high-resolution data that can be used for quantitatively analyzing the fast kinetics of changes in intracellular calcium and membrane potential triggered by electrical stimuli. Initiated by a software signal, the high-speed camera starts automatic repeating sequences of image acquisition involving image exposures and image data transfer using its onboard clock that can achieve sampling rates of 110 Hz (Fig. 2C). This approach can capture a higher number of images in a given time than using software triggers for each sampling. We compared the different sampling rates by capturing hiPSC CM APs and calcium transients at low-speed (27 Hz) and high-speed (>110 Hz) sampling rates. Although low sampling rates may be sufficient to capture spontaneous beating rates (~0.5 Hz), high-speed

Fig. 2. EFS parameters and software trigger. (A) Computational simulations of voltage fields between a center electrode and surrounding cylindrical electrode. X axis is the distance from the center; the center electrode has 1 mm diameter. Y axis is the field voltage when applying 10 V. Higher: bottom of central electrode is higher compared with outer electrode; lower: bottom of center electrode is the same as outer electrode. (B) Schematic diagram of center and cylindrical electrodes. (C) Schematic for comparing data sampling rates between software triggering each sampling (top) versus triggering once at the beginning and using the internal clock to trigger exposure. Color images available online at www.liebertpub.com/adt

sampling rates may be necessary to accurately capture the shape of the AP or calcium transient at higher beating rates (>0.5 Hz) triggered by EFS.

EFS Application in Neuroscience

Human iPSCs offer a new model system to analyze cellular function with normal and disease conditions, and an opportunity to discover and validate new pharmacological agents. In this study, we describe a HTP phenotyping platform for analyzing the human neuron activity using a calcium-sensitive fluorescent dye.

Human iPSC-derived motor neurons (BrainXell, Inc.) were plated on poly-D-lysine-coated 96-well microwell plates. By treating with motor neuron maturation supplement (BrainXell, Inc.) for 7 days, motor neurons showed mature neuronal morphology (Fig. 3A). The neurons were cultured in a neural differentiation medium and loaded with a calcium assay buffer containing 5 μ M calcium-sensitive fluorescent dye, Cal-520/AM (AAT Bioquest). After incubating for 30 min at 37°C in 5% CO₂, the motor neurons were successfully loaded with Cal-520 indicated by the appearance of green fluorescence in the cytoplasm and neurites (Fig. 3B). However, after

triggering with EFS, the motor neurons showed only a minor calcium change (amplitude of calcium wave <1.5 fluorescence ratio [FR]) in the neural differentiation medium, similar to a recent article¹⁶ that showed that the traditional neural differentiation medium impairs AP generation and calcium imaging. To improve neural function, we applied ACSF (126 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1 mM MgSO₄, 1.2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose), similar to a recently described BrainPhysTM Neuronal Medium.¹⁶ The ACSF increased the amplitude of the calcium wave to 3.0 (FR). We examined the EFS calcium change at different time points to see when motor neurons were fully matured. The amplitude of the calcium response was 1.54 (FR) on day 4 post-plating, increased to 2.82 (FR) on day 7, and further increased to 3.39 (FR) on day 10 (Fig. 3C). The EFS calcium response did not increase further when motor neurons were cultured for a longer time. These results suggest that the motor neurons are functionally mature by day 10.

Neural function is regulated by different ion channels and neurotransmitters.¹⁷ Sodium channels are responsible for the rising phase of APs.¹⁸ Gamma-aminobutyric acid (GABA)¹⁹ and glycine²⁰ are the main inhibitory transmitters for motor neurons. We then examined the effects of a sodium channel blocker, and GABA and glycine neurotransmitters on the calcium response in our platform. DMSO (0.1%) (absence of any compound), tetrodotoxin (TTX; 1 μ M) (sodium channel blocker), glycine (100 μ M), GABA (100 μ M), THIP (4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol

Fig. 3. Optimization of EFS for iPSC-derived motor neurons. **(A)** Image of motor neurons showing mature neuronal morphology. **(B)** Motor neurons loaded with Cal-520 displaying green fluorescence in the cytoplasm and neurites. **(C)** Calcium change of motor neurons in response to EFS at different time points; graphs display overlay of traces from six wells (day 4, 1.54 ± 0.12; day 7, 2.82 ± 0.02; day 10, 3.39 ± 0.02; $n=6$, mean ± SEM). iPSC, induced pluripotent stem cell; SEM, standard error of the mean. Color images available online at www.liebertpub.com/adt

hydrochloride; 100 μ M; selective GABA A receptor agonist), and CGP 54626 ([S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid; 50 μ M; selective GABA B antagonist) were added to the motor neuron cultures. The Ca^{2+} response evoked by EFS in the DMSO group had the amplitude of 3.48 (FR), and TTX largely reduced the amplitude of the Ca^{2+} response to 1.51 (FR), although a longer incubation time with TTX may be required for complete inhibition. Alternatively, this may be due to TTX-resistant Na^{+} channels or activation of voltage-gated calcium channels by EFS. The

amplitude of the Ca^{2+} response was also reduced by glycine to 3.05 (FR), GABA to 2.35 (FR), THIP to 2.28 (FR), and CGP to 2.82 (FR) (Fig. 4 and Table 3). Together, these results suggest that the human iPSC-derived motor neurons and their networks are responsive to functional regulators.

EFS Applications in Cardiac Physiology and Pharmacology

In addition to neuronal cell culture analysis, EFS offers a great opportunity to analyze rapidly changing Ca^{2+} transient activity in CMs triggered by periodic electrical stimulations.

Fig. 4. Regulation of neural function by ion channel blockers and neurotransmitters. **(A)** Calcium measurements of motor neuron cultures after EFS in the presence of DMSO (0.1%), TTX (1 μ M), glycine (100 μ M), GABA (100 μ M), THIP (100 μ M), and CGP 54626 (50 μ M). Graphs display overlay of recordings from 6 wells. **(B)** EFS-stimulated neuronal calcium wave after treatment with TTX, glycine, GABA, THIP, and CGP 54626 ($*P < 0.0001$; Table 3). **(C)** Inhibition of EFS-stimulated neuronal calcium wave by TTX, glycine, GABA, THIP, and CGP 54626 ($*P < 0.0001$; Table 3). CGP 54626, [S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid; DMSO, dimethyl sulfoxide; GABA, gamma-aminobutyric acid; THIP, 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride; TTX, tetrodotoxin. Color images available online at www.liebertpub.com/adt

Table 3. Neuron Electric Field Stimulation Assay

Compound	Concentration (μ M)	<i>n</i>	FR		% Inhibition	
			Value $\pm$ SEM	Z'	Value $\pm$ SEM	Z'
TTX	1	6	1.51 $\pm$ 0.01	0.88	56.6 $\pm$ 0.22	0.966
Glycine	100	6	3.05 $\pm$ 0.02	0.33	12.5 $\pm$ 0.50	0.575
GABA	100	6	2.35 $\pm$ 0.02	0.75	32.7 $\pm$ 0.46	0.877
THIP	100	6	2.28 $\pm$ 0.02	0.76	34.7 $\pm$ 0.51	0.874
CGP 54626	50	6	2.82 $\pm$ 0.01	0.64	19.3 $\pm$ 0.31	0.847

CGP 54626, [S-(R*,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid; FR, fluorescence ratio; SEM, standard error of the mean; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride; TTX, tetrodotoxin.

An electrical stimuli faster than a spontaneous rate at which iPSC-derived CMs depolarize their membrane potential was applied to a cell monolayer (Fig. 5A). Shapes of Ca^{2+} transients were analyzed by calculating ensemble averages of each profile (Fig. 5B). The statistical analyses of Ca^{2+} transient amplitudes showed a slight increase in amplitude after the EFS was turned on at our stimulation rate (see Materials and Methods; Fig. 5C). This may indicate that the external electrical stimulation induces more Ca^{2+} entering into the cytoplasm from the extracellular space or its internal store, the sarcoplasmic reticulum.^{21,22} Calcium-store depletion reagents or extracellular buffering agents could be used to further assess this calcium increase.

Average traces of paced CMs show that there is uniform calcium transient shape over the course of several reads. Ca^{2+} transients in paced cells show no significant change in amplitude or duration over several reads in time-matched controls (Supplementary Fig. S1B; Supplementary Data are available online at www.liebertpub.com/adt).

AP of CMs is regulated by various ion channels, including those for Na^+ , K^+ , and Ca^{2+} .²³ Control of the rate of APs by various compounds has been utilized in therapeutics for heart disease.²⁴ Dofetilide is one of the common drugs to treat arrhythmia by inhibiting the IKr (rapid delayed rectifier current) channel activity.²⁵ Similar to dofetilide, E4031 is used as a reference compound to also inhibit the IKr channel activity. The application of dofetilide or E4031 (Fig. 5D, G) increased the duration of Ca^{2+} transients (Fig. 5E, H and Supplementary Fig. S1). The electrical stimulation by EFS this time reduced average amplitudes of Ca^{2+} transients (Fig. 5F, I), which indicates that the calcium regulatory system is unable to keep up with the faster pacing rate. Interestingly, the electrical stimulation also

induced cardiac alternans of Ca^{2+} transients, especially immediately after turning on the EFS.^{26,27} The alternans of cardiac contractions or events leading to contraction (*i.e.*, AP, Ca^{2+} transient) is clinically important because it is a sign of arrhythmia.^{28–30}

Class III antiarrhythmic drugs (dofetilide) and its reference compound (E4031) have been shown to lengthen APs in a reverse rate-dependent manner.³¹ Significant differences in calcium transient duration between paced and unpaced cells were seen at higher concentrations (>10 nM) of dofetilide or E4031 (Supplementary Fig. S1 and Table 4). By using EFS to stimulate cells at different rates, one can investigate the rate-dependent effects of pharmaceuticals to induce calcium alternans, indicating the propensities of CMs to arrhythmia.³² To demonstrate the potential of the FDSS/ μ CELL EFS system for investigating rate-dependent changes in the presence of pharmaceuticals, we stimulated CMs at different frequencies (0.5–2 Hz) with and without dofetilide (Supplementary Fig. S2).

Application of the Assays in High-Throughput

The HTP application of phenotypic assays requires assay reproducibility and a large enough range of signal windows and high SNR. For calcium assays using CMs plated in 96-well plates, we measured variabilities in the amplitude and duration of calcium transients using two different calcium dyes (FLIPR Calcium 6 and Cal-520). Scatter plots of the amplitude and CaTD-90 measurements indicate there is little edge or drift effects (<20%), demonstrating good spatial uniformity in the assay (Supplementary Fig. S3A, B). The standard deviation (SD) for fluorescence amplitude and CaTD-90 was low (<10%) across the plate (amplitude, 7.6% SD; CaTD-90, 5.0% SD). Application of the drugs dofetilide and E-4031 resulted in

Z' -values ≥ 0.5 , which are good criteria values for a HTP assay (Supplementary Fig. S1 and Table 4). Interplate variability was assessed by determining the average, SD, and coefficient of variation (CV) of the CM CaTD-90 for three separate 96-well plates. The average and SD for each plate was 1.14 ± 0.13 , 1.13 ± 0.14 , and 1.25 ± 0.12 s, and the CV for each plate was 11.4%, 12.3%, and 9.6%, respectively. This indicates that there is minimal interplate variability for the assay.

DISCUSSION

The recent development of EFS technologies for high-throughput optical assays allows for new ways of studying the effects of chemicals and pharmaceuticals on iPSC-derived neurons and CMs. EFS allows for the direct application of a precise voltage and frequency to a cell monolayer. EFS applied to iPSC-derived neurons, combined with an optical calcium dye, allows for rapid assessment of their activity and response

Fig. 5. The effect of EFS pacing on the frequency, amplitude, and duration of calcium transients in cardiomyocytes. **(A)** Trace of cardiomyocytes unpaced from 0 to 30 s and paced from 30 to 60 s at 0.5 Hz. Upon application of pacing, the calcium transient frequency speeds up from 0.17 to 0.5 calcium transients/s. **(B)** Comparison of the average trace of unpaced and paced cardiomyocytes. **(C)** Amplitude changes in unpaced and paced cardiomyocytes (unpaced, $2,292 \pm 19$, $n=4$; paced, $3,201 \pm 88$, $n=29$; $P < 0.05$). **(D)** Trace of cardiomyocytes treated with dofetilide unpaced from 0 to 30 s and paced from 30 to 60 s at 0.5 Hz. **(E)** Comparison of the average trace of unpaced and paced cardiomyocytes treated with dofetilide (unpaced, mean $\pm$ SD, $n=6-8$; paced, mean $\pm$ SD, $n=27-29$). **(F)** Amplitude in paced cardiomyocytes treated with dofetilide (unpaced, $n=6-8$; paced, $n=27-29$; $P < 0.05$). **(G)** Trace of cardiomyocytes treated with E-4031 unpaced from 0 to 30 s and paced from 30 to 60 s at 0.5 Hz. **(H)** Comparison of the average trace of unpaced and paced cardiomyocytes treated with E-4031 (unpaced, mean $\pm$ SD, $n=6-8$; paced, mean $\pm$ SD, $n=27-29$). **(I)** Amplitude in paced cardiomyocytes treated with E-4031 (unpaced, $n=6-8$; paced, mean $\pm$ SD, $n=27-29$; $P < 0.05$). SD, standard deviation. Color images available online at www.liebertpub.com/adt

Table 4. Cardiomyocyte Electric Field Stimulation Assay

DMSO			Dofetilide				E-4031					
Dose	Unpaced CaTD- 90 ± SD	Paced CaTD- 90 ± SD	Dose (nM)	Unpaced		Paced		Dose (nM)	Unpaced		Paced	
				CaTD- 90 ± SD	Z'	CaTD- 90 ± SD	Z'		CaTD- 90 ± SD	Z'	CaTD- 90 ± SD	Z'
1	0.99 ± 0.05	0.97 ± 0.03	0	0.97 ± 0.06	<0	0.91 ± 0.07	<0	0	0.93 ± 0.02	<0	0.88 ± 0.06	<0
2	1.11 ± 0.24	1.10 ± 0.01	0.1	1.12 ± 0.09	<0	1.06 ± 0.07	<0	0.1	1.03 ± 0.23	<0	1.04 ± 0.05	<0
3	1.02 ± 0.08	1.05 ± 0.03	1	0.99 ± 0.17	<0	1.05 ± 0.07	<0	1	1.02 ± 0.03	<0	1.02 ± 0.07	<0
4	1.16 ± 0.08	1.13 ± 0.02	3	1.21 ± 0.17	<0	1.21 ± 0.09	<0	3	1.18 ± 0.11	<0	1.17 ± 0.10	<0
5	1.15 ± 0.01	1.13 ± 0.02	10	1.72 ± 0.13	0.24	1.58 ± 0.06	0.44	10	1.59 ± 0.11	0.43	1.55 ± 0.07	0.44
6	1.08 ± 0.05	1.17 ± 0.02	30	2.34 ± 0.16	0.52	1.74 ± 0.03	0.64	30	2.13 ± 0.17	0.54	1.71 ± 0.03	0.67

CaTD-90, calcium transient duration-90; SD, standard deviation.

to chemical modulators. In particular, our results show that functional changes in iPSC-derived motor neurons in response to stimuli can be readily detected by fluorescence imaging-based HTP assays, highlighting the possibility of establishing patient iPSC-based HTP phenotyping platforms for drug testing. Indeed, motor neuron diseases, including spinal muscular atrophy³³ and amyotrophic lateral sclerosis,³⁴ exhibit changes in excitability. The HTP phenotyping platform described in this study will help develop drugs that alter neuronal excitability. Similarly, many psychiatric disorders, such as epilepsy and schizophrenia, are associated with changes in neuronal excitability. Hence, modification of our current HTP platform by using neurons affected by motor neuron diseases will enable the establishment of diversified screening platforms.

Drug development requires the testing of all new pharmaceuticals for cardiotoxicity. EFS application to iPSC-derived CMs allows the assessment of cardiac function at beat rates faster than the spontaneous rate and can reveal effects such as cardiac alternans. Uniform electrical stimulation and frequency across the entire 96-well plates allow an easier comparison of CM activity between wells. The effects of drugs and chemicals on CMs may differ depending on the beat rates of the CMs and application of EFS may allow a better assessment of proarrhythmic compounds on CMs. Rapid assessment of cardiotoxicity using iPSC-derived CMs and EFS in a high-throughput fluorescence assay will accelerate the development of new pharmaceuticals.

Alternative methods for AP detection include using multielectrode arrays (MEA) and manual/automated patch clamping. In contrast to MEA, optical detection allows the

analysis of AP profiles using VSD. Manual patch clamping is tedious and can vary depending on personal skill. Although automated patch clamping systems can increase the number of samples and reduce the dependence on personal skill, the critical limitation is that it requires suspended cells instead of active neurons or contracting CMs on plates.^{35,36}

Robust SNR of the Ca²⁺ transient measured by optical signal analysis in HTP format for both neuronal and cardiac cells derived from iPSCs enables us to analyze the effects of drugs and small molecules quantitatively. The good dynamic range of amplitude and duration of Ca²⁺ transients is important for HTP analysis. The latest calcium indicator dyes FLIPR Calcium 6 and Cal-520 exhibited robust cytoplasmic entry and detection of calcium transients in CMs in 96-well plates, and both dyes are sufficient for HTP assays³⁷ (comparison in *Supplementary Fig. S3*).

Our quantitative analyses of Z-factors indicate that optical measurement of Ca²⁺ signals is a good assay system for HTP screening. The application of EFS enabled further improvement of the reproducibility of cardiac Ca²⁺ transient profiles and neuronal stimulation without the use of chemical stimuli. Observation of Ca²⁺ transients before and after EFS stimulation also allows us to observe cardiac arrhythmia phenomena *in vitro*. Finally, this HTP assay system will provide opportunities for applying these techniques toward the development of precision medicine by using patient-specific model systems that mimic the healthy and diseased state of human cells and organs, providing a method for HTP physiology-based compound screening in disease models.

SUMMARY

Neurons and CMs generated from human iPSCs are becoming a prevalent and useful *in vitro* model for cardiac safety assessment, and high throughput optical plate readers, such as the FDSS/ μ CELL system, are being increasingly utilized in recent studies.^{38,39} The addition of EFS to high-throughput optical drug screening systems will greatly enhance the ability to investigate and discover new pharmaceuticals and therapies for disease.

ACKNOWLEDGMENTS

This work has been supported, in part, by NIH grants R44GM087784, R43GM109735, and R01HL109505 to T.W. at InvivoSciences, Inc. The FDSS/ μ Cell instrument and software were kindly provided by Hamamatsu Photonics.

DISCLOSURE STATEMENT

N.J.D. is an employee of InvivoSciences, Inc., and T.W. is the CSO of InvivoSciences, Inc. Z.-W.D. is an employee of BrainXell, Inc.

REFERENCES

- Draper MH, Weidmann S: Cardiac resting and action potentials recorded with an intracellular electrode. *J Physiol* 1951;115:74–94.
- Efimov IR, Nikolski VP, Salama G: Optical imaging of the heart. *Circ Res* 2004;95:21–33.
- Sudhof TC: Neurotransmitter release: The last millisecond in the life of a synaptic vesicle. *Neuron* 2013;80:675–690.
- Nakanishi S: Synaptic mechanisms of the cerebellar cortical network. *Trends Neurosci* 2005;28:93–100.
- Stam CJ, van Straaten EC: The organization of physiological brain networks. *Clin Neurophysiol* 2012;123:1067–1087.
- Stevens JL, Baker TK: The future of drug safety testing: Expanding the view and narrowing the focus. *Drug Discov Today* 2009;14:162–167.
- Himmel HM: Drug-induced functional cardiotoxicity screening in stem cell-derived human and mouse cardiomyocytes: Effects of reference compounds. *J Pharmacol Toxicol Methods* 2013;68:97–111.
- Chi KR: Revolution dawning in cardiotoxicity testing. *Nat Rev Drug Discov* 2013;12:565–567.
- Miller EW, Lin JY, Frady EP, Steinbach PA, Kristan WB, Jr, Tsien RY: Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. *Proc Natl Acad Sci U S A* 2012;109:2114–2119.
- Grenier V, Walker AS, Miller EW: A small-molecule photoactivatable optical sensor of transmembrane potential. *J Am Chem Soc* 2015;137:10894–10897.
- Ren Y, Ridsdale A, Coderre E, Stys PK: Calcium imaging in live rat optic nerve myelinated axons in vitro using confocal laser microscopy. *J Neurosci Methods* 2000;102:165–176.
- Gee KR, Brown KA, Chen WN, Bishop-Stewart J, Gray D, Johnson I: Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium* 2000;27:97–106.
- Taylor DL, Woo ES, Giuliano KA: Real-time molecular and cellular analysis: The new frontier of drug discovery. *Curr Opin Biotechnol* 2001;12:75–81.
- Chiu LL, Iyer RK, King JP, Radisic M: Biphasic electrical field stimulation aids in tissue engineering of multicell-type cardiac organoids. *Tissue Eng Part A* 2011;17:1465–1477.
- Kavanagh KM, Duff HJ, Clark R, Robinson KV, Giles WR, Wyse DG: Monophasic versus biphasic cardiac stimulation: Mechanism of decreased energy requirements. *Pacing Clin Electrophysiol* 1990;13:1268–1276.
- Bardy C, van den Hurk M, Eames T, et al.: Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro. *Proc Natl Acad Sci U S A* 2015;112:E2725–E2734.
- Rutecki PA: Neuronal excitability: Voltage-dependent currents and synaptic transmission. *J Clin Neurophysiol* 1992;9:195–211.
- Kress GJ, Mennerick S: Action potential initiation and propagation: Upstream influences on neurotransmission. *Neuroscience* 2009;158:211–222.
- Obata K: Synaptic inhibition and gamma-aminobutyric acid in the mammalian central nervous system. *Proc Jpn Acad Ser B Phys Biol Sci* 2013;89:139–156.
- Betz H, Gomez J, Armsen W, Scholze P, Eulenburg V: Glycine transporters: Essential regulators of synaptic transmission. *Biochem Soc Trans* 2006;34(Pt 1):55–58.
- Fast VG, Cheek ER, Pollard AE, Ideker RE: Effects of electrical shocks on Cai2+ and Vm in myocyte cultures. *Circ Res* 2004;94:1589–1597.
- Schlottthauer K, Bers DM: Sarcoplasmic reticulum Ca(2+) release causes myocyte depolarization. Underlying mechanism and threshold for triggered action potentials. *Circ Res* 2000;87:774–780.
- Shih HT: Anatomy of the action potential in the heart. *Tex Heart Inst J* 1994;21:30–41.
- Redfern WS, Carlsson L, Davis AS, et al.: Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: Evidence for a provisional safety margin in drug development. *Cardiovasc Res* 2003;58:32–45.
- Roukoz H, Saliba W: Dofetilide: A new class III antiarrhythmic agent. *Expert Rev Cardiovasc Ther* 2007;5:9–19.
- Euler DE: Cardiac alternans: Mechanisms and pathophysiological significance. *Cardiovasc Res* 1999;42:583–590.
- Surawicz B, Fisch C: Cardiac alternans: Diverse mechanisms and clinical manifestations. *J Am Coll Cardiol* 1992;20:483–499.
- Fast VG, Cheek ER: Optical mapping of arrhythmias induced by strong electrical shocks in myocyte cultures. *Circ Res* 2002;90:664–670.
- Merchant FM, Sayadi O, Puppala D, Moazzami K, Heller V, Armoundas AA: A translational approach to probe the proarrhythmic potential of cardiac alternans: A reversible overture to arrhythmogenesis? *Am J Physiol Heart Circ Physiol* 2014;306:H465–H474.
- Zhou X, Bueno-Orovio A, Orini M, et al.: In vivo and in silico investigation into mechanisms of frequency dependence of repolarization alternans in human ventricular cardiomyocytes. *Circ Res* 2016;118:266–278.
- Jurkiewicz NK, Sanguinetti MC: Rate-dependent prolongation of cardiac action potentials by a methanesulfonanilide class III antiarrhythmic agent. Specific block of rapidly activating delayed rectifier K+ current by dofetilide. *Circ Res* 1993;72:75–83.
- Edwards JN, Blatter LA: Cardiac alternans and intracellular calcium cycling. *Clin Exp Pharmacol Physiol* 2014;41:524–532.
- Liu H, Lu J, Chen H, Du Z, Li XJ, Zhang SC: Spinal muscular atrophy patient-derived motor neurons exhibit hyperexcitability. *Sci Rep* 2015;5:12189.
- Wainger BJ, Kiskinis E, Mellin C, et al.: Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. *Cell Rep* 2014;7:1–11.
- Rajamohan D, Kalra S, Duc Hoang M, et al.: Automated electrophysiological and pharmacological evaluation of human pluripotent stem cell-derived cardiomyocytes. *Stem Cells Dev* 2016;25:439–452.
- Yu HB, Li M, Wang WP, Wang XL: High throughput screening technologies for ion channels. *Acta Pharmacol Sin* 2016;37:34–43.

37. Daily NJ, Santos R, Vecchi J, Kemanli P, Wakatsuki T: Calcium transient assays for compound screening with human iPSC-derived cardiomyocytes: Evaluating new tools. *J Evol Stem Cell Res* 2017;1:1–11.
38. Lu HR, Whittaker R, Price JH, et al.: High throughput measurement of Ca^{++} dynamics in human stem cell-derived cardiomyocytes by kinetic image cytometry: A cardiac risk assessment characterization using a large panel of cardioactive and inactive compounds. *Toxicol Sci* 2015;148: 503–516.
39. Zeng H, Roman MI, Lis E, Lagrutta A, Sannajust F: Use of FDSS/ μ Cell imaging platform for preclinical cardiac electrophysiology safety screening of compounds in human induced pluripotent stem cell-derived cardiomyocytes. *J Pharmacol Toxicol Methods* 2016;81:217–222.

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Abbreviations Used

ACSF = artificial cerebrospinal fluid
 AP = action potential
 Ca^{2+} = calcium
 CaTD-90 = calcium transient duration-90
 CGP 54626 = [S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid
 CM = cardiomyocytes
 CV = coefficient of variation
 DMSO = dimethyl sulfoxide
 EFS = electric field stimulation
 FDSS = functional drug screening system
 FR = fluorescence ratio
 GABA = gamma-aminobutyric acid
 HTP = high-throughput
 iPSC = induced pluripotent stem cell
 MEA = multielectrode arrays
 SD = standard deviation
 SNR = signal-to-noise ratio
 THIP = 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride
 TTX = tetrodotoxin
 VSD = voltage-sensitive dyes