Simultaneous Voltage and Calcium Mapping of Genetically Purified Human Induced Pluripotent Stem Cell–Derived Cardiac Myocyte Monolayers

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Rationale: Human induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) offer a powerful in vitro tool to investigate disease mechanisms and to perform patient-specific drug screening. To date, electrophysiological analysis of iPSC-CMs has been limited to single-cell recordings or low-resolution microelectrode array mapping of small cardiomyocyte aggregates. New methods of generating and optically mapping impulse propagation of large human iPSC-CM cardiac monolayers are needed.

Objective: Our first aim was to develop an imaging platform with versatility for multiparameter electrophysiological mapping of cardiac preparations, including human iPSC-CM monolayers. Our second aim was to create large electrically coupled human iPSC-CM monolayers for simultaneous action potential and calcium wave propagation measurements.

Methods and Results: A fluorescence imaging platform based on electronically controlled light-emitting diode illumination, a multiband emission filter, and single camera sensor was developed and utilized to monitor simultaneously action potential and intracellular calcium wave propagation in cardiac preparations. Multiple, large-diameter (∼1 cm), electrically coupled human cardiac monolayers were then generated that propagated action potentials and calcium waves at velocities similar to those commonly observed in rodent cardiac monolayers.

Conclusions: The multiparametric imaging system presented here offers a scalable enabling technology to measure simultaneously action potential and intracellular calcium wave amplitude and dynamics of cardiac monolayers. The advent of large-scale production of human iPSC-CMs makes it possible to now generate sufficient numbers of uniform cardiac monolayers that can be utilized for the study of arrhythmia mechanisms and offers advantages over commonly used rodent models. (Circ Res. 2012;110:1556-1563.)

Key Words: arrhythmia research | bioengineering | cardiac regeneration | induced pluripotent stem cells | optical mapping

The advent of induced pluripotent stem cell (iPSC) technology has increased the impetus to improve bioengineering and imaging methodologies to enable the mechanistic study of human cardiac preparations manifesting disease phenotypes and for drug testing in vitro.1,2 However, the laborious nature of single-cell electrophysiological recordings commonly used as an electrophysiological assay precludes efficient screening of electric wave propagation through a functional syncytium of cardiac tissue.2 Microelectrode arrays and optical mapping have been used on small iPSC cell-derived cardiomyocyte (iPSC-CM) aggregates, but observed conduction velocities have been very low (<2.5 cm s⁻¹).3,4

A new method is needed to enable the generation of large electrically coupled human iPSC-CM monolayers that propagate action potentials and calcium waves with much higher speeds. Using genetically selected and purified iPSC-CMs, we report here a new method for the generation of electrically coupled human cardiac monolayers (∼1 cm diameter) that uniformly conduct action potentials and calcium waves at velocities comparable with those commonly observed in neonatal rat ventricular myocyte monolayers (>20 cm s⁻¹). This new cell and bioengineering technology enables future studies of arrhythmia
mechanisms and drug screening using patient-specific cardiac monolayers rather than just rodent model systems.

Methods
Reference Applications of the Imaging Platform for Simultaneous Voltage and Ratiometric Calcium Mapping in Cardiac Tissue
Detailed Methods are provided in the Online Supplement. Here, we use a novel optical mapping system for simultaneous \(V_m\) and ratiometric intracellular calcium (\([Ca^{2+}]_i\)) imaging of cardiac preparations, including human iPSC-CM monolayers. The optical mapping system presented here (Figure 1) offers several advantages over most existing systems for multiparametric imaging. First is the use of multiple light-emitting-diodes (LEDs) as excitation light sources, which provide a stable, flexible, and economical alternative to established methods. Unlike traditional light sources, LED light intensity can be modulated with exceedingly fast response times, thus making high-speed excitation light switching possible without the need for moving parts. Based on recent advances in LED fabrication technology, powerful LEDs are now available that cover the entire spectrum from deep ultraviolet to infrared, making them an increasingly attractive alternative for multiparametric cardiac imaging. Second, the imaging method presented here provides a means for quantitative (ratiometric) calcium wave imaging in cardiac tissue of various levels of structural complexity. The use of fluorescence for quantitative measurement is difficult because fluorescence intensity is affected by a range of ill-controlled factors, such as regional differences in dye loading, internalization in subcellular compartments, and photon-bleaching. These limitations are particularly relevant when utilizing single-excitation/single-emission fluorescent dyes. To address this, ratiometric Ca\(^{2+}\) dyes are utilized that, on binding Ca\(^{2+}\), display spectral shifts with opposite polarity on exposure to different excitation wavelength. The ratio of the two emission intensities is independent of fluorescence signal intensity, thereby providing a means of quantifying [Ca\(^{2+}\)]\(_i\), and allowing reliable comparison between regions and samples. Third, we have utilized a low-affinity ratiometric Ca\(^{2+}\) dye, fura-4F. To minimize perturbation of the [Ca\(^{2+}\)]\(_i\) dynamics in cardiac tissue, the choice of Ca\(^{2+}\) dye is critical for acquiring accurate measurements of the amplitude and time course of [Ca\(^{2+}\)]\(_i\) transients. For cardiac myocytes and tissue, which show large and rapid changes in [Ca\(^{2+}\)]\(_i\), a low-affinity and rapidly responding dye is needed. Other commonly used ratiometric Ca\(^{2+}\) dyes, such as fura-2, have a relatively high-affinity for Ca\(^{2+}\). This can lead to an artificial prolongation of the Ca\(^{2+}\) transient and confound interpretation (ie, the dye acts as a chelator that releases Ca\(^{2+}\) with delay). Fourth, the new system uses a single sensor with a multiband transmission filter and no moving parts. The use of a single sensor obviates the technical complexity of multisensor alignment for simultaneous multiparametric imaging. More complete details of the optical mapping set-up and electronics are available in the Online Supplement.

We have thoroughly validated the performance of this optical mapping system using cardiac tissue of various levels of structural complexity from monolayers of cardiac myocytes to native cardiac tissue slices and the whole heart (Figures 2, 3). Details about each cardiac preparation can be found in the Online Supplement. Figure 2A demonstrates the utility of the system for studying cardiac arrhythmia mechanisms in neonatal rat ventricular myocyte monolayers, a commonly used in vitro model. Using our mapping system, we were able to monitor simultaneously \(V_m\) and [Ca\(^{2+}\)]\(_i\), wave propagation of electric rotors (re-entrant electric activity that can underlie cardiac arrhythmias; Online Movie I). Comparison of the data, also illustrated in the 2 snapshots presented in Figure 2A, reveals that the rotating \(V_m\) wavefront precedes the Ca\(^{2+}\) wavefront by approximately 10 ms. Next, we demonstrate simultaneous \(V_m\) and [Ca\(^{2+}\)]\(_i\), imaging of thin (\(\approx 350\)–\(150 \mu m\)-thick) ventricular tissue slices (Figure 2B; Online Movie II). Cardiac tissue slices represent an exciting pseudo-two-dimensional model that balances structural complexity (cells within the slice are in an environment that is as close to native as possible in vitro) and ease of data interpretation (observed signals can be related more reliably to their histological sources). As illustrated in Figure 2C, we also used the same imaging system to map \(V_m\) and [Ca\(^{2+}\)]\(_i\), wave propagation in Langendorff-perfused rat hearts (Online Movie III). Normalized fluorescence intensity maps for \(V_m\) and [Ca\(^{2+}\)]\(_i\) were recorded during sinus rhythm and are shown at two time points 9.72 ms apart. The entire anterior epicardial surface of the ventricles activated almost simultaneously, whereas the peak of [Ca\(^{2+}\)]\(_i\), occurred with a delay of approximately 16 ms.

Generation of Human iPSC-CM Monolayers
Next, we generated human cardiac monolayers to enable the simultaneous mapping of \(V_m\) and [Ca\(^{2+}\)]\(_i\), wave propagation in a human model system. Human iPSC-CMs were obtained from Cellular Dynamics International (Madison, WI). The iCell cardiomyocytes are highly purified human cardiac myocytes (>98% pure cardiomyocytes) derived from iPSC cells using cardiac-directed differentiation and purification protocols. Importantly, the high purity of these cardiomyocyte cultures remains unchanged over the course of at least 2 weeks in culture. The iCell cardiomyocytes are differentiated from human iPSCs reprogrammed from a nonembryonic terminally differentiated cell source, thus avoiding the ethical controversy surrounding embryonic stem cell use. Cryopreserved vials (liquid nitrogen) of iCell human cardiac myocytes were thawed and subsequently plated on bovine fibronectin-coated (20 μg/mL; Invitrogen) transparent silastic membranes at a density of 125,000 cells per monolayer in differentiation media (embryoid body differentiation media, commonly referred to as embryoid body-20, comprising 80% Dulbecco Modified Eagle Medium [DMEM/F12], 0.1 mmol/L L-glutamine, 1% nonessential amino acids, 1 mmol/L L-glutamine, 0.1 mmol/L L-β-mercaptoethanol, and 20% fetal bovine serum; Gibco) supplemented with 10 μmol/L L-blebbistatin. After 24 hours in embryoid body-20, the medium was switched to iCell maintenance medium, supplemented with 10 μmol/L L-blebbistatin, and cells were cultured for an additional 96 hours at 37°C, in 5% CO\(_2\), with the medium changed once daily. Monolayers (n=4) were subsequently processed for electrophysiological analysis by optical mapping as described for neonatal rat cardiomyocyte monolayers or for immunocytochemistry analysis (n=7). The cellular structure and composition of typical monolayers generated in this study are presented in Figures 3 and 4.

Results
Human iPSC-CM and Monolayer Structure
Cardiomyocytes were plated on fibronectin-coated transparent silastic membranes as described. Immunostaining was performed essentially as described recently. For immunostaining, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 10 minutes, and rinsed twice before blocking with 5% donkey serum in phosphate-buffered saline plus 0.1% Triton X-100 (Sigma) for 1.5 hours at room temperature. Primary antibodies including mouse monoclonal anti-MLC-2a (IgG2b, 1:200 dilution; Synaptic Systems), rabbit polyclonal anti-MLC-2v (IgG, 1:100 dilution; ProteinTech Group), mouse monoclonal α-actinin (1:500; Sigma), and rabbit polyclonal connexin 43 (1:100;
Millipore) were added to 5% donkey serum in phosphate-buffered saline plus 0.1% Triton X-100 and incubated overnight at 4°C with constant agitation. Subsequently, samples were washed three times in phosphate-buffered saline plus 0.1% Triton X-100. The secondary antibodies donkey anti-rabbit DyLight 488 and donkey antimouse DyLight 594 (1:500 dilution; Jackson ImmunoResearch) were diluted in the same solution as primary antibodies and incubated at room temperature in the dark for 1.5 hours. Negative control experiments using only the secondary antibodies were performed to ensure specificity of immunostaining and fluorescence detection. Samples were then washed three times with phosphate-buffered saline plus 0.1% Triton X-100 and once with phosphate-buffered saline only. Finally, nuclei were stained with DAPI (1:1000 dilution; Invitrogen) for 10 minutes at room temperature in the dark. Cover slips were mounted on microscope slides for confocal imaging.

Immunofluorescence imaging was performed using a 20× or 60× objective on a Nikon A1R laser scanning confocal microscope system (Nikon Instruments, Melville, NY). Figure 3 shows the cellular and subcellular structure of representative cardiac monolayers that were used in this study. Figure 3A shows a cardiomyocyte monolayer immunostained for α-actinin (red) and nuclei (DAPI, blue). Consistent with a recent report using iCell cardiomyocytes,12 nuclei were evenly distributed and α-actinin positive staining was present throughout the field of view. Higher magnification (60×) image in Figure 3B shows the sarcomere structure of the Z-line protein α-actinin (red) and also shows that the gap junction protein connexin 43 (green) is localized at the points of myocyte–myocyte contact. This suggests that impulse propagation in these human monolayers may be mediated through cell-to-cell communication via connexin 43. Figures 3C to 3E show the result of coimmunolabeling for α-actinin (sarcomeric Z-line) and MLC-2v (sarcomeric A-band).
Figure 3C demonstrates that not all myofilaments stained positive for the ventricular-specific myosin light chain (MLC-2v). For MLC-2v-positive myofilaments, alternating Z-line and A-band staining was observed (Figure 3D). A fluorescence intensity plot over the distance denoted by the white arrow in Figure 3D is shown in Figure 3E. Proper spatial organization of the sarcomeric Z-line (red) and A-band (green, MLC-2v staining is localized in each half of the sarcomeric A-band) suggests a degree of functional maturity of the cardiac sarcomere. The average sarcomere length measured by the average Z-line to Z-line distance is 1.98 ± 0.03 μm, n=51 cells.

Flow Cytometry Analysis of Cardiac Myocyte Population

Next, we used immunofluorescence and flow cytometry to quantify the cardiomyocyte population in iCell cardiomyocyte monolayers. Expression of the two cardiac MLC-2 isoforms of the heart, MLC-2a and MLC-2v, provides information about the diversity of the cardiomyocyte population. The MLC-2a expression is detected in all chambers of developing mouse and human hearts. Postnatally, MLC-2a expression is restricted to the atria in mice, whereas MLC-2a expression is found in both the atria and, to a lesser extent in the ventricles in humans. However, MLC-2v expression is restricted to the ventricular chambers in humans, and this chamber specificity persists into adulthood.

In mouse and human embryo bodies, MLC-2a expression precedes MLC-2v expression, thereby suggesting that MLC-2v expression is also a marker of myocyte maturity. Immuno-fluorescence and laser confocal scanning analysis demonstrate that these monolayers are comprised of at least three phenotypes expressing primarily MLC-2a (red), MLC-2v (green), and both MLC-2a and MLC-2v (yellow in Figure 4A, B). Cells expressing MLC-2v likely represent ventricular-like cells, whereas MLC-2a–only expressing cells can identify a range of potential cardiomyocyte types, including atrial-like cells.

Results of flow cytometry analysis using MLC-2a–specific and MLC-2v–specific antibodies are shown in Figures 4C and 4D. For flow cytometry, cells were washed with phosphate-buffered saline and then incubated with 1 mL/well of 0.25% Trypsin-EDTA plus 2% chicken serum for 5 minutes. Equal volume of embryoid body-20 medium, containing 20% fetal bovine serum, was subsequently used to inhibit trypsinization. Cells were detached from the cover slips and aggregates were disrupted to singularize the cells by pipetting up and down. Cell suspensions were centrifuged for 5 minutes at 1,000 rpm; the
supernatant was discarded and cell pellets were resuspended in 1 mL 1% paraformaldehyde and incubated at 37°C for 10 minutes to fix the cells. Samples were centrifuged and pellets were resuspended in 1 mL ice-cold methanol before incubation on ice for 30 minutes to permeabilize the cells. Cells were washed once with FACS buffer (phosphate-buffered saline without Ca\(^{2+}\) and Mg\(^{2+}\), 1% bovine serum albumin) plus 0.1% Triton and centrifuged, and the supernatant was discarded, leaving a volume of 100 μL. Primary antibodies for MLC-2a (Synaptic Systems, mouse monoclonal, dilution 1:200) and MLC-2v (ProteinTech Group, rabbit polyclonal, dilution 1:400) were dissolved in 100 μL/sample FACS buffer plus 0.1% Triton for total sample volume of 200 μL. Samples were incubated with primary antibodies overnight at 4°C. Cells were washed twice in 3 mL FACS buffer plus 0.1% Triton and centrifuged, and supernatant was discarded, leaving approximately 100 μL. Secondary antibodies (donkey antimouse Dylight 488 and donkey antirabbit Dylight 594, dilution 1:500; Jackson ImmunoResearch) were dissolved in 100 μL/sample FACS buffer plus 0.1% Triton for total sample volume of 200 μL and incubated for 45 minutes in the dark at room temperature. Cells were washed twice in FACS buffer plus 0.1% Triton, centrifuged, resuspended in 200 μL FACS buffer plus 0.1% Triton, and stored on ice until FACS analysis. To define the thresholds for positive 488 and 594 fluorescence, negative control samples were incubated with 488 or 594 secondary antibodies only. Black peaks in Online Figure I represent both the background fluorescence of the secondary antibodies and the autofluorescence of the cells. Green and red peaks show, respectively, 488 and 594 fluorescence for positive control samples (Online Figure I). Both negative and positive control samples allow gating precisely for 488 and 594 positive fluorescence thresholds. Data were collected on a FACS system (MoFlo XDP) and analyzed using FlowJo version 9.4.11. Flow cytometry confirms the immunofluorescence staining of MLC-2a and MLC-2v as shown in Figures 4C and 4D. Three distinct populations of CMs were identified: MLC-2a^+/MLC-2v^- (upper left quadrant, 8.4%); MLC-2a^-/MLC-2v^+ (upper right quadrant, 13.6%); and MLC2a^-/MLC2v^+ (lower right quadrant, 44.6%). This is similar to the subpopulations of atrial and ventricular myocytes identified using electrophysiology criteria of action potential (AP) morphology of iCell cardiomyocytes.\(^{12}\)

**Simultaneous Voltage and Calcium Mapping in Human iPSC-CM Monolayers**

We generated large (approximately 1-cm diameter) human iPSC-CM monolayers for optical mapping using genetically selected iPSC-CMs (iCell; Cellular Dynamics International). These iPSC-CM monolayers exhibit spontaneous pacemaker activity (1.22±0.09 Hz; n=4; Figure 5A and Online Movie IV), and they also can be electrically stimulated (Figure 5A and Online Movie V). Uniform propagation of V_m and [Ca\(^{2+}\)]_i through the entire monolayer was observed in each case (n=4), as shown in the representative activation maps of Figure 6 (conduction velocity=21.9±2.47 cm/s^-1 and a V_m to [Ca\(^{2+}\)]_i peak delay of approximately 33 ms). The V_m activation maps of spontaneous pacemaker activity recorded in two separate monolayers are shown in Figures 6A and 6C. Figures 6B and 6D show activation maps of paced beats in where the stimulus was placed approximately at the center of the monolayer, and where the V_m wave spread uniformly through the monolayer in all directions.

Figure 7 shows the uniformity of AP and [Ca\(^{2+}\)]_i characteristics across the monolayer. The action potential duration (APD) at 90% repolarization (APD\(_{90}\)) and the [Ca\(^{2+}\)]_i transient duration at 90% return to baseline (CaT\(_{90}\)) are shown from four spatially distinct locations during spontaneous and electrically paced propagation (indicated by blue, red, green, and cyan squared regions). The right panel of Figure 7A presents APD\(_{90}\) and...
CaT\textsubscript{90} during spontaneous activity (cycle length of 1,267\,ms), the left panel of Figure 7B presents APD\textsubscript{90} and CaT\textsubscript{90} during electrically paced activity (cycle length of 500\,ms), and the right panel of Figure 7B presents APD\textsubscript{90} and CaT\textsubscript{90} during electrically paced activity (cycle length of 750\,ms). As can be seen, the transient duration characteristics for each parameter are uniform across the plate and increase with cycle length. Three consecutive transients, after equilibration, were taken for each data point. The same analysis for another representative monolayer can be found in Online Figure II.

**Discussion**

Novel bioengineering techniques are continuously being developed to create human cardiac tissue constructs that can be used to improve cardiac research and for development of cardiac regeneration therapies.\textsuperscript{17} The novel imaging and bioengineering approaches presented here offer the potential to increase throughput quantification of action potential and Ca\textsuperscript{2+} wave propagation in iPSC-CM multicellular tissue constructs. We generated large electrically coupled human iPSC-CM monolayers for optical mapping using genetically selected iPSC-CMs (iCell; Cellular Dynamics International; Figure 3). Although previous studies have explored V\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} (nonratiometrically) by imaging, each parameter individually (using a different set of voltage and calcium dyes in small [diameter \(\leq 1\) mm] human iPSC-CM monolayers),\textsuperscript{3} simultaneous V\textsubscript{m} and ratiometric [Ca\textsuperscript{2+}]\textsubscript{i} imaging have not yet been reported to our knowledge.

To date, action potential conduction velocity of iPSC-CM aggregates measured using multiple electrode array technology has been very slow (1–2.5 cm/s\textsuperscript{1}).\textsuperscript{4} Similarly, higher spatial resolution optical mapping of small iPSC-CM aggregates (approximately 400 \(\mu\)m diameter) have shown very slow (1–2 cm/s) action potential spread.\textsuperscript{3} These conduction velocities pale in comparison to the more commonly used neonatal rat ventricular myocyte monolayer model in which conduction velocities are typically an order of magnitude faster.

**Figure 5.** Simultaneous fluorescence imaging of V\textsubscript{m} and ratiometric [Ca\textsuperscript{2+}]\textsubscript{i} in human induced pluripotent stem cell–derived cardiomyocyte (iPSC-CM) monolayers. A, Normalized fluorescence intensity maps (color bar shown) of spontaneous and electrically paced (at the center of the monolayer) activation. Black arrows show the direction of activation wave propagation. The leftmost panel shows a grayscale image of the fluo- rescing monolayer. B, Left panel shows camera signals (on a 16-bit scale) of Em\textsubscript{1}, Em\textsubscript{2}, and Em\textsubscript{3} fluorescence from the blue squared region in panel A over the course of 2 seconds. Right panel shows normalized ratiometric calcium signal (obtained by dividing Em\textsubscript{1} by Em\textsubscript{2}, followed by normalization) and normalized voltage signal (Em\textsubscript{3} normalization). Scale bar: 5 mm.

**Figure 6.** Activation maps (from 2 monolayers) of spontaneous and electrically paced propagation in human induced pluripotent stem cell–derived cardiomyocyte (iPSC-CM) monolayers. A, Spontaneous activity in dish 1. The spatial distribution of the activation timing, derived from maximum voltage upstroke velocity, with isochronal lines at each 2-ms interval. B, Electrically stimulated (at the monolayer center) activity in dish 1. The spatial distribution of the activation timing, derived from maximum voltage upstroke velocity, with isochronal lines at each 2-ms interval. The 2 white circles are separated by a 5-mm distance. At a pacing frequency of 2 Hz, it took 24.9\,\pm\,2.3\,ms (from 3 stimulations) for the activation wave to propagate from the inner to the outer circle. This translates to a conduction velocity of 20.1 cm/s\textsuperscript{1} for this monolayer. C, Spontaneous activity in dish 2. Isochronal lines at each 2-ms interval as for panel A. D, Electrically stimulated (at the monolayer center) activity in dish 2. Conduction velocity=23.6 cm/s\textsuperscript{1} calculated as for panel B. Scale bar: 5 mm.
tachycardia have been studied recently using patient-specific therapies. Genetically linked cardiomyopathies technology for the development of in vitro patient-specific therapies, and for the study of arrhythmia mechanisms.

rodent monolayer models for in vitro testing of potential drug human iPSC-CM monolayers provide an attractive alternative to dishes or multielectrode dishes. Regardless of the mechanism, these of flexible silastic membranes as opposed to rigid plastic Petri dishes. Regardless of the mechanism, these human iPSC-CM monolayers provide an attractive alternative to rodent monolayer models for in vitro testing of potential drug therapies, and for the study of arrhythmia mechanisms.

Human iPSC-derived cardiac myocytes are emerging as a technology for the development of in vitro patient-specific disease models for drug testing and for the development of regenerative therapies. Genetically linked cardiomyopathies such as long QT syndrome, Timothy syndrome, LEOPARD syndrome, and catecholaminergic polymorphic ventricular tachycardia have been studied recently using patient-specific iPSC-derived cardiac myocytes. Each of these reports, the patient-specific cardiomyocytes recapitulated the electrophysiological phenotype of the specific disease. For example, myocytes obtained from long QT syndrome patients exhibit APD prolongation, development of early depolarization, and triggering of spontaneous beats. Indeed, human iPSC-CMs have ionic currents and channel gating properties underlying their APs and after depolarizations that are quantitatively similar to those reported for human cardiac myocytes. The iCell cardiomyocytes have more negative maximum diastolic potential values for atrial-like and ventricular-like APs compared with reports with human embryonic stem cell-derived cardiomyocytes, and the AP durations are within the normal range of the human ECG QT interval. Ma et al reported that the maximum diastolic potential of ventricular-like iCell CMs is -76 mV, and the maximum diastolic potential of atrial-like CM is -73 mV. This is hyperpolarized compared with the average resting membrane potential of rodent monolayer systems, which is -65 mV. The AP amplitude of iCell CMs was similar for atrial and ventricular cells (100 mV and 104 mV, respectively) in that study, as was the dV/dt max (26 V/s and 28 V/s). The APDs reported by Ma et al differed between atrial and ventricular CMs (286 ms versus 414 ms, respectively). Thus, the electrophysiological characteristics of the cardiomyocytes used here have some properties of more mature human cardiac myocytes and offer advantages over commonly used rodent model systems. Here, we present, using optical mapping, that the APD90 of human iPSC-CM monolayers is approximately 340 ms (Figure 7), which is in between the APD of human iPSC ventricular and atrial myocytes. This APD is anticipated in an electrotonically interconnected mix of the two electrophysiologically distinct CM types. Furthermore, the APD90 of human iPSC monolayers recorded here is two-times longer than the APD90 of neonatal rat ventricular myocyte monolayers (approximately 170 ms at 1-Hz pacing). The spontaneous beating rate of human iPSC-CM monolayers observed here (1.2 Hz) is representative of the resting adult human heart rate (60–70 bpm). However, the spontaneous beating rate of neonatal rat ventricular myocyte monolayers varies widely, typically between 1 and 4 Hz, which is much slower than the heart rate of adult rats. Therefore, besides the human source of iPSC-CMs being an advantage over using rodent systems, the electrophysiological phenotype of these human myocytes offer a model system that cannot be recapitulated using rodent systems.

As pointed out recently, the transition from “bench to bedside” and realization of the full potential of human iPSC-CM technology will require more time and work to overcome other limitations of the technology, including the heterogeneity of the cell types (Figure 4). Nevertheless, the use of human iPSC-CM monolayer systems as presented here offers a novel model for the mechanistic study of human cardiac impulse generation and propagation for the mechanisms involved in arrhythmogenesis. Furthermore, the use of patient-specific iPSC-CMs and monolayers offers a potentially unique ex vivo approach to obtaining clinically relevant information.

Sources of Funding
Clarendon Fund Scholarship (P.L.), British Heart Foundation (C.B., P.K.), National Institutes of Health grants P01-HL087226 (J.J.), and the Leducq Foundation (J.J.) all contributed to this work.

Disclosures
None.

References
Novelty and Significance

What Is Known?

- Human-induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) represent a new model for personalized study of heart disease.
- Transmembrane voltage, intracellular calcium homeostasis, and impulse propagation are three key parameters of interest in the study of heart disease and drug effects; however, a simple and scalable imaging platform for simultaneously measurement all three parameters in macroscopic human iPSC-CM tissue constructs is needed.

What New Information Does This Article Contribute?

- Large electrically coupled human cardiac monolayers can be produced using genetically purified human iPSC-CMs, permitting the study of the mechanisms of arrhythmia in human cells.
- The results demonstrate the potential of the system for simultaneous mapping of the three key parameters, transmembrane voltage, intracellular calcium concentration, and excitation propagation, for studying cardiac physiology and pathology and for high-throughput drug testing.

Human iPSC-CMs can be used to study human disease mechanisms to investigate genetic disorders and to perform patient-specific drug testing. To date, most studies using human iPSC-CMs have measured cellular phenotypes, and there is a paucity of experimentation reported at the multicellular level. To take advantage of this powerful tool, large electrically coupled tissue constructs must be developed and new scalable multiparametric imaging platforms are needed. This study describes the production of large electrically coupled monolayers using genetically purified human iPSC-CMs and an imaging platform capable of simultaneously measuring action potential and intracellular calcium wave propagation. Human cardiac monolayers were formed on elastic membranes using readily accessible commercial iPSC-CMs and the imaging platform was constructed using a single camera and off-the-shelf light-emitting-diodes, multiband optical filters, and electronics. These human cardiac monolayers yielded impulse conduction velocities significantly higher than previously reported, highlighting the importance of the mechanical environment and cell purity. The use of human iPSC-CM cardiac monolayers offers several advantages over commonly used rodent monolayer systems for the study of disease and arrhythmia mechanisms. This approach could be scaled-up for high-throughput investigations into the mechanisms of human arrhythmias or for studying patient-specific drug effects.
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*Circ Res.* 2012;110:1556-1563; originally published online May 8, 2012; doi: 10.1161/CIRCRESAHA.111.262535

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIAL

Non-standard Abbreviations:

iPSC=Induced Pluripotent Stem Cells
iPSC-CM=Induced Pluripotent Stem Cell-Derived Cardiomyocyte
LED=Light Emitting Diode
UV=Ultraviolet
V_m=Membrane Voltage
[Ca^{2+}]_i=Intracellular Calcium Concentration

Instrument design details

The imaging system (Fig. 1A) comprises readily-available optical filters, lenses, LEDs (both UV and visible), custom-built electronics and a single, high-performance high-speed electron-multiplied charge-coupled-device (EMCCD) camera (both the Evolve™ 128 and the Cascade® 128+ were tested, Photometrics, AZ USA). We measure V_m using di-8-ANEPPS, and [Ca^{2+}], using fura-4F. These dyes show no optical cross-talk and are well-suited for loading in cardiac tissue.¹

A schematic illustration of the imaging system is shown in Figure 1A. Fura-4F was excited with two UV LEDs: (1) Ex1: UVMAX325-HL-15 (peak power output 15mW; peak wavelength 330 nm; Roithner LaserTechnik, Vienna, Austria) and (2) Ex2: NCSU034B (peak power output 400mW; peak wavelength 385 nm; Nichia, Tokushima, JAPAN). The light from the LEDs for Ex1 and Ex2 was collimated with a UV fused silica bi-convex lens (LB4879; Thorlabs) and plano-convex lens (LA1951; Thorlabs), respectively. Di-8-ANEPPS was excited with a blue LED; Ex3: CBT-90-B (peak power output 53W; peak wavelength 460 nm; Luminus Devices). Light from the LED for Ex3 was collimated with a plano-convex lens (LA1951; Thorlabs) and passed through an excitation filter (D470/20x; Chroma Technology). Fluorescence emission from the dye-loaded preparations was passed through one multi-band emission filter (59022m; Chroma Technology), with high-percentage transmission spectra for both bands (~500-545nm & ~600-670nm) (Fig. 1B), and collected with a fast camera-suitable lens (f/# 0.95; DO-2595; Navitar). Ex1 and Ex2 produce fura-4F emission (Em1 & Em2) in the first band of the emission filter (colored green in Fig. 1B); Ex3 produces di-8-ANEPPS emission in the second band of the emission filter (colored red in Fig. 1B).

During any frame-exposure-period of the camera, the tissue is illuminated with only one of the three excitation sources. Because of the lack of cross-talk between the dyes, the emitted fluorescence at any time represents one of the two ratiometric [Ca^{2+}]_i emissions, or V_m. Because the camera frame rate is higher than the smooth signal dynamics one can measure all three parameters in a straightforward fashion (Fig. 1C). A high-speed microcontroller coordinates the fast-response-time LEDs (on the order of microseconds) with the frame-exposure-signal from the camera. A standard desktop computer is used for the camera system and to communicate with
the microcontroller (e.g. to change the LED output power through a custom-built digital-to-analog-converter).

The microcontroller-based interface was implemented to 1) synchronise excitation light switching with EMCCD camera frame exposure periods, and 2) control the timing of local electrical stimulation. The LEDs were controlled with a custom-built high-power LED driver circuit that enables illumination power to be tuned, and light output to be switched on or off in the kHz range. The circuitry for the electrical biphasic stimulator was also custom-built to optimise control and flexibility. A monopolar stimulation electrode (Lohmann Research Equipment, Castrop-Rauxel, Germany) was used to apply local electrical stimuli. Custom software was used to design experimental protocols, direct communication with the microcontroller, and for image processing as before.²

**Neonatal rat ventricular myocyte monolayers**

Isolation and culture techniques for neonatal rat ventricular myocytes and monolayer formation was done as described before.³⁻⁵ Briefly, following cervical dislocation and decapitation, hearts from 1- to 2-day-old neonatal Sprague–Dawley rats (Charles River Laboratories) were removed and collected in calcium and magnesium free Hanks' balanced salt solution (HBSS) at room temperature. All animal handling was in accordance and with full approval of the University Committee on Use and Care of Animals (UCUCA) of the University of Michigan.

Then, the ventricles were isolated, minced, and enzymatically digested in a solution that contained 0.06% trypsin (weight/volume, w/v, Gibco, 15090) and 0.15% pancreatin (w/v, Sigma, P3292) at 37°C. Supernatant enzyme solutions with digested cells were collected every 15 min and replaced with fresh enzyme solutions (30mL) for 6 to 7 times until tissue blocks disappeared. Collected enzyme solutions were centrifuged, cells were resuspended in 20mL fresh culture medium (10%FBS in M199 medium), and plated in two 10cm dishes and stored in the incubator for pre-plating. After a 2-hour pre-plating period to allow non-cardiomyocyte attachment, the supernatant containing mainly cardiomyocytes was collected, centrifuged, and cells were resuspended for culture in M199 medium (Cambrex) with 10% FBS (Cellgro), 20 U/mL penicillin, 20 µg/mL streptomycin, and 100 µmol/L bromodeoxyuridine (Sigma).

Finally, myocytes were plated in 35-mm tissue culture dishes at a density of 1x10⁶ cells per dish, to allow formation of confluent monolayers. To facilitate cardiomyocyte attachment, dishes were coated with human placental collagen type IV (Sigma), and cells were cultured in an incubator (37°C, 5% CO₂).

Monolayers formed within 4 days (n=5) and were stained in HBSS (in mmol/L: 1.6 CaCl₂, 5.4 KCl, 0.8 MgSO₄, 0.4 KH₂PO₄, 4.2 NaHCO₃, 136.9 NaCl, 0.3 NaHPO₄, 5.5 D-Glucose, and 10 HEPES; pH 7.4) with fluorescent probes for Vₘ and intracellular Ca²⁺ recordings. Monolayers were stained by immersion in media at 37°C, containing (1) fura-4F AM (Ca²⁺ sensitive probe, Molecular Probes) at 10µmol/L for 30 minutes and (2) di-8-ANEPPS (Molecular Probes) at 40µmol/L for
10 minutes. Experiments were performed at 36±1°C. The Evolve™ 128 camera was used for all measurements (2x2 binning; full 128x128 pixel region; 992 frames per second).

**Rat ventricular tissue-slices**

Hearts were isolated from female Wistar rats (n=3), weighing 250 – 350 g, after cervical dislocation and in accordance with Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act of 1987, and swiftly connected to a Langendorff perfusion setup. Hearts were perfused at a constant rate of 5 mL min⁻¹ with Tyrode’s solution (containing, in mmol L⁻¹: NaCl 140, CaCl₂ 1.8, KCl 5.4, MgCl₂ 1, Glucose 11, HEPES 5; bubbled with O₂). Contractile activity was suppressed with 2,3-Butanedione 2-Monoxime (BDM, 10 mmol L⁻¹, Sigma-Aldrich).

Fluorescent dyes were sequentially injected into the aortic cannula for coronary perfusion. Hearts were first stained by re-circulating perfusion with 50 mL of 10 µmol L⁻¹ fura-4F AM for 30 minutes. The myocardium was then stained by delivering, without recirculation, a 20µL bolus of 40µmol L⁻¹ (in DMSO) di-8-ANEPPS, applied over 5 minutes (i.e. diluted in 25 mL perfusate). To load di-8-ANEPPS, Pluronic F-127 (Sigma-Aldrich) was added to the bolus, to a final concentration of 0.2 - 0.5%.

Ventricular tissue slicing techniques are described elsewhere. Briefly, a tissue chunk of the left ventricle was glued, epicardium up, with histoacryl tissue adhesive (Aesculap AG & Co KG, Tuttlingen, Germany) onto a 4% agarose gel (NuSieve GTG Agarose) block that was fixed on top of the vibratome cutting stage (Integraslice, Campden Instruments Ltd., UK). Slices, 350µm thick, were cut in cold (0°C) Tyrode’s solution (with 10 mmol/L BDM) with a steel blade at a progression speed of 0.03 mm s⁻¹, vibration amplitude 2 mm and vibration frequency of 80 Hz. The slices, cut in what would have been a roughly tangential plane to the ventricle before tissue excision, were transferred to a custom imaging chamber and pinned down onto a PDMS block in order to prevent curling. Tissue slices (at least 4 generated per left ventricle) were imaged at 36±1°C in Tyrode's solution, with 10 µmol L⁻¹ blebbistatin (Sigma-Aldrich) to prevent contraction. The Cascade® 128+ camera was used for all tissue slice measurements (2x2 binning; full 128x128 pixel region; 926 frames per second).

**Isolated perfused rat whole-heart**

Whole-heart imaging was performed on each of the hearts used for the tissue-slice work described above. All experiments were conducted at 36 ± 1 °C with the hearts positioned in a cradle and perfused at a constant rate of 5 mL min⁻¹ with Krebs-Henseleit solution. Contractile motion was minimized by application of the contraction-uncoupler blebbistatin (10 µmol L⁻¹). The Cascade® 128+ camera was used for all isolated heart measurements (2x2 binning; full 128x128 pixel region; 926 frames per second).
Flow cytometry of human iPSC-CM monolayers

To define the thresholds for positive 488 and 594 fluorescence, negative control samples were incubated with 488 or 594 conjugated secondary antibodies only. Black peaks in online figure I represent both the background fluorescence of the secondary antibodies and the auto-fluorescence of the cells. Green and red peaks show respectively 488 and 594 fluorescence for positive control samples (see Online Figure I). Both negative and positive control samples allow gating precisely for 488 and 594 positive fluorescence thresholds. Data were collected on a FACS system (MoFlo™ XDP) and analyzed using FlowJo V 9.4.11.

Image processing

Custom software written in MATLAB (The MathWorks) was used to perform optical mapping image processing. Signals were filtered (in time) with local regression using weighted linear least squares and a 1st degree polynomial model (MATLAB’s built-in smooth function) and images were filtered using 2D median filtering (MATLAB’s built-in medfilt2 function). Speed was measured using the maximum voltage upstroke velocity and delays between \( V_m \) and \([Ca^{2+}]_i\) were measured using the peaks of \( V_m \) and \([Ca^{2+}]_i\). Confocal images were acquired and analysed using Nikon Elements Software.

References

Online Figures

Online Figure I. Threshold determination for positive 488 (left, green) and 594 (right, red) fluorescence staining for flow cytometry. Positive immunostaining for MLC-2v (green) or MLC-2a (red) is shown by the shift of the fluorescence of CMs counted. Black peaks show the background fluorescence.
Online Figure II. Action potential duration (APD\textsubscript{90}) and calcium transient duration (CaT\textsubscript{90}) during spontaneous and electrically-paced propagation in human iPSC-CM monolayers.

**A, Left panel:** Fluorescence image of a human iPSC-CM monolayer. The white circle indicates the location of the point electrical stimulator. APD\textsubscript{90} and CaT\textsubscript{90} are taken from the four squared-regions (blue, red, green and cyan). **Right panel:** APD\textsubscript{90} and CaT\textsubscript{90} during spontaneous activity (average cycle length of 1332 ms).

**B, Left panel:** APD\textsubscript{90} and CaT\textsubscript{90} during electrically-paced activity with a cycle length of 500 ms. **Right panel:** APD\textsubscript{90} and CaT\textsubscript{90} during electrically-paced activity with a cycle length of 750 ms.

Scale bar: 5mm.