ELECTROPHYSIOLOGICAL SORTING OF PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES IN A MICROFLUIDIC PLATFORM

F.B. Myers[1], O.J. Abilez[2,3], C.K. Zarins[3], and L.P. Lee[1*]

[1]*Department of Bioengineering, Biomolecular Nanotechnology Center, Berkeley Sensor and Actuator Center, University of California, Berkeley, USA*

[2]*Department of Bioengineering, Stanford University, USA*

[3]*Department of Surgery, Stanford University, USA*

ABSTRACT

We are developing a microdevice for label-free cell sorting which sorts stem cells and their differentiated progeny based on their response to electrical stimulation. Specifically, we are interested in purifying ventricular-like cardiomyocytes from induced pluripotent stem cell (iPSC) derived populations for cardiac tissue replacement therapies. Electrophysiological measurements are commonly used to identify subpopulations of electrically-excitable cells and to determine the degree to which stem cells have differentiated into these cell types. However, there is currently no way to sort cells based on electrophysiological parameters. Label-free, non-genetic cell purification methods are ideal for tissue replacement therapies because labeling molecules and antibodies may be toxic to the patient or interfere with the integration of the graft tissue. Furthermore, for certain cell types, such as cardiomyocytes, there are no reliable molecular surface markers available. Our recent efforts have focused on developing a microsystem and instrumentation to measure extracellular field potentials (FPs) from cells, and we have made a number of technical innovations to reduce feedthrough stimulus artifact and enhance FP signal amplitude. We are able to distinguish clusters of iPSC-derived cardiomyocytes from undifferentiated embryoid bodies with very good contrast. Our eventual goal is to develop an automated system capable of analyzing and sorting individual cells.

KEYWORDS: stem cells, cardiomyocytes, electrophysiology, sorting

INTRODUCTION

One of the major challenges in translating stem cell biology into tissue replacement therapy will be the establishment of effective separation methods which specifically isolate differentiated cells and exclude cells which may hamper graft performance or lead to teratoma formation.[1] Conventional separation techniques for stem cells require exogenous labeling or genetic modification, neither of which is ideal for clinical applications. However, many of the cell populations relevant for therapy are electrically-excitable (e.g. cardiomyocytes, neurons, skeletal muscle, and vascular smooth muscle), meaning they produce transmembrane ion currents in response to electrical stimulation. Microelectrode recordings of these signals can provide rich phenotypic information non-invasively and without labeling.[2,3] Furthermore, when applied to cardiac tissue engineering, we hypothesize that electrophysiological homogeneity of implanted cardiomyocytes will lead to improved graft viability, improved sarcomeric alignment and electromechanical coupling within the host myocardium, and reduced incidence of arrhythmias.

THEORY

All animal cells maintain concentration gradients of certain ions across their plasma membranes through the use of active ion transport proteins. Electrically-excitable cells also feature voltage-gated ion channels which, upon activation by sufficient transmembrane electric fields, transiently open and allow ions to flow across the membrane down these concentration gradients. These ion currents lead to a voltage signal in the resistive medium surrounding the cell. This extracellular field potential (FP) signal can be detected with a nearby microelectrode. Each cell type has a characteristic expression pattern including many different ion channels, each with unique gating kinetics. Therefore, each cell type also has a characteristic FP. Furthermore, FP signals change as a cell matures from an embryonic to an adult phenotype during stem cell differentiation.[2,3]

Microelectrode field potential signals are notoriously weak and field stimulation produces dramatic artifacts in the recording which can obscure these signals. Our system addresses these problems in three ways. First, because the cells are confined in a microchannel, the ohmic voltage drop in the vicinity of the cells increases since current is confined to the cross-section of the channel. Second, we employ a differential electrode geometry which dramatically reduces the stimulus artifact seen by the amplifier electrodes. Finally, we have developed an artifact suppression algorithm which eliminates artifact through a combination of template subtraction, linear filtering, and least squares exponential curve fitting/subtraction.

Figure 1 depicts the operation of the device. Individual cells or cell aggregates are introduced into the device as a dilute suspension through a central channel and hydrodynamically focused over a detection region using co-laminar sheath flows. When a cell enters the detection region it causes a drop in impedance between two detection electrodes in the center of the channel (similar to electrodes in a Coulter counter). The flow is immediately stopped when a cell is detected. One of these detection electrodes is positioned directly under the cell and the other is positioned just to the side of the cell (transverse to the flow). These two electrodes differentially measure the voltage signal generated by the cell using a high impedance, low noise instrumentation amplifier. When the cell is directly over the central detection electrode, a short electrical pulse will be delivered through two large stimulus electrodes positioned directly upstream and downstream of

the detection electrodes. Due to their geometry in the channel, the stimulus and detection electrodes form a balanced bridge circuit. Because the stimulus electrodes are positioned perpendicularly to the detection electrodes, the stimulus artifact seen by the amplifier is common-mode and thus mostly rejected. The cell's field potential signal , however, will be detected differentially since the cell is only near one of the electrodes. Based on automated analysis of this signal, the outlet flow will be switched to one of several output reservoirs using external electromechanical valves.

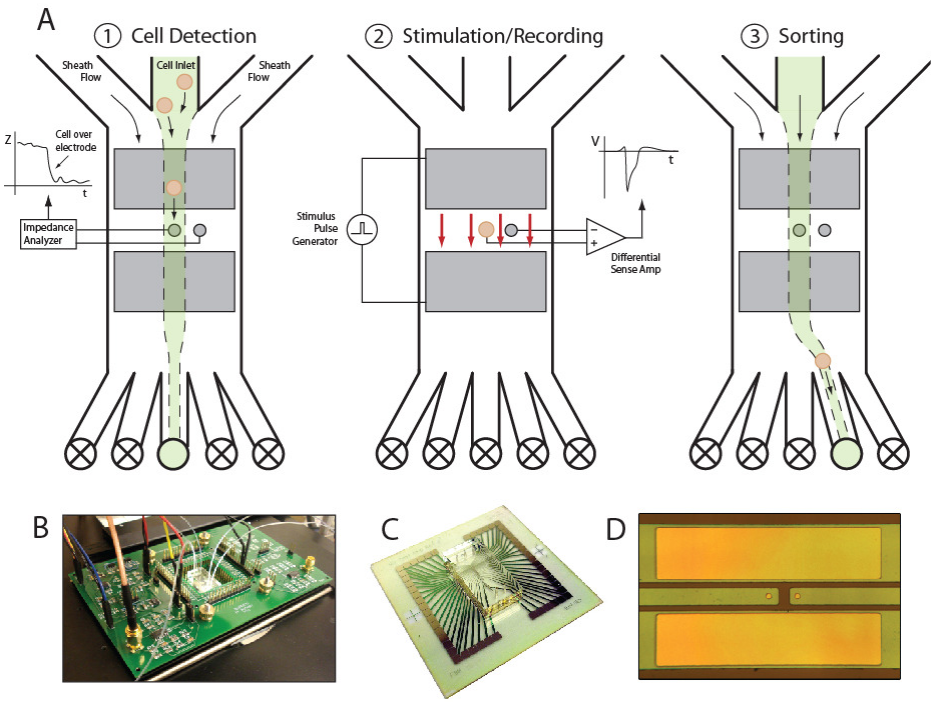

Figure 1: (A) Conceptual diagram of microfluidic electrophysiological cell sorter. (1) Cells are hydrodynamically focused over detection electrodes. The presence of the cells is indicated by a drop in impedance, and when this occurs, the flow is stopped. (2) Once stopped, cells are stimulated and the differential signal between the two detection electrodes is recorded. Because the detection electrodes are equidistant between the stimulus electrodes, the stimulus artifact is common mode and thus rejected. (3) The field potential signal is analyzed and the cells are sorted accordingly. (B) Custom instrumentation amplifier PCB. (C) Assembled microfluidic device consisting of a PDMS microchannel on glass with Pt electrodes. (D) Photo of fabricated electrodes.

EXPERIMENTAL

Human iPSCs (IMR90 line, WiCell, Madison, WI) were maintained in mTeSR1 medium on Matrigel-coated plates. These cells were differentiated into cardiomyocytes using Activin A and BMP-4 in an RPMI/B27 differentiation medium. During differentiation, cells gradually formed three-dimensional clusters, and after 9 to 20 days, some of these clusters began showing spontaneous beating, characteristic of cardiomyocytes. After differentiation, cardiomyocytes were maintained in DMEM/FBS. These spontaneously beating clusters were manually scraped off the plates and immediately introduced into the microfluidic device. As a negative control, embryoid bodies were formed by manually scraping undifferentiated iPSCs off of the plates, triturating them, and plating them out ultra-low attachment plates. These cells formed 3D aggregates and were analyzed in the microfluidic device within 2 days.

Microfluidic devices with channel widths of 400-1000 µm and heights of 100-500 µm were fabricated from PDMS using photolithographically-patterned SU8 molds on silicon wafers. 100nm Pt electrodes (with 20nm Ti adhesion layer) were patterned on glass slides using a metal evaporation and lift-off process. 400nm of PECVD Si_3N_4 was deposited as a passivation layer, and this was etched using SF_6 plasma to define the electrode contacts. Pt black was electrochemically deposited on the electrodes using a potentiostat, and this resulted in electrode impedances around 100 kΩ at 1 kHz (40 µm electrode diameter). The PDMS devices were bonded to the electrode slides using oxygen plasma after manual alignment under a stereoscope.

We measured both evoked and spontaneous field potentials arising from stationary cardiomyocyte clusters 100-300 µm in diameter. We chose clusters of cardiomyocytes for our initial experiments rather than single cells because they are more robust (we have recorded from clusters for several hours), and they visibly contract, which gives us an easy indication that they are responding to stimulus. In our experiments, we delivered short pulses (500 µs to 5 ms) of current stimulus (50-200 µA) using a custom stimulator which was optoisolated from the detection circuitry. We also varied the stimulus frequency (0.5-4 Hz). We used an instrumentation amplifier with high CMRR to measure the field potential signals from the cells.

RESULTS AND DISCUSSION

Figure 2 shows exemplary stimulus responses from both differentiated cardiomyocyte clusters and undifferentiated embryoid bodies. The cardiomyocyte clusters consistently produce FPs in response to field stimulation and many clusters also spontaneously produce FPs at a characteristic time interval. Undifferentiated cells, in contrast, produce no signals whatsoever. In this example, 4 stimulus pulses are shown. The first two do not result in FPs from the cardiomyocytes because they are administered while the cells are in the refractory period following a recent spontaneous contraction. The last two pulses do result in FPs. The delay between spontaneous contraction and stimulation can be adjusted to measure the refractory duration.

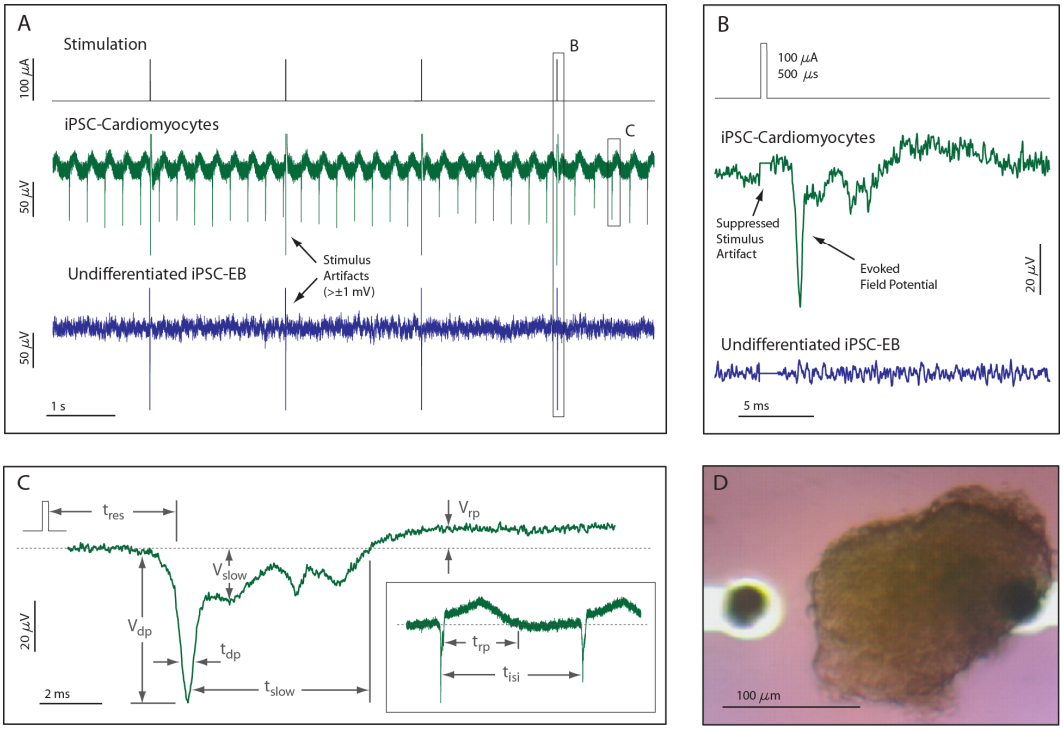

Figure 2: (A) Stimulus response of differentiated cardiomyocytes and undifferentiated embryoid bodies. (B) Closeup of evoked FP after stimulus artifact suppression. ~60μV FP clearly visible from cardiomyocytes while undifferentiated cells produce no signal. (C) Spontaneous FP averaged over 10 cycles to reduce noise. Multiple amplitude and timing parameters can be measured: response time (t_{res}), depolarization time (t_{dp}), slow current time (t_{slow}), repolarization time (t_{rp}), interspike interval (t_{isi}), depolarization amplitude (V_{dp}), slow current amplitude (V_{slow}), and repolarization amplitude (V_{rp}). Inset shows two successive spontaneous FPs. (D) A cardiomyocyte cluster positioned over one detection electrode, with the other electrode on the left.

We have demonstrated that the FPs from non-adhered cells (and even cells in motion) can be detected with excellent signal-to-noise ratio (SNR>50 in many cases) when the cells are confined to microchannels with dimensions approaching the cells' diameter. These signals can also be averaged over multiple repetitions to further enhance SNR. With higher SNR, one can quantify a number of features of the FP which indicate ion channel expression levels and cell maturity/phenotype. The field potentials observed in our experiments appear more complex than typical single cell recordings, reflecting the possibility that multiple cardiomyocyte phenotypes within the cluster are being recorded. Furthermore, these signals are often highly localized within a cluster. From these observations, we believe that single cell recordings should be possible.

CONCLUSION

We have demonstrated that it is possible to distinguish differentiated cardiomyocytes from undifferentiated stem cells using electrophysiology. While more work needs to be done to statistically quantify the specificity/sensitivity of these signals with respect to well-known protein markers, these results are nevertheless encouraging. Teratoma formation is currently a major risk in stem cell therapies, and this technique will likely provide a very low false positive rate, as it is unlikely that undifferentiated cells would produce signals resembling FPs. As this technique does not require labeling or genetic modification of cells, it is highly relevant for regenerative medicine.

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CONTACT

*L.P. Lee, tel: +1-510-642-5855; lplee@berkeley.edu