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Optogenetic LED Array for Perturbing Cardiac Electrophysiology*

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Abstract— **Optogenetics is the targeted genetic introduction of light-sensitive channels, such as Channelrhodopsin, and pumps, such as Halorhodopsin, into electrically-excitable cells that enables high spatiotemporal electrical stimulation and inhibition by optical actuation. Technologies for inducing optogenetically-based electrical stimulation for investigating *in vitro* and *in vivo* neural perturbations have been described. However, modification of existing technologies or creation of new ones has not been described for chronic cardiac applications. Here, an LED array system for optogenetically perturbing cardiac electrophysiology is described. The overall layout of the system consists of an LED holder containing six LED's that deliver pulsed ~470 nm light to pluripotent stem cell-derived cardiomyocytes cultured in a 6-well tissue culture plate. The response of the cardiomyocytes is monitored by microscopy and the system is enclosed within a standard incubator. This system is relatively simple to create and uses mostly off-the-shelf components. The overall function of the system is to deliver chronic light stimulation over days to weeks to differentiating stem cell-derived cardiomyocytes in order to investigate perturbations in their electrophysiology.**

I. Introduction

Optogenetics is the targeted genetic introduction of light-sensitive channels and pumps into cells that enables their high spatiotemporal control by optical actuation, leading to downstream generation of electrical action potentials [1-3]. This technology overcomes the limitation of low spatial control of traditional electrical stimulation; this limitation is due in part by electrode size and spacing, which hinders single cell control within a population of cells [4, 5].

To date, optogenetics has been demonstrated in various cellular types including central neurons [1-3], peripheral neurons [6], skeletal myocytes [7], and pluripotent stem cells (PSC) [8-10]. In the cardiac field, optogenetics has been used to apply long-term depolarization to murine stem cell-derived cardiomyocytes [8], to localize zebrafish pacemaker development [11], to control light-activated behavior of human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) [10, 12, 13], and to stimulate cardiac activity by various other techniques [14, 15].

Human pluripotent stem cells (hPSC) have the potential to be differentiated into every cell type in the body and can be differentiated into cardiomyocytes (hPSC-CM) [16]. However, differentiation protocols for hPSC-CM have resulted in CM with an immature phenotype and with heterogeneous electrical properties [16]. Recent evidence has shown that electrical stimulation causes alignment of cultured CM along the electrical conduction axis *in vitro* [17] and that electrical conduction during development is required to preserve cardiac morphology *in vivo* [18]. As such, chronic electrical stimulation may be needed for inducing a mature CM fate and for directing CM towards a specific atrial, pacemaker, or ventricular subtype [19].

Technologies for applying optogenetically-based electrical stimulation for neural *in vitro* perturbation [20, 21] and *in vivo* perturbation [22-24] have been described. However, modification of existing technologies or creation of new ones has not been described for chronic (several days to weeks) cardiac applications. Here, an optogenetic six (6)-LED array for perturbing cardiac electrophysiology is described. Fig. 1 shows the overall layout of the system where an LED assembly consisting of six (6) LED's and an LED holder delivers ~470 nm light to CM's cultured in a 6-well tissue culture plate. The response of the CM's is monitored by microscopy and the system is enclosed within a standard incubator kept at 37° C and 5% CO_2. This system is relatively simple to create and uses mostly off-the-shelf components as described below.

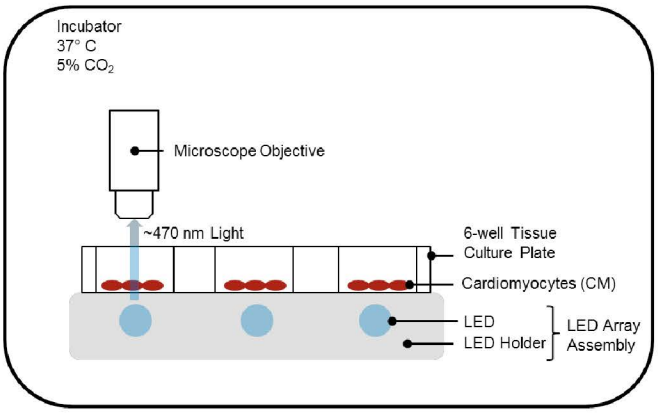

Figure 1. LED array for chronic perturbation of cardiac electrophysiology. The array assembly consists of six (6) LED's and an LED holder that delivers ~470 nm light to CM's cultured in a 6-well tissue culture plate. The response of the CM's is monitored by microscopy and the array is enclosed within a standard incubator

II. Methods

All experiments, methods, and protocols for this study were approved by the Stanford University Stem Cell Research Oversight (SCRO) committee.

A lentiviral vector containing a combination of channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR), pLenti-EF1α-ChR2-NpHR-eYFP-WPRE (pLECNYT) with a puromycin resistance gene was constructed and packaged into non-replicating virus as previously described [3, 10, 13]. All constructs have been fully sequenced and vector maps are available at http://www.optogenetics.org.

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An hPSC line carrying both ChR2 and NpHR was created as previously described [10, 13]. Briefly, a stable human induced pluripotent stem cell (hiPSC) line (IMR90, WiCell) was created by transduction with the pLECNYT packaged virus. ChR2-NpHR-eYFP expressing cells were isolated by selection pressure via addition of puromycin. For this hPSC line, only ChR2 can be activated by the LED system described below and, therefore, this line is further referred to as $hPSC^{ChR2+}$ for simplicity.

Pluripotency of $hPSC^{ChR2+}$ was maintained through daily feeding with mTeSR1 (StemCell Technologies, Vancouver, Canada) and were grown on hPSC-qualified Matrigel (BD Biosciences, San Jose, CA) in 6-well tissue culture plates (BD Biosciences).

For CM differentiation, hPSC were grown in 6-well tissue culture plates containing RPMI-1640 media with B27, 1x non-essential amino acids, 1x antibiotics, and 0.1 mM β-mercaptoethanol (all Invitrogen, Carlsbad, CA). The differentiation method utilized 50 ng/mL Activin A, 5 ng/mL BMP-4, and 37.5 ng/mL concentrated Dkk-1 as from our previously described methods [10, 13]. Cardiomyocytes generally began beating between days 10 and 14.

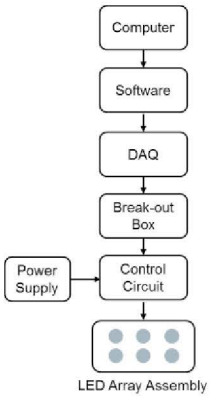

Figure 2. System layout for the LED array. Computer-control of the array is achieved via data acquisition and control (DAQ) software and hardware interfaced with a simple circuit that controls the array frequency, amplitude, and duration output.

As shown in Fig. 2, the LED array system consists of a dedicated computer, control software, data acquisition (DAQ) hardware, a break-out box, a control circuit and power supply, and six (6) LED's within an LED array assembly. The components are described in detail below.

A Dell XPS L502X laptop with 8 GB RAM and a 750 GB hard drive (Dell, Austin, TX), running Windows 7 Ultimate (Microsoft, Redmond, WA) is used. Data acquisition (DAQ) hardware is connected to any free USB port.

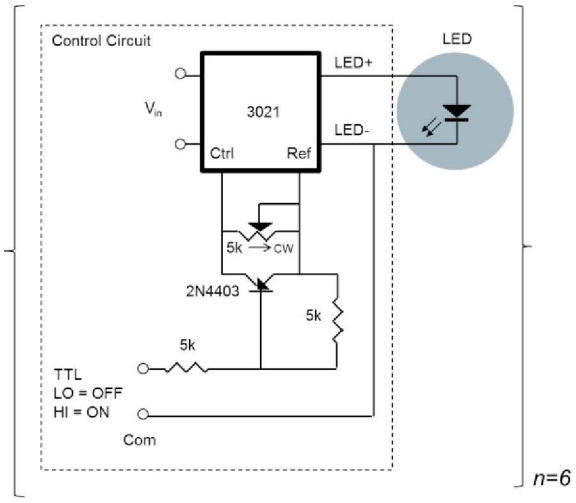

Figure 3. LED control circuit.

A custom virtual instrument (VI) software application was created using LabVIEW 2010 (National Instruments, Austin, TX). The VI independently controls each LED of the LED array described below via a digital TTL output signal that can be varied in duration and frequency.

A USB-6009 DAQ or a USB-6225 DAQ (National Instruments, Austin, TX) relays a commanded TTL output signal from the VI software application to the LED control circuit via a break-out box. Depending on need, the USB-6225 allows for more precise timing and synchronization of the TTL signals.

The break-out box consists of stripped addressed wires for input connection from the DAQ hardware and six (6) BNC connectors for output connection to the control circuit below. The stripped wires and BNC connectors are directly connected and contained within a 11.5 x 7.5 x 4.5 cm plastic box (BUD Industries, Willoughby, OH).

As shown in Fig. 3, the control circuit consists of a TTL input from the break-out box above into a 2N4403 transistor (Jameco, Belmont, CA). Six (6) 1000 mA internally dimmable 3021 Buck Puck DC drivers (Luxeon, Ontario, Canada) control the output of six (6) blue (~470 nm) Rebel Star LED's mounted on 20 mm Star CoolBases (Luxeon). A six-wire 3021HEP wiring harness (Luxeon) connects one adjustable 5 K potentiometer, one driver, and one LED. 24 V power is supplied to six (6) drivers. 5 K resistors are used as shown. Components are soldered to a Vector 8001 PCB with 0.042 inch diameter holes and cut to fit within a 15 x 11.5 x 5.5 cm plastic box (BUD Industries, Willoughby, OH). Input to the circuit is via six (6) panel-mount BNC connectors and output is via two (2) panel-mount male DB-9 connectors leading to the LED array assembly below.

As shown in Fig. 4, the LED array assembly consists of a custom designed LED holder, six (6) 25 mm square x 20 mm high Alpha heat sinks (Luxeon), six (6) blue (~470 nm) Rebel Star LED's (Luxeon) attached to the heat sinks, and six (6) Fraen 9° 20 mm optic lenses with flat bottom holders (Luxeon) used for focusing the LED light output onto the bottom surface of a mounted 6-well plate. Six (6) set screws

hold the heat sink-LED assembly within the LED holder. The wires from the LED's terminate in two (2) female DB-9 connectors. The LED holder was designed with SolidWorks (Dassault Systèmes, Vélizy, France) and fabricated with a uPrint Plus 3D printer (Stratasys, Eden Prairie, MN).

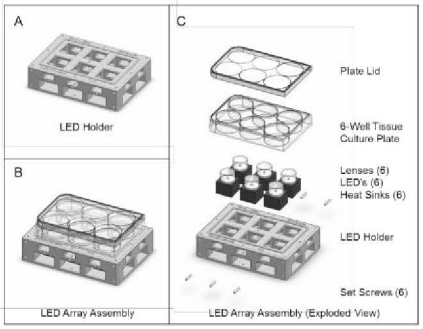

Figure 4. LED array assembly. (A) An LED holder was designed and fabricated with a 3D printer. (B) The LED array assembly with a 6-well tissue culture plate in place. (C) An exploded view of the LED array assembly showing the various components as labeled.

III. Results

Undifferentiated hPSC were transduced with lentivirus, sorted, and expanded to create a hPSC^{ChR2+} line. As shown in Fig. 5, PCR confirmed the presence of pluripotency markers along with the expression of ChR2, NpHR, and eYFP.

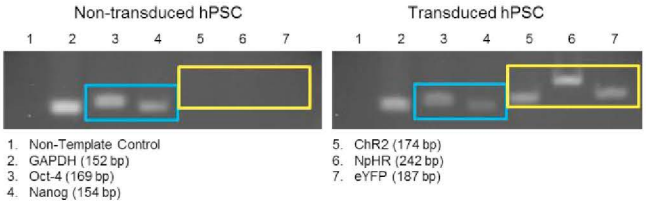

Figure 5. PCR comparing presence of pluripotency markers Oct-4 and Nanog along with the expression of ChR2, NpHR, and eYFP in non-transduced and transduced hPSC (bp = base pair; GAPDH = glyceraldehyde 3-phosphate dehydrogenase).

The hPSC^{ChR2+} line were differentiated into hPSC^{ChR2+}-CM. These cells spontaneously contracted and showed no differences from wild-type hPSC (hPSC^{WT}-CM) (data not shown). In addition, these CM expressed ChR2 as confirmed by the presence of eYFP signals under standard fluorescence microscopy.

Upon stimulation with a Lambda DG-4 300 W Xenon light source filtered for ~470 nm blue light (different from the LED array), it was confirmed that hPSC^{ChR2+}-CM were responsive to blue light and could be activated at frequencies ranging from 0.5 to 1.5 Hz.

As shown in Fig. 6, the LED array assembly holds a 6-well tissue culture plate as designed. The 6-well plate is kept in place by a ~2 mm lip on the LED holder that prevents the plate from moving in any lateral direction. Each of the LED's focuses ~470 nm blue light onto the bottom of the 6-well plate. Each LED produces enough power (> 5 mW/mm^2) to stimulate hPSC^{ChR2+}-CM. Power can be adjusted with each potentiometer attached to each individual LED driver. Each LED can be controlled independently, with pulse widths typically ranging from 1-100 ms, and frequencies ranging from 0-10 Hz, making it suitable for mimicking cardiac rates in rodents and humans. Different phases between LED stimulation can also be achieved.

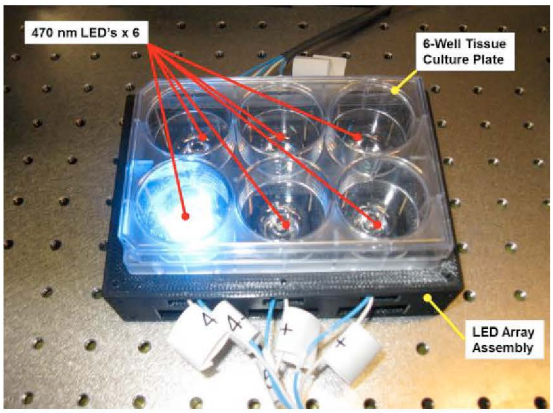

Figure 6. The LED array assembly with a 6-well tissue culture plate in place. Blue light (~470 nm) is shown emanating from one LED directly under one well of a 6-well tissue culture plate.

IV. Discussion

At the gene, protein, and functional levels, undifferentiated hPSC stable lines transduced with ChR2 and NpHR allow for downstream optical control of hPSC-CM. The blue light LED array described here only stimulates ChR2, but the addition of ~580 nm yellow light LED's should allow for NpHR activation. These LED's are commercially available and could easily replace the blue ones described here.

Since optical control has been achieved with other electrically-active cells such as neurons and skeletal muscle cells, the LED array system described here could also be used to study chronic electrophysiological perturbations in these cell types. Previous studies suggest that electrical stimulation and maturation of stem cell-derived cells which are electrically-active will be required for optimal function and subsequent engraftment into host tissues to both avoid arrhythmias and restore function in damaged recipient tissues and organs [17, 18, 25].

Although an LED array for stimulating cells in a 6-well tissue culture plate is described here, there is no reason that array systems cannot be made for standard 12-, 24-, 48-, and 96-well plates. In fact, arrays for these higher well formats would be welcomed since they would enable higher throughput, use of more samples to increase statistical power, and use of fewer cells per well. Furthermore, integration of LED arrays into microfluidic cell culture systems would also allow for more flexibility in combining optical stimulation with other modes of stimulation from the mechanical and biochemical domains.

Finally, the LED holder described here was fabricated using 3D printing technology. This is not a requisite for this system and a simpler holder could likely be fabricated by traditional milling.

It is hoped that the technology presented here will inspire more refined and accessible optical stimulation technology. It is also envisioned that application of optogenetics to the cardiac system will create new avenues for basic and translational studies in stem cell biology, electrophysiology, and regenerative medicine.

V. Conclusion

Genetically targeted light sensitive proteins can enable *in vitro* optogenetic actuation of hPSC-CM. An LED array has been designed, fabricated, and tested for delivering the blue light required for activating $hPSC^{ChR2+}$-CM. The overall function of the system is designed to deliver chronic light stimulation to differentiating stem cell-derived cardiomyocytes, which will enable the investigation of perturbations in their electrophysiology.

Acknowledgment

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