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Fluorescence-based system for measurement of electrophysiological changes in stretched cultured cardiomyocytes

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Abstract—Acute or sustained stretch of cardiac tissue is known to play a key role in arrhythmogenesis. Using a fluorescence approach, we designed a system measuring calcium transients and transmembrane potential changes in monolayers of cultured cardiomyocytes under uniaxial elongation and electrical stimulation. Cardiac myocytes are seeded on a rectangular PDMS template held and stretched by a motorized linear guide system. Electrical stimulation is performed with two parallel carbon electrodes supplied by amplified pulses from a digital-to-analog converter. The cells are stained with either voltage- or calcium-sensitive dye (di-4-ANEPPS and Fluo-4 AM respectively). The two available excitation light sources are both current-controlled LED arrays ($\lambda = 523 \pm 45$nm for di-4-ANEPPS and $\lambda = 505 \pm 15$nm for Fluo-4 AM). The filtered emitted fluorescence ($\lambda > 610$nm for di-4-ANEPPS and $\lambda = 535 \pm 25$nm for Fluo-4 AM) is transduced to current with a photodiode, converted to amplified voltage signals and digitized. The design and preliminary validation results are presented.

I. Introduction

ELECTRICAL and mechanical activities of the heart are closely interrelated. Electrical excitation controls cardiac contractions through the calcium-induced calcium release mechanism [1]. Conversely, changes in cell length and/or tension play a powerful feedback role on the process of electrical excitation [2]. It has been shown that mechanical stimulation results in activation of the stretch-sensitive ion channels [3], a phenomenon termed mechano-electric feedback (MEF). In the presence of acute or sustained stretch, MEF may increase susceptibility to atrial and ventricular arrhythmias [4]. Therefore, an in depth knowledge of MEF is crucial to understand the role of mechanical constraints in arrhythmogenesis.

Intact tissue (including whole heart) or single cells are the most common experimental models for the study of MEF. Confluent monolayers of cultured cardiomyocytes (CMs) are increasingly used as a gold standard because they mimic the main features of intact tissue function while allowing precise experimental control, an advantage associated with single cells [5]. Many devices have already been engineered to stretch cell monolayers ([6]–[9]), however, in addition to being expensive (for commercially available ones), they are not all-in-one systems incorporating simultaneous electric field stimulation and fluorescence imaging capabilities.

The objective of the present project is to design a low-cost, open-source system measuring electrophysiological changes in monolayers of electrically stimulated cultured CMs under controlled uniaxial linear elongation. As a first step, the focus is on transmembrane potential and calcium transient recordings. Fig. 1 illustrates the overall approach. In this proceeding, we present the design and preliminary results obtained to validate the approach.

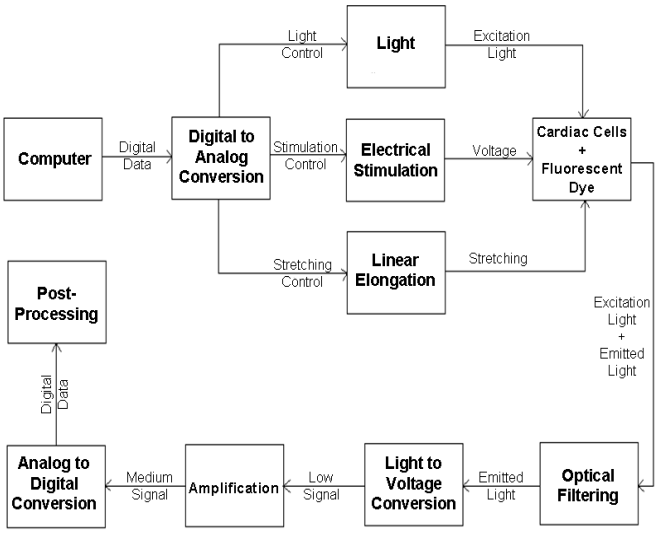

Fig.1. Data flow diagram of the system. User-determined digital data are converted into signals controlling light, electrical stimulation and elongation subsystems. The generated stimuli are all applied to CMs previously stained with a fluorescent dye (di-4-ANEPPS/Fluo-4 AM). The emitted fluorescence is isolated by an optical filter, converted to voltage, amplified, digitized and saved for post-processing analysis.

II. Design and Validation

A. Computer Control and Electrical Conversions

The user controls the system via a custom-written Matlab (R2008b) program processing the following inputs:

--Excitation light intensity (Level 1 – Level 6).
--Electrical stimulation amplitude (V), frequency (Hz), pulse width (s), and duration (s).
--Uniaxial linear elongation direction (stretch/unstretch), length (mm), and speed (mm/s).
--Acquisition sampling rate (samples/s) and duration (s).

The parameters are converted to digital data and sent to a National Instrument (NI) multifunction data acquisition

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module (NI USB-6221). The NI card controls each subsystem with digital or analog signals, as appropriate:
--Excitation light: TTL signal.
--Electrical stimulation: analog pulse at 1/10 of final amplitude.
--Uniaxial linear elongation: TTL signal.

B. Cardiomyocytes Culture Setup

Cells are cultured on a molded rectangular Polydimethylsiloxane (PDMS) template. The template has T-shaped endings that are thick enough to be kept in place by pliers (see Fig. 2). The center of the template forms a clear-bottom (12mm x 12mm x 0.5mm) well containing medium and is dedicated to the culture of CMs and imaging experiments.

PDMS was chosen because of its biocompatibility and the possibility of easily obtaining a range of physiologically relevant Young's modulus values [10]. A 30:1 (ratio of silicone elastomer to curing agent) solution of PDMS (Sylgard 184, Dow Corning Inc) is poured into a custom aluminum mold, placed under vacuum for air removal and curing for 48 hours at 25°C. Fig. 2 shows a simulation of the mechanical stress distribution of the template undergoing stretch. The Young's modulus (500kPa) and Poisson ratio (0.49) were chosen to fit the material properties [11]. The von Mises stress has been found to be uniformly distributed in the culture region located in the center area of the template.

Coating of the PDMS is required to ensure cell adhesion. Adhesion and viability of HEK293 and H9C2 cells have been qualitatively evaluated for different types of coating: gelatin-fibronectin (G1890, F1141, Sigma-Aldrich), collagen (C0543-1VL, Sigma-Aldrich), polylysine (P4707-1VL, Sigma-Aldrich). Preliminary observations show that gelatin-fibronectin (GF, 0.2% gelatin and 0.00125% fibronectin in distilled water) enables a greater viability and optimal adhesion of cells. An example is shown in Fig. 3 for HEK293 cells cultured for two days on a GF-coated PDMS template before (panel a) and after stretch (panel b).

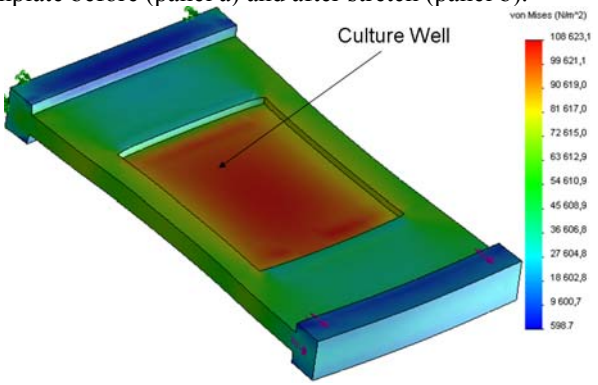

Fig. 2. PDMS template. A 30:1 (ratio of silicone elastomer to curing agent) solution of PDMS cures (under vacuum for 48 hours at 25°C) inside a custom aluminum mold. The resulting template has a central clear-bottom well (12mm x 12mm x 0.5mm) for CMs culture and imaging. The simulated stress (N/m^2) given by the color code is maximum and uniformly distributed in the center area of the culture well where cells are seeded.

C. Excitation Source

The purpose of the light source is to generate the photons required to excite the dyes molecules (di-4-ANEPPS or Fluo-4 AM). Two light sources (green and cyan) have been built and are described below:

--The green (λ = 523 ±45nm) light source, for di-4-ANEPPS, is an array of six LEDs (NTE 30038, NTE Electronics Inc.) electrically coupled in parallel. The light intensity is proportional to the amplitude of the current flowing through the LEDs. This amplitude is set via TTL signals controlling a Darlington transistor array (ULN2003A, STMicroelectronics) dedicated to the selection of current limiting resistors as depicted in Fig. 4.

--The cyan (505 ± 15nm) light source is an array of three high-power pre-mounted LEDs (Rebel, Luxeon Star LEDs) coupled in series. The source can be used for excitation of both di-4-ANEPPS and Fluo-4 (calcium dye). The wavelength spectrum is limited with a band-pass filter (455nm < λ < 499nm). The principle for controlling light intensity is the same as previously described. Different values of resistances (11Ω, 20Ω, 40Ω, 80Ω) and a high current Darlington transistor array (ULN2064B, STMicroelectronics) were chosen due to the increased current requirements of the cyan LEDs.

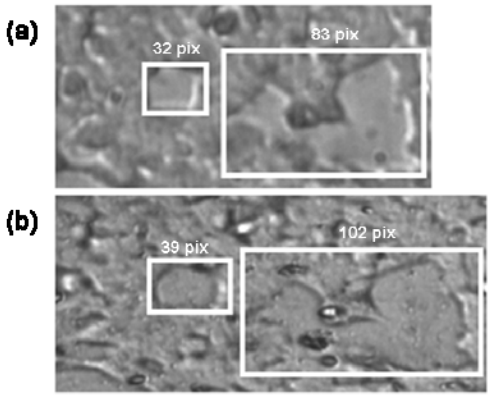

Fig. 3. Stretching attached HEK293. (a) The cells adhered to PDMS template coated with gelatin-fibronectin and were kept in culture for two days. (b) Application of a 1.8mm elongation to the template resulted in ~20% stretch (along the direction of the deformation) for the two areas of the culture well.

D. Electric Field Stimulator

A computer-controlled subsystem delivering a biphasic electric field was built. As illustrated in Fig. 5, the NI card outputs analog stimulation pulses at 1/10 of the amplitude set by the user to bypass its voltage limitation. The gain (x10) of the custom-made non-inverting power amplifier increases the DAQ output voltage (Stim/10) to the level selected by the user (Stim).

The electric field is applied to the cell monolayer via a pair of plate electrodes. Carbon electrodes (SK-05 ISO Graphite Plates, Industrial Graphite Sales LLC) were selected since these were found to have the best current injection characteristics compared to several other usual types of material (stainless steel, titanium, etc.) [12]. The system allows voltage amplitudes of up to 17V (250mA limited by R_3).

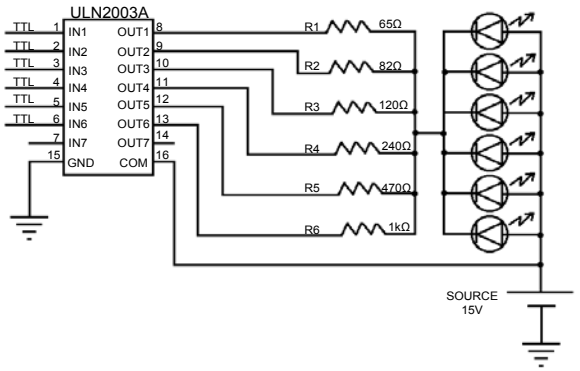

Fig. 4. Electrical circuit for the excitation green light source. TTL signals from the NI card control the Darlington transistor array (ULN2003A, Texas Instruments). When TTL = 1 on the input pin INn, the voltage of the output pin OUTn drops from 15V to 0.7V inducing a current flow through the resistance between the 15V voltage source and OUTn. The value of this current is limited by the resistance Rn (n = 1 to 6).

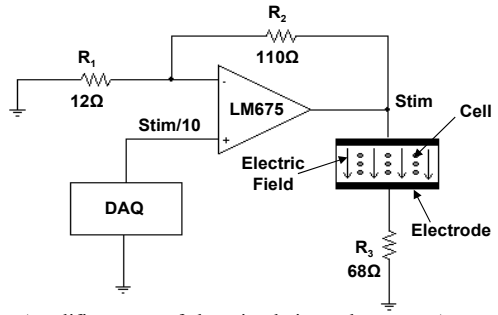

Fig. 5. Amplifier stage of the stimulation subsystem. A power amplifier chip (LM675, National Semiconductor) is used with resistors R1 and R2 to create a non-inverting amplifier configuration. The gain (x10) of the amplification increases the DAQ output voltage (Stim/10) to the level selected by the user. The system allows voltage amplitude up to 17V (250mA limited by R_3).

E. Uniaxial Linear Stretcher

Computer-controlled elongation of the PDMS template applies a deformation to the attached cardiomyocytes with variable length and speed. Our design is illustrated in Fig. 6.

The PDMS template is held horizontally by a pair of custom-made aluminum pliers. Each of the pliers is coupled to a linear guide (a carriage sliding on a rail) driven by a linear stepper motor (15000 Series, Haydon Kerk Motion Solutions). The NI card sends TTL signals to a dual full-bridge driver (L298N, SGS Thomson Microelectronics Inc) that changes the direction of the currents flowing through the motor internal coils and movement. For each specific set of TTL signals (sequence), the chip drives the motors one step (20µm) forward or backward. The speed of movement is thus controlled by the frequency of the input sequences.

F. Optoelectronics and Data Acquisition

Excited molecules of di-4-ANEPPS emit a red (λ_{max} = 630nm) fluorescence light proportional to the transmembrane voltage of the cells. Isolated with a long-pass optical filter (λ > 610nm, Edmund Optics Inc), the fluorescence is transduced into current by a fast photodiode (S1226-5BK, Hamamatsu) (Fig. 7). The current is then converted to small voltage by a load resistance R (4.7MΩ) following Ohm's law (V = RI). The resistor and the capacitor (C = 100pF) implement a low-pass (anti-aliasing) filter of 340Hz cutoff frequency. The small voltage is amplified by a variable gain low-noise amplifier (AD524, Analog Devices). The digitization is performed by the NI card at a sampling rate (0.5 to 1kHz). The subsystem has been experimentally validated on stained (di-4-ANEPPS) ex-vivo whole rat heart. An example of ventricular recording with pacing period of 300 ms is shown in Fig. 7b.

To study calcium dynamics in post-cultured cell, Fluo-4 AM was chosen. Excited molecules of Fluo-4 AM emit a light (λ_{max} = 516nm) proportional to calcium transients. The emission filter (λ = 535 ± 25nm, Chroma Technology) was chosen to optimize photon transmission to the photodiode. Experimental validation is underway.

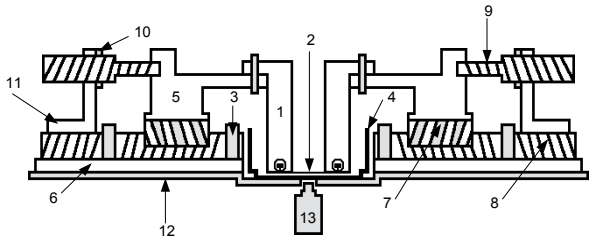

Fig. 6. Schematic of the stretcher apparatus. The PDMS sheet (2) is held by a pair of pliers (1), coupled to a stretcher apparatus made of two linear guide systems (5,7,8) and two computer controlled linear stepper motors (9). The stretcher is placed on a stage (12) on which the optical lens is focussed (13).

III. DISCUSSION

Our proposed approach together with PDMS template design offer the possibility of studying electrophysiological responses of cultured monolayers of CMs under mechanical and electrical stimulation. The system can integrate common setups for rapid superfusion of medium to ensure cell viability and study pharmacological responses. Thus far we have developed: (1) a Matlab control program, (2) two excitation light sources for di-4-ANEPPS and Fluo-4 AM, (3) an electric field stimulator, (4) a uniaxial linear stretcher, (5) and an acquisition circuit. A PDMS template that includes a specific region for culture of cell monolayers has also been designed. We have presented interesting preliminary results; however, the validation process is still in progress.

The system permits integrated programmed electrical stimulation protocols which allow conditioning of cells before and during fluorescence acquisitions as required for precise electrophysiological studies. Cell excitation is sensitive to changes in electrical field, therefore a fixed electrode position was chosen. Implementation of mechanical stimulation led to positioning the electrodes transverse to the axis of elongation, maintaining a constant inter-electrode distance. The choice of linear stepper actuators was based on (1) better control of the template deformation and (2) relatively low cost of the motors. As

implemented in bipolar full-step drive, the applied stretch force is maximal but translation resolution is limited to step changes of 20 μm. Thus, the possibility to integrate piezoelectric actuators for increased stretch resolution and subsequent smoother changes in elongation could be evaluated in future experiments.

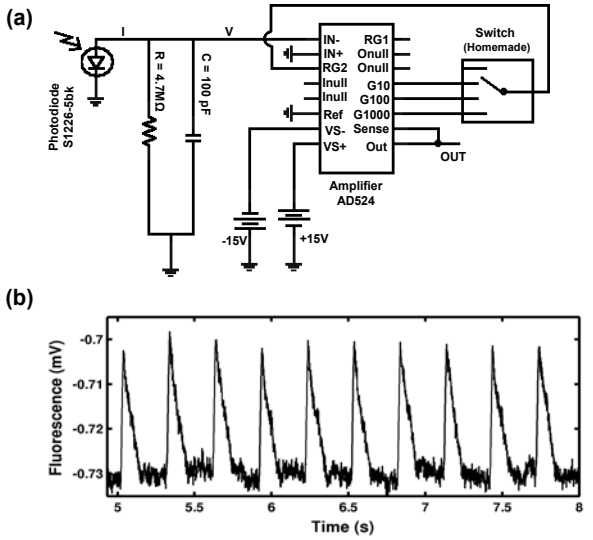

Fig. 7. Light-to-voltage conversion and amplification. (a) Electrical circuit. First, the photodiode converts the light into a current that is converted into small filtered (340 Hz cutoff frequency) voltage by a resistance R (4.7MΩ) in parallel with a capacitor C (100pF). The signal is then amplified with a variable gain low-noise amplifier (AD524, Analog Devices). (b) Experimental validation of the light-to-voltage conversion circuit. Signals have been acquired from ventricular surface of whole rat heart paced with 300 ms period of stimulation. After DC removal, the raw signal was filtered (Butterworth) to attenuate its 60 Hz component (electromagnetic noise). The unit of the fluorescence (mV) corresponds to the measured voltage following amplification (gain = 10).

Electrophysiological recordings from cells can be obtained by different methods: microelectrodes [13], multielectrode arrays [14], and fluorescence mapping [15]. Compared to microelectrodes, the fluorescence approach is non-invasive and allows stable acquisition during mechanical stimulation. Optical mapping also presents the advantage of providing direct access to transmembrane voltage variations or intracellular calcium changes while multielectrode arrays yield only extracellular potentials and are sensitive to electrical stimulation artifacts. However, the spatial resolution of optical mapping cannot be fully exploited with a single photodiode. In the present study, the measured signal is the total fluorescence from all electrically-stimulated cells included in the effective field of view focused on the active area (1.2 mm^2) of the photodetector. Thus, electrical propagation studies are not yet feasible but will be considered at a later stage with either a photodiode array, CMOS or charge-coupled device (CCD) camera, as described in other studies [16].

We opted for an "open-source" system to study MEF in cultured monolayers of CMs. Codes, detailed circuit layout, and item list needed for the setup will be available once the final validation is completed. Furthermore, the system could be adapted easily to meet other purposes. For example, alternative LEDs and resistances could be selected to accommodate different excitation light requirements based on the simple design presented in Fig. 4. Similarly, amplifiers and resistance (R_3) could be selected to obtain desired voltage and current properties of the electric field stimulator (Fig. 5). In summary, with small changes, the system may be used to probe any stretch-sensitive electrophysiological parameter that is measurable using the fluorescence approach.

After final improvements and validation, our system will be an interesting all-in-one tool for MEF studies allowing electrophysiological recordings on monolayers of cells being simultaneously stretched and electrically stimulated.

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