Journal of
Bioengineering & Biomedical Science

Daily et al., J Bioengineer & Biomedical Sci 2015, 5:3
DOI: 10.4172/2155-9538.1000168

Research Article Open Access

Improving Cardiac Action Potential Measurements: 2D and 3D Cell Culture

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Abstract

Progress in the development of assays for measuring cardiac action potential is crucial for the discovery of drugs for treating cardiac disease and assessing cardiotoxicity. Recently, high-throughput methods for assessing action potential using induced pluripotent stem cell (iPSC) derived cardiomyocytes in both two-dimensional monolayer cultures and three-dimensional tissues have been developed. We describe an improved method for assessing cardiac action potential using an ultra-fast cost-effective plate reader with commercially available dyes. Our methods improve dramatically the detection of the fluorescence signal from these dyes and make way for the development of more high-throughput methods for cardiac drug discovery and cardiotoxicity.

Keywords: Action potential; iPSC; Cardiomyocyte; Tissue; 3D

Abbreviations: iPSC: induced Pluripotent Stem Cell; CM: Cardiomyocyte; AP: Action Potential; EHT: Engineered Heart Tissue; 2D: Two-dimensional; 3D: Three-dimensional; Ca^{2+}: Calcium

Introduction

One of the challenges in analyzing action potential (AP) of biological samples is to record rapidly changing electrical activities in live biological samples. A gold standard for AP measurements is to place electrodes into a biological sample, which was established more than 50 years ago [1]. As an alternative to the electrode techniques, optical mapping of AP has been applied to analyze electrophysiology of various sample types including whole hearts [2]. However, until recently, the weak sensitivity of optical probes to detect APs was hampering the ability to carry out the measurements in many laboratories. Improved sensitivity of the probe to measure AP enabled us to use a plate reader with a rapid optical acquisition feature (CLARIOstar®, BMG LABTECH, Cary, NC) for measuring AP of cardiomyocytes derived from human induced pluripotent stem cells (iPSCs).

Here, we demonstrate an application of microplate readers in detecting APs in high throughput, exhibiting technical considerations to improve signal-to-noise (SN) ratios. The opportunities to improve SN ratios include 1) improving sensitivity of optical probes, 2) increase sensitivity of detection system, 3) increasing the amount of optical probes in an optical focal volume. One of the widely used optical probes for AP is an electrochromic potentiometric dye, di-4-ANEPPS, which shifts fluorescent spectra depending on its electromagnetic environment. While its response time is ultra-fast (fs-ns), the sensitivity of probe is low; ~8% of change in signal is detected with 100 mV [3]. A recently developed potentiometric dye, FluoVolt (Molecular Probes/ Thermo Fisher Scientific, Waltham, MA) changes signals by photo-induced electron transfer within the dye molecule. The response time of the probe is still good and can register ~28% of change in signal with 100 mV [4]. In addition to the sensitivities of optical probes, total amount of dye within the focal volume can influence the ability to detect signals. While potentiometric dyes accumulate only on the plasma membrane, most of the biological sensors, such as Ca^{2+} indicators, distribute throughout the cytoplasm. Hence, a larger amount of Ca^{2+} indicators are localized within a focal volume for the photo-detector compared to potentiometric dyes. The detection of potentiometric dyes normally requires an optical system with higher numerical aperture gathering more light from dyes than that with diffused focus. Therefore, a microscope using a high magnification objective is suitable to detect relatively weak changes in fluorescent signals. An instrument with high spatial resolution, including high-power microscopes, coupled with a sensitive detection system can measure localized changes in APs. Alternatively, even with an instrument with lower sensitivity, AP can be measured from multiple electrically connected cells including cells in mono- and multi- layer(s) as well as cells in tissues. However, the users should be aware of potential artifacts that influence AP profiles, which will be discussed below.

Materials and Methods

Cell and tissue culture

Human cardiomyocytes were derived from commercially available human induced pluripotent stem cells (iPSCs, Applied Stem Cell, Milpitas, CA) using a protocol published previously [5,6]. RPMI media 1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with B27 (Gibco/Thermo Fisher Scientific, Waltham, MA) were used to maintain functional contracting cardiomyocytes. Differentiated cardiomyocytes were harvested from the plates by treatment with 0.25% Trypsin (Thermo Fisher Scientific, Waltham, MA) and Accutase(Innovative Cell Technologies, San Diego, CA) and seeded onto 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) coated, 96-well half-area plates (Corning Inc., Corning, NY) for monolayer culture and seeded at 10 K, 20 K, 30 K, 40 K cells per well densities. Three-dimensional tissues were fabricated using a protocol established previously [7]. The resulting tissue solution was aliquoted into 96-well plates with scaffold inserts (MC-96, InvivoSciences, Madison, WI) and incubated at 37°C. The tissues were cultured in Advanced RPMI1640 (Thermo Fisher Scientific, Waltham, MA) at 37°C and 5% CO_2 for at least four days. During this time, the cardiomyocytes remodel 3D cell

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Received: September 29, 2015; **Accepted:** October 05, 2015; **Published**: October 16, 2015

Citation: Daily NJ, Yin Y, Kemanli P, Brian Ip, Wakatsuki T (2015) Improving Cardiac Action Potential Measurements: 2D and 3D Cell Culture. J Bioengineer & Biomedical Sci 5: 168. doi:10.4172/2155- 9538.1000168

culture into a compact tissue and resume spontaneous contractions in unison.

Loading cells and tissues with optical sensors

Cells or tissues were incubated at 37°C with FluoVolt™ (Molecular Probes/Thermo Fisher Scientific, Waltham, MA,1:500 dilution with Powerload™) and FLIPR 6 (Molecular Devices, Sunnyvale, CA, 1:1 dilution) a potentiometric and calcium indicator dye respectively for ~45-60 min in Advanced RPMI (Thermo Fisher Scientific, Waltham, MA). The cells or tissues were washed 2x with Advanced RPMI. The optical signals were stabilized for recording spontaneous contraction after incubation for an additional 15-30 min at 37°C.

Fluorescence measurements

Fluorescence signals were recorded at 0.01 second intervals for 10 seconds from stained cells or tissues using the microplate reader CLARIOstar® (BMG LABTECH, Cary, NC) using the linear variable filter (LVF) monochromator (excitation/emission, 482-16/530-40 nm) or the addition of filters(excitation/emission,482-16/530-40 nm). The samples were pre incubated at 37°C in the plate reader chamber before and during fluorescence measurements. Signals from different sample types (cell and tissue) and filter system were normalized to background recordings of wells filled with Advanced RPMI with phenol red. The same cell culture media was used during the recording for cells and tissues.

Data analysis

Custom software developed using MATLAB®(MathWorks, Natik, MA)computed an ensemble average of multiple cardiac action potential and calcium transient profiles. The software calculates amplitudes and duration of action potential and calcium transient.

Results

First, we demonstrated the effects of sensitivity of the optical detection system on signal to noise ratios for measuring action potential of cardiomyocytes with the ultra-fast microplate reader. The monochromator based optical detection system is advantageous for flexibly adjusting excitation and emission wavelength for various fluorophores with different optical properties. However, some of the photons may not reach the photoreceptors. By using a traditional filter system, the signal to noise ratio was improved to analyze action potential profiles (Figure 1). We saw a vast reduction in the background noise from the fluorescence signal when the fluorescence readings were taken with dedicated filters (Figure 1B) as compared to a monochromator based approach at the same excitation and emission wavelengths (Figure 1A). The amplitude of the signal was increased by ~2-fold when filters were used.

Secondly, we demonstrated the effects of changing the amount of optical probes within the focal volume of the optical detection system. To change the number of dye-loaded cells within the focal volume, the density of cardiomyocytes cultivated on cell culture plates was varied. An increasing number of cells (10 K, 20 K, 30 K, and 40 K cells per well in a half area 96-well plate) was cultured until they formed a spontaneously contracting layer (~7-12 days after seeding) (Figure 2). The amplitude of the action potential increased dramatically from 10 K cells per well (Figure 2A) at the lowest signal that is barely detectable to fully resolved action potential recordings at 40 K cells per well (Figure 2D). Although the changes in action potential fluorescence increased dramatically at the highest seeding densities (40 K cells/well, Figure

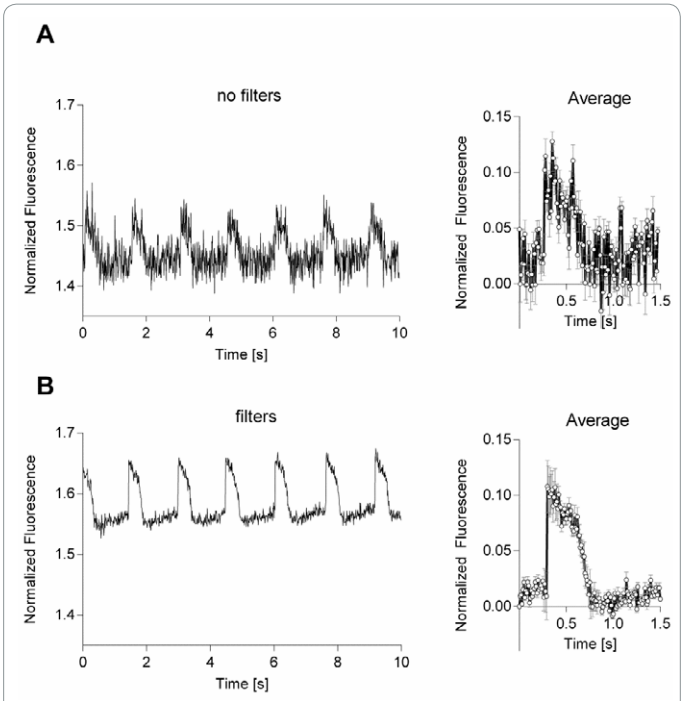

Figure 1: Measurement of Action Potentials of 2D Cardiomyocyte Cultures with an LVF Monochromator or Optical Filters.

A) Action potentials from 2D monolayer cardiomyocyte culture using the CLARIOstar® with LVF Monochromator without filters. Average trace on the right with error bars (SEM). B) Action potentials measured from 2D monolayer cardiomyocyte culture using the CLARIOstar® with an excitation filter at 482-16 nm and emission filter at 530-40 nm with a long-pass dichroic filter at 504 nm. Average trace on the right with error bars (SEM).

2D) as compared to the lowest density (10 K cells/well, Figure 2A), the seeding density does not drastically improve the background noise in the signal seen with the addition of filters. The overall fluorescence signal increased as well with the higher seeding densities from 1.30 to 1.56 normalized fluorescence units. This is due to the increased number of cells stained with the dye in the well.

Next, we demonstrated effects of using optical sensors with higher sensitivity than FluoVolt on signal to noise ratios for analyzing profiles of calcium transients. By seeding increasing numbers of cardiomyocytes (10 K, 20 K, 30 K and 40 K cells into wells of a half area 96-well plate), effects of varying the amount of optical sensor within a detection volume on signal to noise ratios were demonstrated (Figure 2E-H). At 10 K cells/well (Figure 2E), the signal did not exhibit typical periodic Ca^{2+}-transients in cardiomyocytes. At seeding densities at or above 20 K cells/well (Figures 2F-2H), typical Ca^{2+}-transients in contracting CMs were immediately visible; indicating a requirement of a minimum number of cells for detecting synchronous Ca^{2+}-transients. Increasing the cell seeding density to 20 K – 40 K cells per well did not significantly improve the signal. In addition, frequency of contractions seemed lower at the higher numbers of seeded cardiomyocytes (40 K cells/well, Figure 2H) than those of mid-range densities (20 K and 30 K cells/well, Figures 2F and 2G). This may indicate that seeding density can influence electrophysiological properties of cardiomyocytes.

Lastly, we demonstrated that the amount of optical sensor within a focal volume can be increased by culturing cardiomyocytes in three dimensional (3D) tissue constructs. The frequency of contractions between monolayer cells and cardiac tissue constructs was similar,

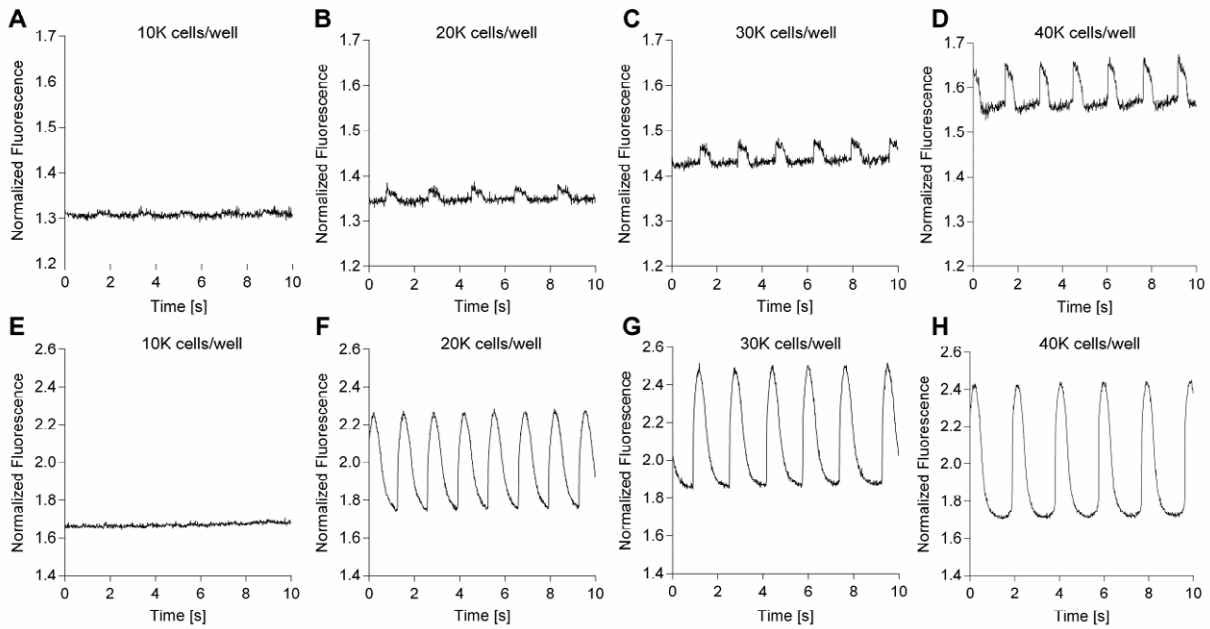

Figure 2: Measurement of Action Potentials and Calcium Transients with Different Densities of Cardiomyocytes Cultures.

Action potential recordings from cardiomyocytes seeded at (A) 10K cells/well, (B) 20K cells/well, (C) 30K cells/well, and (D) 40K cells/well. Calcium transient recordings from cardiomyocytes seeded at (E) 10K cells/well, (F) 20K cells/well, (G) 30K cells/well, and (H) 40K cells/well.

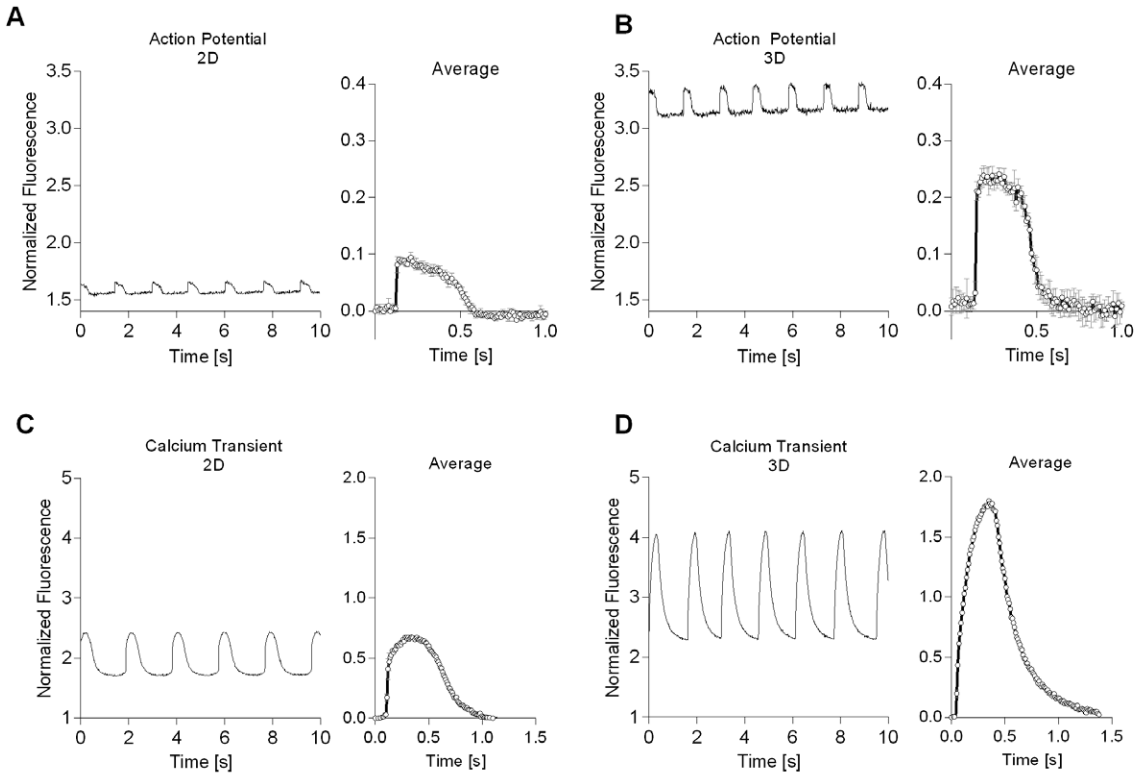

Figure 3: Comparison of Action Potential and Calcium Transients in 2D and 3D Cultures.

(A) Action potentials recorded from 2D cardiomyocyte monolayer with 40K cells/well with average trace shown on the right with error bars (SD) (B) Action potentials recorded from 3D EHT with average trace shown on the right with error bars (SD). (C) Calcium transients recorded from 2D cardiomyocyte monolayer with 40K cells/well with the average trace shown to the right with error bars (SEM)(D) Calcium transients recorded from 3D EHT with the average trace shown on the right with error bars (SEM).

but signals indicating action potentials and calcium transients were dramatically increased in 3D cardiac tissue constructs (Figures 3B and 3D) as compared to 2D monolayer cell cultures (Figures 3A and 3C). As previously demonstrated using 3D tissue constructs culture [8] detection of biological optical sensors can be improved as compared to a system using 2D cell cultures.

Discussion

Signal to noise ratio improvement strategy

The recently developed potentiometric optical sensor has better sensitivity than those used traditionally. Here, we demonstrated three ways to improve signal to noise ratio so one can analyze changes in profiles in response to drug treatments for cardiac safety analysis and efficacy of ion channel regulators that may have clinical potential [9]. The three methods include;

1. Improving sensitivity of instruments,
2. Increasing the dye amount within the focal volume by

a. increasing the number of cells in 2D cell culture,

b. using 3D tissue constructs.

When 3D EHTs were used to measure action potential, even with limited sensitivity of the potentiometic dye, we were able to analyze shapes of the action potential profiles. Alternatively, measurements of Ca^{2+} transient can be performed to obtain similar information since AP and Ca^{2+}-transient is connected intimately (i.e., excitation-contraction coupling) (Figures 4A and 4B).

Limitations

One of the potential limitations of detecting action potential dyes is their potential interference with the biochemical properties of cells.

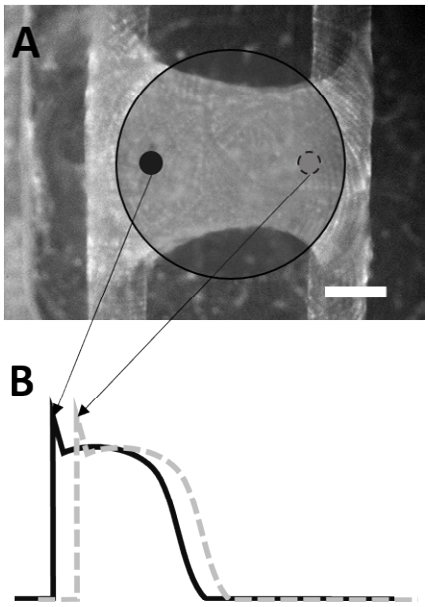

Figure 4: A Potential Artifact of Action Potential Profile.

(A) Engineered heart tissues loaded with FluoVolt and schematically illustrated two different spots within an area of optical detection illuminated by a diffused excitation focus. Bar = 1mm (B) Delayed action potential profiles from two spots in (A).

Different concentration of dye molecules can affect native membrane potential as well as potential interaction with test compounds. A solution to this issue is to validate a new method using dyes by a classical approach using electrodes [9]. As indicated we were able to improve signal to noise ratios of AP measurements by increasing amount of dye molecules within an optical focal volume. However, an optical detection system using a diffused focal volume collects photons from points that are spatially separated apart (Figure 4). Therefore, the detection system collects all the AP profiles from all the points of the focal volume but they could be delayed from one another. This could result in generating AP profiles not as sharp as expected. For instance, the detection could be less sensitive to detect a rapid depolarization (phase 0) of AP that is driven by a transient increase in fast-Na^+-channel conductance.

Summary

Despite the potential limitations in using optical dyes with diffused focus detection system to analyze action potential profiles, increasing sensitivity with various approaches will allow us to analyze effects of compounds on AP profiles in high-through put. By knowing the limitations, one can analyze data accordingly to draw a reasonable conclusion. Hence, the presented approaches are useful for a high-throughput compound screening.

Disclosure

The authors NJD, YY, PK, BI are at the time of development employees of InvivoSciences, Inc. which conceived of and developed the protocols described here. TW is the CSO for InvivoSciences Inc.

Acknowledgements

This work has been supported in part by NIH grants, R44GM087784, R43GM109735 and R01HL109505.

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