Non-invasive Video Image-based Analysis Method Coupled to Field Potential Recording for Evaluation of the Drug-induced Effect in Cardiac Tissue

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ABSTRACT

Reports on the use multi electrode arrays (MEA) systems for screening the electrophysiological effects of drug candidates on cardiac tissues as a response to the need of the pharmaceutical industry for a high-throughput screening method have increased in the past decades. Though the field potential (FP) measured in MEA have been reported coherent to the progression of the well-established progression of action potential (AP), certain pharmacological effects do not accurately reflect in the observed FP waveform alteration. Here, we revisit the imaging analysis algorithm developed in our lab based on the pixel intensity variation that can be utilized to improve the FP measurement and interpretation. Imaging analysis was done on videos recorded with a microscope camera at 30 Hz frame rate and 680 × 510 pixel resolution resulting to a relative lower system requirement compared to other methods. It is expected that combining our method with MEA systems can greatly aid in developing a screening platform for cardiovascular effect profiling of candidate drugs.

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1. Introduction

The framework on cardiac muscle signal propagation and contraction-relaxation motion has been studied intensively with the aim of, but not limited to, building a sound clinical prediction model to identify the factors that can pose possible risks to cardiac safety. It has been established that the electrical membrane potential of an excitatory cell, like most type of cardiac cells, rise and fall to activate intracellular processes and is commonly termed as action potential (AP).1

A series of depolarization phase, plateau phase, and repolarization phase are involved in the action potential that progresses over the course of 200–300 ms. When a non-pacemaker cardiomyocyte is significantly stimulated externally, the cell membrane potential raises from the resting potential (−90 mV) to a point that the voltage gated Na+ channel opens for about 0.1 ms producing a brief depolarization phase; Na+ entering the cell. The Na+ channel closes as soon as it opens for about 1 to 2 ms and transitions to a closed state that prevents the activation of the cell for a certain period (effective refractory period). Consequently, the cell is repolarized briefly due to the activation of a transient outward current (iTo) and concentration gradient brought by the efflux of K+. In addition, when the action potential upstroke reaches ~20 mV, L-type Ca2+ channels open and inactivate slowly providing a long lasting Ca2+ current. The influx of Ca2+ induces the release of Ca2+ from the sarcoplasmic reticulum and causes muscle contraction. The efflux of K+ counterbalances the influx of Ca2+ with the help of the delayed rectifier (iK) channels that are activated very slowly by the potential at the end of the depolarization phase. This gradual increase of conductance to K+ is responsible to the plateau phase. The duration of the plateau phase is determined partially by the distribution of the type of iK; iKs (slow activating) and iKr (rapid activating), that may vary from one cardiomyocyte to another. As the membrane potential becomes more negative and the Ca2+ channels are closed, the conductance to the inwardly rectifying K current (iK1) increases leading to the rapid depolarization phase. The delayed rectifier K+ channels close when the membrane potential is restored to about

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−85 to −90 mV, while iK1 remains conducting that sets the resting membrane potential. Within these phases, the ion concentration is restored through the Na+−Ca2+ exchange current and Na+−K+ pump current.2,3

The potassium ion channel therefore plays a salient role in the progression of AP, repolarization in particular, which is why for many years the protein hERG (human Ether-a-go-go related gene) also known as Kc.11.1 that is the alpha unit of a potassium ion channel was viewed relevant for drug-induced channel blockade test.4 However, measuring only one type of current for screening drug-induced effects for a range of channels is expected to give faulty results. An in vitro cardiac safety assay that can integrate more than only the hERG ion channel current is, therefore, a great concern of the pharmaceutical industry to achieve a better drug regulatory program.5,6

Intracellular recording of AP by patch clamp is the standard method; however, it is unsuitable for the rapid screening of chemical entities in the initial drug discovery phase since it demands adept technical conduct and is labor intensive and has low throughput.7 Extracellular recordings using multi-electrode arrays (MEAs) have gain a lot of attention and been utilized for a range of drug-induced response profiling.8−11 Having considerable amount of cells grown directly on the recording electrodes and the gap between electrode surface and cell surface is sufficiently small, the dynamic processes in the tissue at different positions in the array can be assessed.12 More importantly, the extracellular field potential recordings have been shown correlated to the progression of the cardiac action potential and the field potential duration (FPD) correlates closely with the QT-interval utilized in electrocardiogram (ECG) assessment.13 Though the relationship to arrhythmogenesis is not clear, the prolongation of the QT interval is considered a risk factor and an immediate concern for early identification in drug development.2

However, certain effects brought by the presence of some drugs do not accurately reflect in the observed field potential (FP) waveform alteration.7 The FPD does not reveal either the dynamics of different ion channels or the magnitude of the response as an effect of drug treatment. There was a difficulty in distinguishing repolarization wave prolongation for different drug concentration since the offset of the field potential cycle or initiation of repolarization phase could produce the same prolongation.14 In addition, the contraction-relaxation motion of the cardiomyocyte and the type and amount of the cell population in the tissue may affect the shape of the waveform recorded. It is highly suggested to conduct preliminary detection between electrodes to correctly assign the originating peacemaker and determine the propagation sequence.12 Still, due to the relatively big electrode spacing, FP does not allow an up-front determination of conduction velocity that can further lead to false interpretation of extrapolated data.

Thus, a careful consideration should be given to the interpretation of FP waveform. With improvements to the MEA systems and combination to other evaluation methods and systems, a better data analysis and profiling platform can be realized. In this report, we are revisiting the utility of the developed platform that uses video microscopy and image-analysis algorithm for total pixel intensities in derivative images together with MEA recordings. Video microscopy is a non-invasive and relatively easy method to investigate the contractile motion of the tissue or the cell that can be correlated to FP waveform for electrophysiological response-drug administration relationship. Unlike other video microscopy platforms;14−20 specifically block matching method, the effect of non-moving areas for determining the beat profile is not a constraint in this method. In addition, the utility of this platform is not limited by high speed camera and higher resolution requirement. Having a lower system requirement for data processing and storage, this platform is expected to be more accessible and applicable to major users and for the advancement of a sound interpretation of FP recordings.

2. Experimental Aspect

2.1 Cardiac cell culture

Mouse ES cells (mESCs) B6G2 (Riken cell bank, Ibaraki, Japan) were cultured at 37°C in an atmosphere of 5% CO2 on mitomycin C treated STO (ECACC, Salisbury, UK) feeder layer in culture medium. The culture medium consisted of DMEM high glucose (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 15% FBS (Gibco, Life Technologies, CA, USA), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin, 0.1 µM β-mercaptoethanol; purchased from Invitrogen (Life Technologies, CA, USA), and 1000 U/mL recombinant Leukaemia inhibitory factor (LIF, Chemicon, Merck Millipore, Darmstadt, Germany). STO cells (ECACC) were grown at 37°C in an atmosphere of 5% CO2. For STO cell culture, the medium containing DMEM high glucose supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin was used. As they reached confluent stage, STO cells were exposed to 10 μg/mL mitomycin C (Wako, Osaka, Japan) for 2.15 h. The mitomycin C-treated STO cells were washed in PBS and plated in gelatin-coated tissue culture dishes to form the feeder layer and incubated overnight before planting the ES cells on a separate dish.

After 2 days of mES cell culture on feeder cells, cells were treated with trypsin (0.05% Trypsin-EDTA, Invitrogen, Life Technologies, CA, USA) to prepare single mES cell suspension and then the number of cells was counted. To induce differentiation of mES cells, cells were cultivated into cellular clusters called embryoid body (EB) by hanging drop method where 1000 cells were cultured in each drop of 20 µL differentiation medium; components similar to mES cell medium without LIF. The drops were put on the inner side of the lid of a 100 mm culture dish (Iwaki, Tokyo, Japan) and the bottom of the dish was filled with an electrochemistry, 84(5), 283–289 (2016)

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autoclaved Milli-Q water to avoid medium dehydration. The cells were cultured under 5% CO₂ at 37°C for 5 days by hanging drop method until it grew up to EB cells. The sizes ranged from 100 μm and 500 μm.

EBs were transferred to 0.1% gelatine-coated MED probes (MED-P515A, Alpha MED Scientific Inc., Osaka, Japan) filled with 2 mL of differentiation medium and then cultured at 37°C in an atmosphere of 5% CO₂. Medium replacement was done every day. The EBs were cultured until they differentiated to stably contracting cardiomyocytes; ~12 days after transferring and plating.

2.2 MEA recording
Synchronized acquisition of the video recording of the multi-electrode array (MEA) recording were controlled by the MED64 system. Sampling frequency of 20 kHz and a bandwidth of 1–1000 Hz were set and were analyzed to detect and average (n = 10) the field potential duration from the Mobius software (Alpha MED Scientific Inc.).

2.3 Imaging of contraction-relaxation motion and analysis algorithm
A digital color CCD camera (DP71, Olympus, Japan) was mounted on the inverted microscope (IX71, Olympus, Japan). The microscope was equipped with a stage top mini-incubator (TOKAI HIT, Japan) which keeps the temperature of the culture dishes at 37°C. The video was recorded with a 10X objective lens at 30 fps with a 1000 Hz were set and were analyzed to detect and average (n = 10) the field potential duration from the Mobius software (Alpha MED Scientific Inc.).

2.4 Drug administration test
Drug test for verapamil (2–200 μM) was performed by comparing the contractile behavior of the cell before and after drug administration in correlation to the measured field potential as what was reported before. As reference value, the beat behavior of the mESC-CMs in 1.5 mL culture medium without drug was recorded for 2 min. Contractile colonies with beat interval ranging from 600 ms (100 bpm) to 2500 ms (24 bpm) were selected for drug effect test. The desired molar concentration of the drug was obtained by diluting it to the culture media; retaining the total volume of 1.5 mL. After 1 min, the beat behavior of the same colonies was recorded for 2 min. To bring the cells back to the original state, the old medium was aspirated and the cells were washed with fresh medium twice and then placed in the incubator for 10 min. The same cells were observed in the similar way for the same drug but with different concentration. A separate set of dish containing cardiac cells were tested for each selected drug. Stock solutions of the drugs were prepared by dissolving them in separate autoclaved Milli-Q water. Other drugs (all purchased from Wako) used and the corresponding final concentration are as follows: propranolol (0.3–30 μM), quinidine (2–200 μM), sotalol (1–100 μM), and acetylsalicylic acid (0.1–10 μM).

3. Results and Discussion
3.1 Field potential and contractile motion comparison
Figure 2A shows the selected area for field potential measurement. Reports have shown that the onset of the AP correlated well with the deviation of the FP from the baseline. The negative peak of the FP preceded the maximal depolarization of the AP ad the succeeding FP peak appeared to depend on the rise time of the AP. However, the onset of the FP varies from one electrode to another and could either be continuous and slow or a more rapid drop to a negative minimum which can be preceded by a positive pre-spike. This is for the fact that the FP waveform shape depends not only on local transmembrane current but also on passive circuit currents that are induced simultaneously by excited tissue connected to the recording site. The intercellular resistance may be unevenly distributed across the connected cells that is can result to regional differences in the waveforms. Thus, selecting the ideal electrode for measurement is, by itself, a biased method. For the sake of argument, the waveform depicted in Fig. 2B was located for the trials since this has been well documented that minimum FP peak corresponds to influx of Na⁺ and the second maximum peak corresponds to the efflux K⁺ brought by the increase in inwardly rectifying current (i_{K1}). A close comparison of the occurrence and interval of the signals generated were coherent in both field potential measurement (Fig. 2C) and total pixel intensity measurement (Fig. 2E). From Fig. 2D and Fig. 2E, it should be noted that the signal duration of the field potential (~400 ms) is shorter than the contractile motion (~540 ms). For drug screening safety, a 30–50 ms prolongation in the contraction-relaxation phase can be considered a significant risk. Thus, measurement of the variation of signal generation in response to administered drug using this platform may be utilized for such purpose.

The positive and negative pixel intensities of the derivative images corresponded respectively to the systolic and diastolic phases of beating. A local peak with small width and high intensities indicated a strong and well synchronized beating.

Unlike FP measurement and other video microscopy algorithm, this platform is not affected by the non-moving areas. Having the ability to ignore the non-moving areas in the derived images and include the entire field of vision for study makes the generated profile highly reliable. In fact, the method has been utilized for single cell analysis (Fig. 2G) with observable high signal to noise ratio (Fig. 2H) and without the need for any noise filtration.
Thus, the method is not significantly affected by the cell density. For FP readings, a relatively high density of cells is needed on the MEA electrode to produce waveforms with higher amplitudes and to have more electrodes significant for the reading which is a technical constraint that remains a problem.

3.2 Drug screening applicability of the combined methods

Figure 3 contains the reported utility of the combined methods. Figure 3A shows the contractile profile and field potential profile of the beating mESC-CM before and after verapamil (1 µM) administration. Before administration, the upstroke signal in the field potential that corresponds to the systolic or contraction process was uniform throughout the recording (max at ~0.4 mV). In contrast, the positive amplitudes of the beat motion which corresponds to the systolic process varies. This indicates that the movement of the cardiomyocytes is not uniform for each signal generation which cannot be reflected in the profile from MEA. In addition, the diastolic or relaxation process, which was undetectable in MEA measurement, was fluctuated in each beat. The electrophysiological and morphological observations were more distinguished after verapamil administration. Since verapamil is a Ca$^{2+}$ blocker, the action potential and, subsequently, the initiation of contraction will be delayed and reduced. Comparing the change in the measured field potential to the change in measured intensity counts, both had a ~50% decrease. This indicates the possible use of this method to quantify the Ca$^{2+}$ present. In addition, after administration of verapamil, the contractile motion profile showed that the diastolic process was about the same as that in the systolic process; indicating a uniform contraction-relaxation motion which also not reflected in the field potential measurement. Therefore, aside from generating the same beat occurrence, the profile generated with the microscopic imaging system provided more information than the profile generated by the MEA.

The pharmacological response of the cardiac tissue can be evaluated by measuring the change ratio (\%), defined as:

$$\text{change ratio} (%) = 100 \times \frac{(a - b)}{a}$$

where a and b are the beat intervals before and after drug administration, respectively. A positive change ratio indicates an increase in beat rate and a decrease in beat interval while a negative change ratio indicates a decrease in beat rate and an increase in beat interval. The observed change ratio for different concentrations of the administered drugs were plotted as seen in Fig. 3B–3C. The arrow represents the observed chronotropic effect based on the maximum concentration of administered drug where the direction signifies the type of chronotropic effect (downward is for negative or for decrease in beat rate and upward for otherwise) and the number and the length of the arrows represent the relative magnitude of the effect (2 arrows signify a chronotropic effect of ~100%).

Figure 3B shows the chronotropic effect profile of verapamil. Verapamil blocks L-type Ca$^{2+}$ channels which affects the Ca$^{2+}$-
induced and Ca\textsuperscript{2+} release mechanism manifested by the contraction-relaxation motion.\textsuperscript{16} This explains the observable progressive increase in beat interval with increased verapamil concentration which is expected to be a concentration-dependent inhibition.\textsuperscript{16,26,27}

Other negative chronotropic agents used aside from verapamil were quinidine (Na\textsuperscript{+} channel blocker), propranolol (\(\beta\)-adrenergic receptor blocker and Na\textsuperscript{+} channel blocker), and sotalol (\(\beta\)-adrenergic receptor blocker and K\textsuperscript{+} channel blocker). All are known to decrease the beat rate but have different modes of action associated to each drug. Based on Fig. 3C, the potency of the agents can be ranked accordingly. Altering the Ca\textsuperscript{2+} concentration in the cells proves to have the greatest chronotropic effect since verapamil showed to be the most potent (followed by propranolol, quinidine, and sotalol, in order). This is expected since Ca\textsuperscript{2+} release from the sarcoplasmic reticulum which underlies cardiac contraction in general is triggered and coordinated with the entry of Ca\textsuperscript{2+} from the cytoplasm.\textsuperscript{28}

As Na\textsuperscript{+} channel blocker, Quinidine affects the inward Na\textsuperscript{+} flux and the fast depolarization phase of cardiac action potential which delays the activation of K\textsuperscript{+} channels and Ca\textsuperscript{2+} channels. Thus, it prolongs the cardiac potential and subsequently prolongs the beat interval. Figure 3C shows that 200 µM of quinidine is required for a change ratio of \(\sim\)100% which is \(\sim\)200x higher than verapamil.

Propranolol, on the other hand, is a widely-used non-selective selective \(\beta\)-adrenergic receptor antagonist that is discovered to block Na\textsuperscript{+} channels.\textsuperscript{29} The decrease in beat rate is expected since \(\beta\)-adrenergic receptor blocker prevents the ligands to bind and, in effect, the G-protein complex associated with the receptor cannot activate the production of cyclic adenosine monophosphate responsible for turning on the calcium inflow channels.\textsuperscript{30,31} Adding this to Na\textsuperscript{+} blocking channel effect, \(\sim\)86% change ratio at 30 µM of propranolol which is more potent than quinidine as a negative chronotropic agent should be projected (Fig. 3C). However, concentration-dependent inhibition was not observed at a concentration below 3 µM which may indicate a different drug pathway mechanism.

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**Figure 2.** (Color online) Signal profile generation. (A) Cardiac tissue on MEA and selected electrode for FP measurement. (B) Cross section view of the MEA set up and the expected FP waveform. (C) Measured field potential on selected electrode and (D) single signal observed. (E) Contraction-relaxation motion based on derived imaged pixel intensity and (F) single signal observed. (G) Single neonatal cardiomyocyte and (H) observed beat profile for the rod shaped cell.
Another non-selective β-adrenergic receptor blocker agent administered was sotalol that has $K^+$ channel blocking ability. A decrease in beat rate is due to the prolong repolarization phase of the cardiac potential and delay in relaxation of the cell since $K^+$ is related to the resting potential of the cell. Concentration-dependent inhibition was observed but at 100 µM, it only has a change ratio of ~45%. Sotalol is more potent at slower beat rates and binds to channels better at slower rates of activation. Thus, at a normal or higher beat rate, this is the least potent among the negative chronotropic drugs administered.

The results show the applicability of the method to quantitatively profile the administered drugs. In addition, this analytical method made it possible to quantify the beat rate at any place where differentiated EB is located, which cannot be achieved by conventional electrophysiological method. More importantly the coherent response of the measured field potential and contraction-relaxation motion proves the claim the technique can be utilized in improving the analysis of obtained data.

4. Conclusion

Here we report a non-invasive video image-based analysis method as a potential aid to MEA measurements to evaluate the drug-induced effect in cardiac tissue. The beating frequency, strength of beating, and number of distinct beating patches can be obtained which can be utilized for preliminary detection of signal propagation in the cardiac tissue. This is beneficial in easily assigning the electrodes for observation and avoidance of excessive production of toxic gases and pH alteration due to Faradaic interactions. With these findings, it is expected that this approach can be helpful in improving interpretation of FP waveform for in vitro profiling of compound entities in drug discovery and development study.

References


