Automated, Non-Invasive Characterization of Stem Cell-Derived Cardiomyocytes from Phase-Contrast Microscopy

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Abstract. Stem cell-derived cardiomyocytes hold tremendous potential for drug development and safety testing related to cardiovascular health. The characterization of cardiomyocytes is most commonly performed using electrophysiological systems, which are expensive, laborious to use, and may induce undesirable cellular response. Here, we present a new method for non-invasive characterization of cardiomyocytes using video microscopy and image analysis. We describe an automated pipeline that consists of segmentation of beating regions, robust beating signal calculation, signal quantification and modeling, and hierarchical clustering. Unlike previous imaging-based methods, our approach enables clinical applications by capturing beating patterns and arrhythmias across healthy and diseased cells with varied densities. We demonstrate the strengths of our algorithm by characterizing the effects of two commercial drugs known to modulate beating frequency and irregularity. Our results provide, to our knowledge, the first clinically-relevant demonstration of a fully-automated and non-invasive imaging-based beating assay for characterization of stem cell-derived cardiomyocytes.

1 Introduction

Stem cell research holds enormous potential for studying and treating a wide range of human diseases [1]. In recent years, there has been significant progress in using induced pluripotent stem cells (iPSCs) for modeling of human disease. A promising and growing application of iPSCs is the generation of patient-specific cardiomyocytes, which can be used in preclinical testing of new drugs that may cause drug-induced arrhythmia or QT prolongation, as well as post-market safety testing or re-purposing of existing drugs [2, 3]. Due to their important clinical applications, beating characterization of stem-cell derived cardiomyocytes is of great interest.

The characterization of iPSC-derived cardiomyocytes is most commonly performed using electrophysiological signals measured through manual or automated patch-clamp systems as well as micro-electrode arrays (MEA)s [4]. While considered the gold-standard for characterization, patch-clamp methods are expensive, laborious and invasive. MEA systems also require high cell plating density, and due to the direct contact between cells and electrodes, may cause undesirable cellular response.

Several image analysis methods have recently been proposed [5–7] using the apparent cell motion, captured by video microscopy, as an alternative to directly measuring the electrophysiology of the cells. These methods have demonstrated the feasibility of non-invasive characterization of cardiomyocyte beating. In [5] and [6] a motion

P. Golland et al. (Eds.): MICCAI 2014, Part I, LNCS 8673, pp. 57–64, 2014.

field is estimated from the intensity images and then a single one-dimensional time-domain signal that captures the essential feature of the beating of the cardiomyocytes is constructed. Motion field estimation, however, is not accurate when the culture lacks enough texture, or contractions are very strong [7]. Moreover, constructing a meaningful temporal beating signal from the full motion field is impossible unless the cell density is high and uniform across the culture. To address these problems, in a subsequent work [7], an alternative approach has been proposed where images are first segmented into a set of regions, each representing a group of cells that beat together, and a set of features are calculated for each region. Their nearly periodic motion feature, however, is based on an explicit assumption that the beating pattern of each region is periodic or nearly periodic, hence limiting the applicability of the method in studying irregularities of cell beating and arrhythmia.

Although the above works show promising results, in order to successfully capture dynamics of cardiomyocyte beating from microscopy images in clinical setting, several challenges need to be addressed. The method should be able to handle variations in the culture density, ranging from sparse single-cell plating to dense monolayer plating, and work for both healthy and diseased cell lines as well as cell cultures treated by drugs. The plating density of cells affects the synchronicity of beating; densely-plated cells usually beat synchronously, while sparsely plated cells can beat asynchronously and with different frequencies for each cell. In addition, the beating pattern for healthy cardiomyocytes is nearly-periodic (but not perfectly periodic), while the beating pattern for unhealthy or perturbed cells can be highly irregular, and with varying contraction forces. Finally, the cardiomyocyte population is not always pure, and can contain other types of cells that proliferate, thereby changing the behavior and appearance of the culture over time.

In this work, we present a new method that can reliably extract and quantify beating signals from cardiomyocyte cell cultures. Our presented pipeline enables, for the first time, automated extraction of quantitative parameters that are of interest in clinical research from cultures with different cell density and with either regular or irregular beating patterns. The robustness of the presented method has been confirmed by successful analysis of more than 500 videos from different cell lines, and culture conditions. As demonstrated in this paper, our method successfully captures the impact of chemical compounds on the beating rate.

2 Method

Figure 1 shows a block diagram depicting the steps of the proposed method. The input is a phase-contrast image sequence $\{\mathbf{I}_t\}_{t=1,\ldots,N}$, acquired with high capture frequency (e.g. 24 frames/sec) of a cardiomyocyte cell culture. Images are first segmented into regions that consist of cells that exhibit a cyclic motion (beating cells), regions that consist of cells that do not show a cyclic motion (non-beating cells), and background. The result is a mask, consisting of a group of regions, $R_m, m = 1, ..., M$, where each region represents cells that are spaced close to each other and beat at relatively same time. Next a beating signal is calculated for each region R_m. A raw beating signal $u_m(t)$ is constructed by calculating the correlation coefficient of subsequent frames over R_m. A

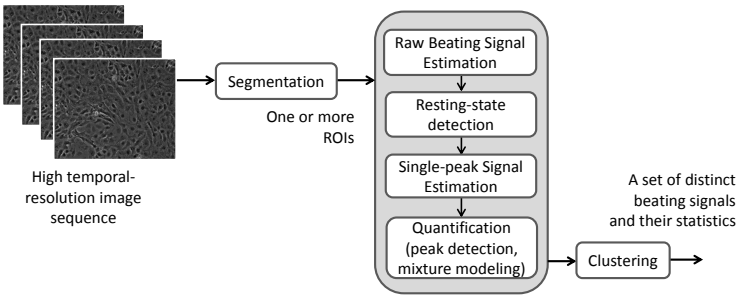

Fig. 1. Block diagram of the proposed method

resting-state reference image is obtained and then a single-peak beating signal $x_m(t)$ is calculated by computing the correlation coefficient of the frames and the resting-state reference image over R_m. Subsequently, features that describe the beating signal, such as its variation of period over time, are extracted. This is achieved by robust peak detection and signal modeling through fitting a mixture of Gaussians to the signal. Finally, a hierarchical clustering algorithm is performed to identify regions with unique beating characteristics and merge adjacent regions that have similar beating characteristics.

2.1 Segmentation

The first step in the proposed image analysis pipeline is segmentation of beating cells from the stationary regions of the image that include non-beating cells and the background. Since there is no difference between beating cells and non-beating cells in terms of their morphology and texture, and there is no clear boundary between the cells in the high density cell cultures, segmentation of individual beating cells is not feasible. Here, we construct a mask of beating regions defined as the intersection of a foreground mask, generated based on the first image in the sequence, and the standard deviation mask, generated over the entire image sequence. Since the cardiomyocyte cells could have a flat structure, the background estimation is done by computing the standard deviation map using a large window. As also proposed in [7], the standard deviation of the image intensity over time is calculated for each location of the image and the resulting standard deviation map is thresholded, followed by morphological operations to fill out holes and remove small segmented regions. The output is a set of M connected regions, $R_m, m = 1, ..., M$. Figure 2 shows two examples of cardiomyocyte image sequences, along with their corresponding computed standard deviation maps and final segmentation.

2.2 Beating Signal Estimation

We estimate a beating signal for each segmented region R_m. We make the assumption that disconnected regions in the image may have different beating signals but each

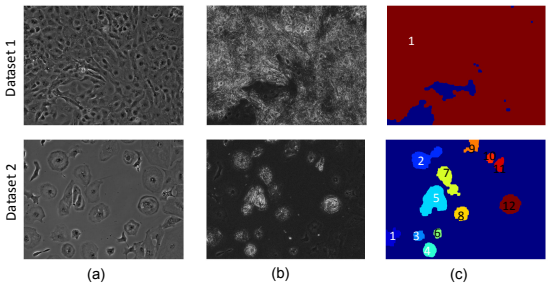

Fig. 2. An example of segmentation of beating regions for two datasets: (a) a frame of the raw image, (b) the corresponding temporal standard deviation map in log scale, and (c) binary segmentation of the beating regions. Top and bottom rows represent an example of high-density and low-density cell plating, respectively.

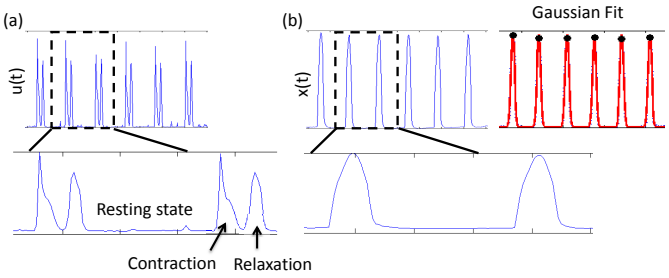

Fig. 3. An example of the beating signal extracted for one of the segmented regions: (a) raw (dual-peak) signal, $u(t)$, and (b) single-peak signal $x(t)$. A zoomed-in version of $u(t)$ and $x(t)$ are shown, along with the Gaussian mixture model fit to $x(t)$ and its peaks.

connected region has a single beating signal. This assumption has worked well in practice. An alternative, without affecting the rest of the pipeline, is to partition each connected region R_m to non-overlapping fixed-sized blocks, derive the beating signal for each block, and then cluster similar beating signals to generate a distinct set of beating signals.

For every image $\mathbf{I}_t$ and each segmented region R_m we form a one-dimensional vector of pixel intensities, denoted by $A_m(t)$. Starting from the second image in the sequence ($t = 2$), the correlation coefficient of intensity vectors of the image and its preceding image in the sequence is computed. We denote the beating signal calculated from the correlation of the successive images for each region R_m by $u_m(t)$, i.e.,

$$u_m(t) = 1 - \mathrm{corr}(A_m(t), A_m(t-1)). \tag{1}$$

where $A_m(t)$ and $A_m(t-1)$ are the intensity vectors corresponding to a segmented region R_m at frame t and $t-1$, respectively.

Figure 3 shows an example of a beating signal obtained for cardiomyocyte dataset 1. This raw signal typically exhibits three states: a resting-state, where the correlation

of successive images is high, a contraction state, and a relaxation state. Although the beating pattern and frequency can be measured from this signal, automatic identification of beating intervals is challenging due to the presence of double peaks and the lack of prior knowledge on their relative magnitude or distances. To obtain a single-peak signal, we first estimate a reference image by taking the median of resting-state images, identified as the frames that have small $u_m(t)$ and $du_m(t)/dt$. A single-peak signal is then generated by computing the correlation coefficient of the intensity vector of the reference image with those of all images in the sequence:

$$x_m(t) = 1 - \mathrm{corr}(B_m, A_m(t)), \tag{2}$$

where B_m is the intensity vector that corresponds to the resting state of the R_m. Figure 4 shows an example of the resulting signal $x_m(t)$.

2.3 Signal Quantification and Modeling

Once the beating signal is estimated, signal processing techniques in the time and/or frequency domains can be applied to calculate quantitative features that describe the beating signal. To capture irregularity and dynamics of beating over time, we perform the analysis in the time domain. We first identify the peaks of $x_m(t)$. A vector of estimated beating intervals τ_m is constructed by calculating the duration between successive peaks. We define the effective beating rate as $f_m = \mathrm{median}(\tau_m)$ and the irregularity of beating pattern as $f_m(\max(\tau_m) - \min(\tau_m))/2$. In addition to the beating intervals, it is important to measure the duration of each beat as well. For robust estimation of this parameter, we model the beating signal with a mixture of Gaussians, $\sum_i w_i \mathcal{N}(\mu_i, \sigma)$, where μ_i's coincide with the location of the extracted peaks and w_i's and σ's are estimated by minimizing the difference between the Gaussian mixture signal and $x_m(t)$. The duration of each beat is estimated by 6σ.

2.4 Clustering

The aim of clustering routine is to extract a distinct set of beating signals for the image sequence. The definition of "distinct" can be application-dependent: one might be interested in grouping the regions that beat at the same frequency but not necessarily in synchrony. Alternatively, in the case of low-density cell plating, it might of interest to identify groups of cells that are in close proximity and beat synchronously. Furthermore, since the number of clusters is unknown *a priori*, clustering approaches that require the number of clusters, such as k-means, are not suitable. Here, we propose a framework which is flexible to accommodate different applications. First, for each time-series signal, $x_m(t)$, we calculate a descriptor vector that contains the application-dependent parameters that will define similarity between the signals. Here, we use the effective beating rate and the time stamp of the first three peaks. To incorporate spatial information, we first calculate the pairwise distance between the regions, using a distance map for each region for efficient computation. Regions with the spatial distance smaller than a given threshold are identified as potential candidates for synchronous beating. We then calculate the pairwise distance between the descriptive vectors and feed these into

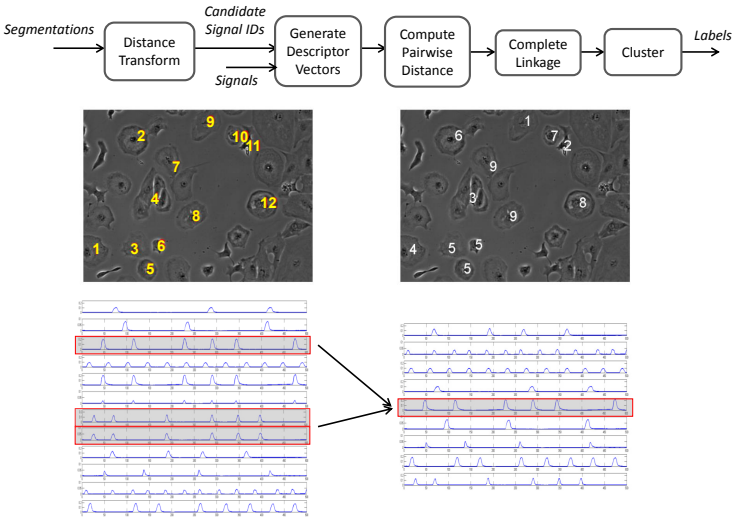

Fig. 4. Clustering of beating regions based on their spatial proximity and similarity of beating signal. The input to the clustering routine is a set of cell segmentations with associated beating signals, some of which may be synchronous, and the output is a set of clusters and associated signals with distinct beating profiles.

an agglomerative hierarchical clustering routine. Figure 4 shows a block diagram of the above flow along with an example of beating regions and their corresponding signals (left) and clustered regions and distinct beating signals (right). As can be seen, regions 3, 5, and 6 are grouped together (cluster 5 on the right) and regions 7 and 8 are grouped together (cluster 9 on the right). Identifying such clusters based on the synchrony of the beating signals provides an indication of the underlying physiological communication between the cells and is of interest for clinical studies.

3 Results

To assess the performance of the presented method, we performed a series of experiments using iPSC-derived cardiomyocytes obtained from commercial vendors. Cardiomyocytes were cultured in multi-well plates following standard culture protocols, with varied plating densities. Imaging data was collected using custom-built 10x phase-contrast microscopes, with capture frequency of 24 frames/sec and typical duration of 15 to 30 seconds. The images were saved in tiff format with 640x480 pixels. The microscopes were constructed using off-the-shelf components, including high-precision multi-well plate scanners, and were configured to work with stage-top incubators that provide precise environmental control during imaging.

We collected and analyzed more than 500 videos of cardiomyocyte cultures from different lines, plated with varied cell culture densities. We observed variation

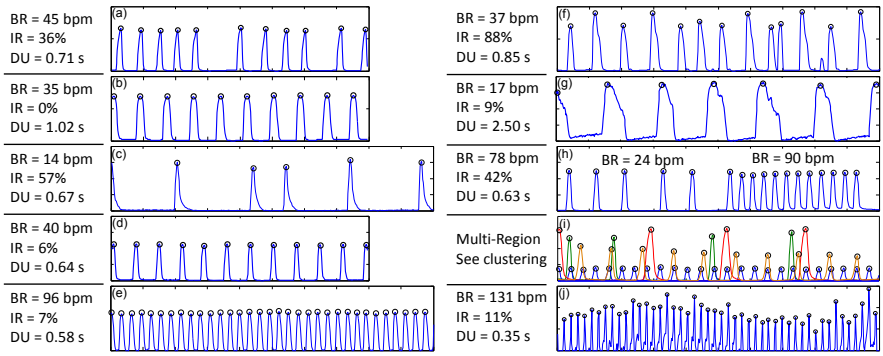

Fig. 5. Examples of estimated beating signals. Effective beat rate (BR), a measure of beating irregularity (IR), and the average beat duration (DU) are automatically measured for each sequence. Signals are extracted from varied cell cultures: Diseased lines (a,h), controls (b,d), after addition of a compound (f), single-cell plating (c, g, i), after media change (e,j).

of beating characteristics over time, after media changes, and with addition of chemical compounds. We used a subset of the videos to experimentally tune parameters such as thresholds for segmentation, peak detection, and cutoff distance in clustering, and the parameters were then fixed for all of our analysis. Figure 5 shows a sample set of beating signals with different profiles, detected and quantified automatically using our method. We confirmed the accuracy of our beating frequency measurements by comparing them to manually derived values from the captured videos. We rendered a movie for each dataset at the frame rate of image capture. A person watched the movie to confirm the number of beating regions and frequencies.

In order to show that our method can characterize the cellular response from different drugs, we performed a set of controlled experiments using Cisapride (a gastrointestinal drug withdrawn from market due to risk of induced arrhythmias) and Norepinephrine (a neurotransmitter used to increase blood pressure and heart rate) applied

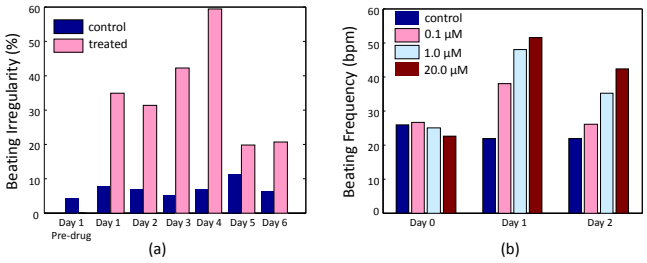

Fig. 6. Characterizing the effect of different compounds: (a) changes in beating irregularity due to addition of Cisapride measured over 6 days, and (b) changes in beating frequency due to addition of Norepinephrine measured over 2 days

to high-confluency cell cultures. As shown in Fig. 6, we measured an average 3-fold increase of beating irregularity with Cisapride-treated cells compared with the controls, observed over 6 days, as well as a dose-dependent increase of beating frequency for Norepinephrine-treated cells, observed over 2 days. These results provide, to our knowledge, the first clinically-relevant demonstration of a fully-automated and non-invasive imaging-based beating assay for characterization of iPSC-derived cardiomyocytes.

4 Conclusion

We presented a new method for non-invasive characterization of stem cell-derived cardiomyocytes using video microscopy and image intensity-based analysis. We described a novel image analysis pipeline for beating signal analysis that enables automated extraction of quantitative parameters that are of interest in clinical research. Our technique accommodates cultures with different cell density and with either regular or irregular beating patterns. We used our method to characterize the effects of two commercial drugs known to modulate beating frequency and irregularity, and showed results for successfully measuring a dose-dependent response. The presented method uses correlation coefficient of images to capture the change in their signal intensity, a simple, yet effective approach in combination with the rest of proposed pipeline in estimating the parameters of interest from varied cardiomyocyte cell cultures. Our future work is focused on estimating additional parameters of interest such as contraction strength, pattern, and accurate shape modeling of the beats.

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