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Multilevel analysis of calcium dynamics in stimulated cultures of cardiomyocytes

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Abstract— **We present an automatic method to characterize calcium activity in a culture of cardiac cells from a sequence of microscopy fluorescence images. The approach quantifies both the response of each individual cell in the culture to external field stimulation and the propagation properties of calcium wave-fronts, thus providing complementary information at different physiological levels. The technique classifies the response of each cell as regular or irregular based on a set of dynamical and morphological features of the calcium transients. Isochronal maps were constructed from the local activation times across the culture, and the front propagation was classified as planar or non-planar. The method has been applied to a set of 35 experiments, and the results indicate a significative connection between irregular behavior at the single-cell level and irregular front propagation.**

I. INTRODUCTION

An increasing number of studies are conducted in order to establish relations between pathological conditions and physiological activity at the cellular level. Such translational research requires interdisciplinary approaches combining knowledge and methods from different fields. In functional cell physiology, for instance, most of the information at the cellular level is obtained by means of cell imaging techniques [1]. Novel image processing methods are hence needed in order to analyze, quantify and classify the spatial and temporal patterns observed.

One of the most active research fields in modern cardiac physiology is the study of calcium regulation in cardiac myocytes. Indeed, calcium fluxes are known to be responsible for heart contraction and constitute one of the most relevant mechanisms underlying the regulation of cardiac function and disease [2].

In the past few years, fluorescence microscopy methods have experienced a considerable improvement in both spatial and temporal resolution [1]. Thus, it is now possible to capture images of a cell culture containing hundreds of cells with sufficient spatial and temporal resolution to resolve the calcium signal for each individual cell at frame rates of 20-100Hz. Such experimental methods allow a simultaneous recording of the overall calcium activity of the culture and the response of the individual cells to external electrical stimulation. Other experimental methods such as optical mapping techniques capture the spatial and temporal behavior of cell cultures or perfused hearts but do not offer the possibility of identifying each of the individual cells within the image field.

The purpose of this work is to present an automatic image and data analysis method that processes a sequence of fluorescence images of stimulated excitable cell cultures and identifies the spatiotemporal dynamics exhibited. The main contribution of our approach is that it characterizes the response of the culture to external electrical stimulation at both the single cell and the whole culture levels. To our knowledge, no previous studies of such automatized multi-scale approach have been reported in the literature. On one hand, the method is capable of characterizing the dynamical response of each individual cell in the culture to the external electrical stimulation. On the other, the approach determines the relevant properties of the propagating front such as velocity, orientation and curvature. The set of attributes provided by the method allows further understanding of the relation between irregular response behavior at the cell level and irregular front propagation at the multicellular level. This connection of different scales is particularly interesting in cardiac tissue, since cells with an irregular calcium handling could induce conduction problems characteristic of arrhythmia.

At the cellular level, the main aim is to distinguish each individual cell in the culture and to classify its physiological response into clinically relevant regimes such as alternans, phase-locking oscillations or irregular patterns. The classification of the calcium dynamics into different groups allows characterizing the response of each cell as either regular or irregular. In this study, we refer to regular cell behavior when the cell responds to each pulse of the external stimulation. We have included in this group the cases in which the cell exhibits an alternating pattern because in such a scenario the cell still responds to each external stimulation. Dynamical regimes such as the occurrence of spontaneous intracellular waves are considered as irregular since the presence of the wave inhibits the response to external stimulation. At the multicellular level, the most relevant information is whether the propagation of the calcium wave across the cell culture occurs in a planar, homogeneous wave or in an irregular, non-planar process. Similarly, we have classified this propagation as either planar or non-planar by considering planar those fronts that do not change significantly the angle of propagation. Propagation events with a significant change in propagation angle such as front breakdown or spiral waves are considered as non-planar.

The paper is organized as follows: In Section II we provide a detailed description of the experimental data used

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in this study and the image and data processing methods. In Section III we report the main results of the study, which establishes a relation between the occurrence of irregular calcium dynamics at the single cell level and the presence of irregular front propagation at the multicellular level.

II. MATERIALS AND METHODS

A. Experimental data

A total of 35 cultures of the murine atrial cell line HL-1 were used for calcium imaging. Each image field contained from 500 to 1000 cells. Each culture dish was submitted to electrical field stimulation using two field electrodes located outside, but parallel to the border of the imaging field. Images were recorded using a confocal resonance scanning confocal microscope or with a conventional fluorescent microscope equipped with a high-speed camera in order. This allowed capturing calcium activity in 512×512 images with a spatial resolution of $1.8\mu m$ at a frame rate of 40 ms, resulting in a set of around 5000 frames per experiment. The stimulation protocol consisted in seven stages, each with a duration of approximately 20s. A first period with no stimulation was followed by 5 periods at increasing stimulation rates of 0.4 Hz, 0.5 Hz, 0.6 Hz, 1 Hz and 1.3 Hz. Finally, the culture was rested for 10 seconds and then stimulated at the initial rate of 0.4 Hz.

B. Image processing and data analysis

The method consists of three main stages, namely cell segmentation, characterization of single cell calcium dynamics and characterization of the front propagation. The general procedure is represented in Figure 1 and each of the stages are described in detail in the following paragraphs. All the data analysis and image processing was implemented using MATLAB (The Mathworks, Boston, MA).

1) Cell Segmentation: First, a cell segmentation procedure was applied to the set of fluorescence images in order to identify the extent of each of the cells in the culture. Our segmentation method is based in the fact that pixels belonging to a location where a cell is present have a higher time variability in the fluorescence signal than pixels within the intercellular region. A variability image is obtained by computing the standard deviation of each pixel in the image. A modified watershed algorithm is then applied to the pixel variability image in order to locate and label each cell in the culture. The standard watershed is modified by setting multiple levels for the starting values of the local extrema and by setting a size stopping rule of the typical cell size. We have compared the performance of our watershed method against a standard biomedical segmentation software CellProfiler [3], always showing a better qualitative performance after exploring different ranges of parameters.

2) Analysis of single cell dynamics: Once all the cells have been identified, a supervised classification algorithm determines the response of each cell as either regular or irregular. Irregular behavior is defined as a situation in which the cell does not respond to stimulation pulses. To this extent, we compute the mean fluorescence of each cell

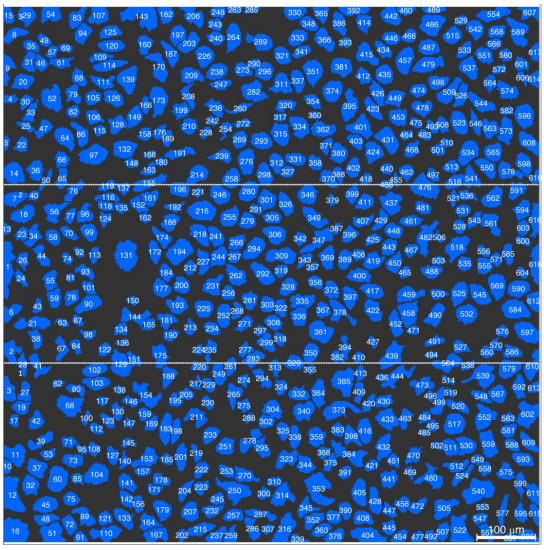


Fig. 2: Segmentation of the cells in the culture.

at frame t, $F(t)$. Then we define the normalized signal $s(t) = (F(t) - F_0)/F_0$, where F_0 is the average baseline value computed as the 1st 10-quantile (lower 10%) of the signal in the section of the experiment where no stimulation is applied. For each experiment, $s(t)$ consists of a series of calcium transients in response to the electrical stimulation, as shown in Figure 3.

Feature extraction: A set of 8 signal attributes were obtained from the signal of each cell $s(t)$: coefficient of variation of the time intervals between signal peaks, ratio between the actual stimulation period and the average time intervals between peaks, fraction of times in which three consecutive peaks alternate its amplitude, location of the maximum value of the Continuous Wavelet Transform of the signal at different scales (Gaussian family, scales from 0.2s to the signal duration), maximum amplitude, mean of the signal, standard deviation of the peak amplitudes and mean baseline level of the signal. In order to classify the cell signals from the set of attributes listed above we use a Random Forest algorithm, an ensemble classifier based on decision trees [4]. The classifier performs a greedy evaluation of the classification attributes and classifies the dynamical response of the cell as regular response (one peak for each stimulation), or irregular (including no correlation between stimulation times and peak locations, presence of waves, absence of response at particular stimulation time and complete absence of cell response). In order to train the classifier we used a set of 509 signals representing all the dynamical regimes.

3) Culture dynamics and Calcium front propagation: In this section we aim to characterize the behavior of the culture as a whole by measuring the propagation velocity of each front as it travels across the culture. We first identify the activation time of each front using the total culture fluorescence signal $SC(t)$. This signal is filtered using a band-pass filter for the stimulation frequencies in the experiment

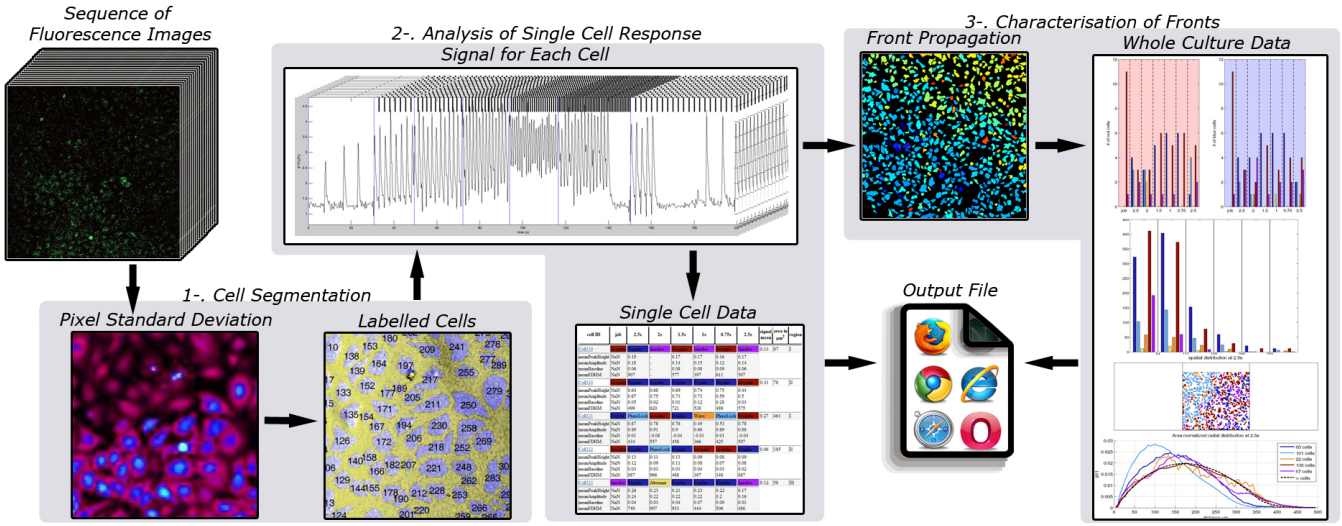


Fig. 1: General data flow of the analysis method: 1. Cell segmentation, 2. Analysis of single cell responses and 3. Characterization of front propagation events.

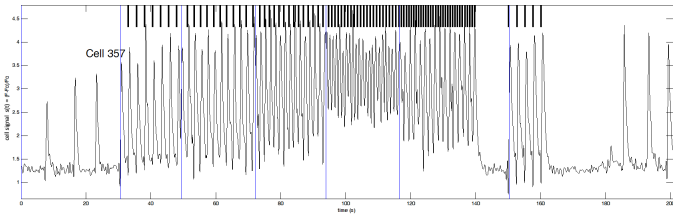
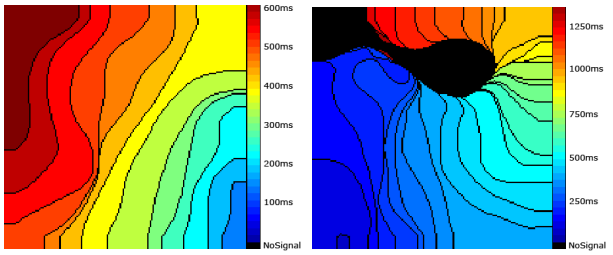


Fig. 3: Fluorescence signal for a representative cell in the culture during the seven stages of the experimental protocol. Stimulation times are marked as ticks at the top.



(a) (b)

Fig. 4: Isochronal maps of two propagation events classified as planar (left) and non-planar (right). The front propagation angle at each frame has been used in order to automatically classify each experiment as either planar or non-planar.

and the maxima and minima of $SC(t)$ are located by finding the frames at which its derivative changes its sign. To characterize front propagation, each of the images in the sequence $I(t)$ is divided into a $N \times N$ squared grid. N is chosen in such a way that each square in the grid fits at least two cells of the culture. Then we construct a filtered image $X(t)$ of size $N \times N$ in which each element i, j corresponds to the fraction of pixels from the original image $I(t)$ that belong to a cell and are inside the grid region i, j that eventually take values over 50% of the maximal value of the signal $s(t)$. Therefore, the image $X(t)$ is reflects the local fluorescence intensity within the squared grid normalized to the local potential maximal fluorescence. The last step is to determine the front location at each frame by binarizing the image $X(t)$ using a heuristic threshold (in our case, 0.4). The binarized image is also used in order to generate isochronal maps of the front propagation (Figure 4). Front propagation velocity is then measured by computing the time gap between two neighboring isochrones in the perpendicular direction of the front. Similarly, the local orientation of the velocity vector can be determined by using the angle of the perpendicular lines to the isochrones. We finally classify the front as either planar or non-planar depending if the evolution of the angular velocity is above or below 125 degrees/s.

III. RESULTS

We analyzed a total of 210 experimental regimes corresponding to the six stimulation regimes of the experimental protocol (0.4, 0.5, 0.6, 1, 1.3 and 0.4 Hz) and each of the 35 cultures in the study.

A. *Distribution of cell responses with pacing frequency*

Once all the cells had been segmented and the corresponding dynamics classified as either regular or irregular, we computed the area ratio for regulars and irregulars cells in the culture. This ratio is defined as the sum of areas of all the cells classified into a particular class (regular or irregular) over the sum of areas of all cells in the culture. In each experiment we computed the area ratio for both regular and irregular responses for each of the pacing frequencies. As it can be observed in Figure 5, the area corresponding to irregularly responding cells increased linearly with increasing pacing frequency (slope 0.77 Hz^{-1}, confidence interval [0.66 0.88]), and the area ratio of cells exhibiting a regular calcium dynamics decreased linearly with stimulation frequency. The

result clearly indicates that at high stimulation frequencies some cells start exhibiting abnormal calcium dynamics.

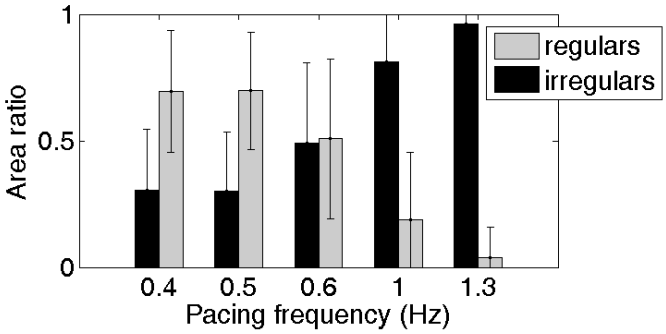


Fig. 5: The area extension of irregularly responding cells increased with pacing frequency, the area ratio of regular cells decreased with pacing frequency.

B. Distribution of fronts with pacing frequency

For each culture and stimulation frequency, each front was classified into either planar or non-planar using the procedure described in section II-B.3. Figure 6 represents the percentage of planar and non-planar fronts observed at low (0.4-0.5 Hz) and high (1-1.3 Hz) stimulation regimes. Comparing the proportion of non-planar fronts at low and high frequency stimulation rates we observed a significant rise in the proportion of non-planar fronts at higher frequencies (Wilcoxon rank-sum test, $p < 0.001$). Equivalently, a decrease in the proportion of planar fronts at low stimulation frequencies can also be observed. The main result indicates that irregular front propagation is more likely to occur under severe stimulation conditions of the cell culture.

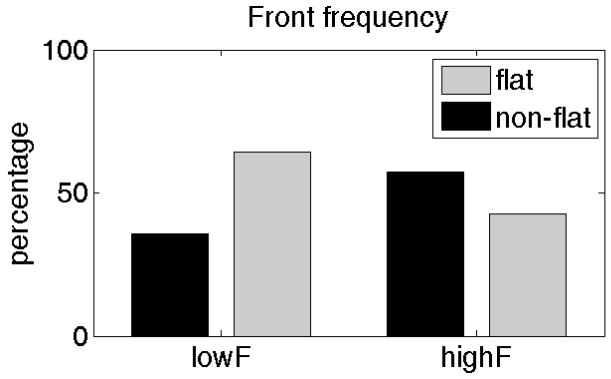


Fig. 6: Percentage of planar and non-planar propagation fronts at high and low stimulation frequencies.

C. Distribution of irregularly responding cells with front propagation

In order to establish a connection between the individual cellular calcium dynamics and the properties of the propagating fronts, we measured the area occupied by irregular cells in two cases: The regimes of the experimental protocol in which all fronts were planar and the cases in which at lease one front was non-planar. Figure 7 represents the boxplot of the comparison of the two measures, indicating that the presence of irregular cells is significantly higher in the pacing regimes presenting non-planar propagation behavior (Wilcoxon rank-sum test, $p < 0.001$). Consequently, our data show that the number of irregular cells is larger in the fronts that exhibit irregular propagation, hence providing a direct connection between the individual response of the cells and the propagation of calcium signal along the culture.

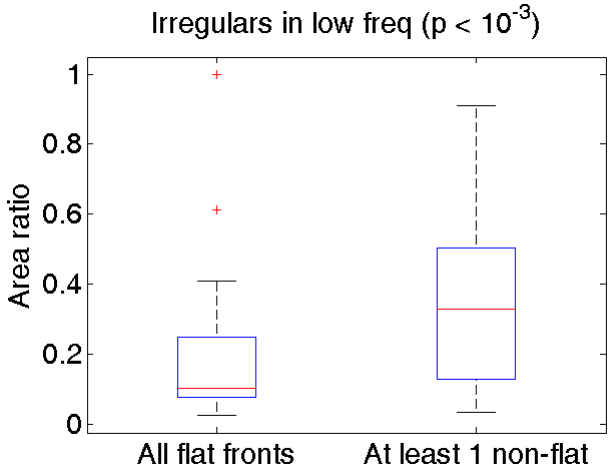


Fig. 7: The area occupied by irregular responding cells is smaller in cases where all fronts were planar (regular) than in cases where at least one front was non-planar (irregular).

IV. CONCLUSIONS AND DISCUSSION

Our next goal was to take into account the spatial distribution of individual cell responses in order to study how regions of high density of irregular cells may affect the propagation as compared to regions with the same fraction of irregular cells scattered homogeneously throughout the culture. This may be of particular relevant to the understanding of the formation of spiral wave patterns in cardiac tissue. We are currently developing an application of the presented method in order to characterize signal propagation in cultures of neurons.

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