

# Identification of intracellular calcium dynamics in stimulated cardiomyocytes

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**Abstract**—We have developed an automatic method for the analysis and identification of dynamical regimes in intracellular calcium patterns from confocal calcium images. The method allows the identification of different dynamical patterns such as spatially concordant and discordant alternans, irregular behavior or phase-locking regimes such as period doubling or halving. The method can be applied to the analysis of different cardiac pathologies related to anomalies at the cellular level such as ventricular reentrant arrhythmias.

## I. INTRODUCTION

There is an increasing number of studies that aim to establish relations between clinical conditions and physiological activity at the cellular level. This kind of research requires an interdisciplinary approach that combines knowledge and methods from different fields. Since most of the information at the cellular level is obtained by means of cell imaging techniques, novel image processing methods are needed in order to analyze, quantify and classify spatial and temporal patterns observed in life science areas such as neuroscience or cardiology [1]. In this context, calcium imaging is particularly relevant because calcium dynamics is a cell regulatory mechanism that plays an important role in many cellular processes such as muscle activation, gene expression or fertilization [2], [3].

In this work we present an automatic image processing method to analyze confocal calcium images of isolated cardiac myocytes. Cardiac myocytes are heart muscle cells that exhibit a variety of dynamical patterns due to the intracellular calcium dynamics [3]. The spatial and temporal distribution of intracellular calcium in cardiac myocytes determines the excitation-contraction coupling of the myocardium and is therefore a basic mechanism underlying heart function [4]. In particular, it is well known that high-frequency pacing of ventricular myocytes leads to the emergence of complex spatiotemporal patterns in the distribution of the intracellular calcium. The apparition of these complex dynamical regimes is a consequence of the nonlinear interplay between different cellular  $\text{Ca}^{2+}$  control mechanisms [3], [5]. Irregular distribution of intracellular

calcium may cause anomalies in the heart function such as T-wave alternans, ventricular fibrillation or conduction problems [6]. In particular, previous studies have established an interrelation between ventricular fibrillation and an overload in the intracellular calcium [7]. Similarly, the presence of spatially discordant alternans, characterized by an out-of-phase activity in different regions of the cell, is known to be related to the apparition of lethal arrhythmias [8]–[10].

The purpose of this work is to present an analysis method that processes a sequence of fluorescence images of stimulated isolated myocytes and automatically identifies the spatiotemporal dynamics exhibited by the cell. The objective is to distinguish physiologically relevant regimes such as spatially concordant and discordant alternans, phase-locking oscillations or irregular patterns. The method uses a feature extraction technique that permits an effective characterization of the experimental sequence allowing for a robust identification of each regime. More specifically, an approach based in the Principal Component Analysis (PCA) is presented to detect the presence of spatial alternans in the experiment. Similar study that addresses this problem in the context of cardiac tissue patterns can be found in the recent literature [11].

The paper is organized as follows: In Section II we introduce the experimental data and provide a detailed description of the processing method. The main capabilities of the technique are described in Section III, where we evaluate its performance and report on several examples of the correct identification of different regimes. Finally, the potentialities of the method and an exposition of further improvements are discussed in Section IV.

## II. MATERIALS AND METHODS

### A. Data acquisition

A total of 22 atrial myocytes were loaded with  $2.5 \mu\text{M}$  fluo-4 for 15 minutes followed by wash and de-esterification for 30 minutes. The myocytes were stimulated intracellularly with an EPC-10 patch-clamp system (HEKA, Germany) as described in [12]. The sequences of confocal images were acquired at a frame rate of 100 Hz with a resonance scanning Leica SP5 AOBs confocal microscope. Ionic currents were recorded simultaneously with a HEKA EPC-10 amplifier.

Synchronization of confocal images and current recordings was achieved using a Leica DAQ box and HEKA patch-master software. Patch-master was used to design electro-

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physiological protocols and to generate triggers for confocal image acquisition and event marking in the stimulation protocols. Local and global changes in cytosolic  $\text{Ca}^{2+}$  levels were detected by quantifying fluo-4 fluorescence in selected regions of interest. The cardiomyocytes were analyzed at different stimulation rates with frequencies ranging from 0.25 to 2 Hz. This resulted in a set of 101 experimental sequences, each consisting in a sequence of  $N$  images of  $512 \times 140$  pixels with a physical pixel size of  $0.28\mu\text{m}$ . All the processing and analysis steps have been implemented in MATLAB<sup>TM</sup> (The Mathworks, Natick MA). The original fluorescence images (24-bit truecolor) are converted to grayscale intensity images by using a weighted sum of the R, G, and B components with weights  $[0.2989, 0.5870, 0.1140]$ . We refer to an experimental sequence of grayscale images as  $\{X_{ij}^k\}$ , where  $k = 1 \dots N$  indexes the frame in the sequence and  $i = 1 \dots N_x, j = 1 \dots N_y$  specify a particular pixel in the image. In order to avoid the presence of static heterogeneities in the spatial distribution of the fluorescence, each pixel is normalized by subtracting its time average activity in the experiment.

### B. Feature extraction

Fig. 1 describes the basic steps of the method, which includes feature extraction and classification. Feature extraction consists of two parts: On the one hand, we determine the temporal properties of the oscillations in the average fluorescence and its correspondence to the stimulation times. On the other hand, we analyze the experimental sequence in order to determine if the images present out-of-phase spatial heterogeneities. These two steps constitute a basis for peak detection and spatial analysis methods detailed below.

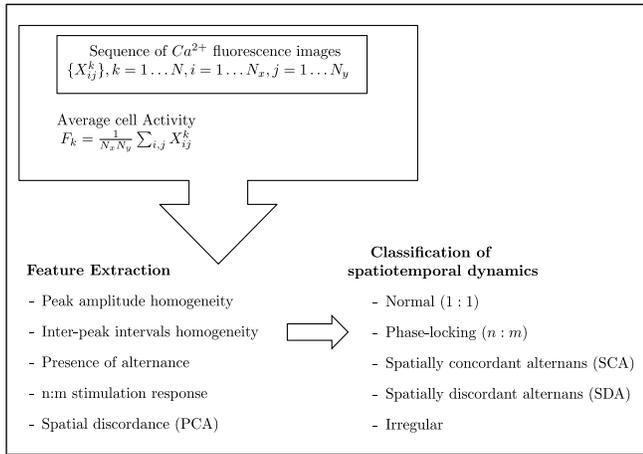


Fig. 1. Schematic description of the method.

1) *Peak detection:* We first compute the average fluorescence cell activity in each frame  $F_k = \sum_{i,j} X_{ij}^k / (N_x N_y), k = 1 \dots N$ , and we identify sequential pairs of local extrema corresponding to the peaks and valleys of  $F_k$ . We then compute the mean and standard deviation of the peaks amplitude  $m_a, \sigma_a$  and of the intervals between consecutive peaks  $m_i, \sigma_i$  (inter-peak intervals).

The distribution of amplitudes is considered homogeneous if the variability of the peaks  $\sigma_a$  is four times smaller than the noise in the signal  $\sigma^1$ . When the distribution of amplitudes is not homogeneous, alternating and irregular regimes are distinguished by testing for the presence of sustained oscillations in the peak amplitude. Similarly, an irregular behavior is identified when the variability in the inter-peak intervals exceeds a certain heuristic threshold  $\sigma_i / m_i > 0.6$ . Finally, the auto-correlation function of  $F_k$  is used to determine the n:m correspondence between the calcium peaks and stimulation pulses.

The previous procedure results in a set of four features, namely amplitude homogeneity, presence of alternance, irregularity of inter-peaks intervals and the n:m stimulation response.

2) *Identification of spatial alternans:* When the peak detection procedure detects the presence of an alternance in amplitude, an additional method is used in order to distinguish between spatially concordant or discordant alternans. To this extent, Principal Components Analysis (PCA) was used to identify the basic spatial modes in the experiment and to identify the existence of regions with an out-of-phase activity [13], [14].

In order to process the data, each image in the sequence  $X_{ij}^k, i = 1 \dots N_x, j = 1 \dots N_y$  was subtracted from its temporal mean and arranged as a d-dimensional column vector  $\mathbf{z}_k = [z_k^1, z_k^2, \dots, z_k^d]^T$  where  $d = N_x N_y$ . The whole experimental sequence was then represented by the  $d \times N$  matrix  $A = [\mathbf{z}_1, \mathbf{z}_2, \dots, \mathbf{z}_N]$ . The principal components are obtained by diagonalizing the  $d \times d$  covariance matrix  $AA^T$ . In our case, since the dimension of the data  $d$  is much larger than the number of observations  $N$  (typical values are  $d \sim 7 \times 10^4$ , while  $N \sim 2 \times 10^3$ ), we reduce the computational cost by using the fact that the largest  $N$  eigenvalues of  $AA^T$  are the eigenvalues  $\{\lambda_1, \lambda_2, \dots, \lambda_N\}$  of the  $N \times N$  matrix  $A^T A$  [15]. The eigenvectors of  $AA^T$  representing the spatial modes  $\mathbf{w}$  can be then obtained from  $\mathbf{w} = A\mathbf{v}$ , where  $\mathbf{v}$  are the eigenvectors of  $A^T A$ .

The main spatial mode in the experiment is found by reconstructing from the eigenvector  $\mathbf{w}_1$  associated with the largest eigenvalue  $\lambda_1$ . PCA reconstruction is achieved by projecting  $\mathbf{w}_1$  to the data matrix  $A$ , which results in an image representing the main spatial variability of the experimental sequence. The histogram of the reconstructed image is then divided in two regions A and B defined by the pixels above and below the average pixel intensity outside the cell (i.e. without calcium activity). The ratio between the pixel count in each region  $\rho = n_B / n_A$  defines a quantity that allows to identify the existence of regions presenting an out-of-phase activity in the sequence. Indeed, in the absence of spatial alternance the first order PCA reconstruction is homogeneous and the number of pixels in region B is low due

<sup>1</sup>Noise is robustly estimated by the median absolute deviation of  $s_k \hat{\sigma} = 1.4826 \cdot \text{median}(|s_k - \text{median}(s_k)|)$ , where  $s_k = F_k - F_k^d$  is a residual constructed from a denoised version  $F_k^d$  obtained by applying a wavelet shrinkage method to the signal  $F_k$  (Symmlet order 8, soft heuristic SURE threshold).

to background fluctuations in fluorescence ( $n_B \ll n_A$ , i.e.  $\rho \sim 0$ ). When the sequence includes a spatially discordant alternant, the PCA projection captures the spatial variability by setting the pixels of the discordant region to negative values, therefore increasing the relative size of region B and consequently the value of  $\rho$ . A heuristic threshold as low as  $\rho = 0.1$  is proven to be sufficient to detect small spatial discordances.

### III. RESULTS

#### A. Identification of $\text{Ca}^{2+}$ dynamical regimes

The information obtained from the peak detection and PCA analysis provide a set of features that allow us to classify the experimental sequences into one of the following cases:

1) *Normal dynamics*: Normal cell response is characterized by a 1:1 stimulation response showing homogeneity in the peak amplitude and a spatial distribution of calcium activity. An example of this behavior is represented in Fig. 4a. As it can be seen, the cell responds to a train of stimulation pulses applied every 4 seconds by generating a calcium transient. This regime is the typical response of a healthy cell and is normally observed at low pacing frequencies.

2) *Spatially concordant alternans*: An example of spatially concordant alternans is depicted in Fig. 2, which shows a 1:1 stimulation response presenting an alternance in peak amplitudes. This temporal alternance appears in the whole cell without spatial inhomogeneities.

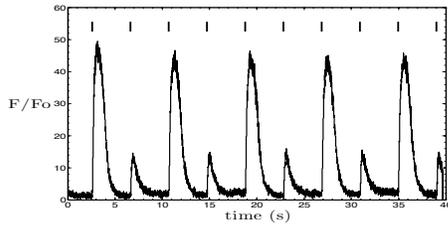


Fig. 2. Example of spatially concordant alternans at stimulation frequency 0.25 Hz. The whole cell responds to the stimulation pulses with alternating amplitudes. In all figures,  $F_0$  corresponds to the background fluorescence of the quiescent cell and vertical marks indicate stimulation times.

3) *Spatially discordant alternans*: Spatially discordant alternans present different regions with out-of-phase activity in response to different stimulation pulses. In Fig. 3a out-of-phase regions A and B are presented. The corresponding average calcium signal of each region is shown in Fig. 3c, exhibiting an alternating behavior in the activity of each zone. The use of the PCA method becomes necessary since this regime cannot be distinguished from a spatially concordant alternant from the average cell activity (see Fig. 3b).

4) *Phase-locking regimes*: Phase-locking is a dynamical regime in which there is a n:m phase synchronization between stimulation pulses and peaks in the signal. In such cases, a nonlinear interaction between stimulation and calcium regulation mechanisms results in the appearance

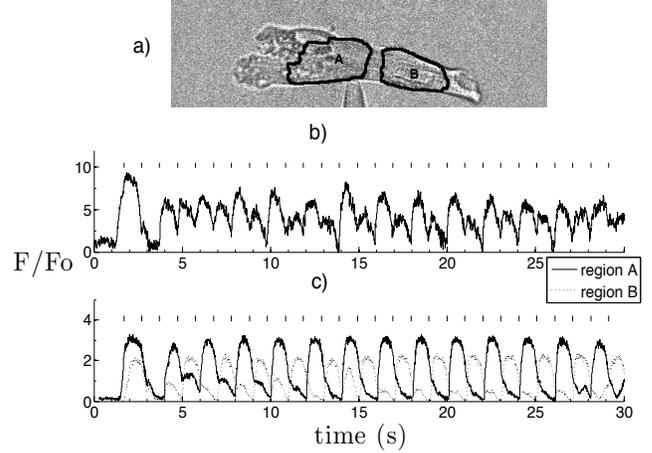


Fig. 3. Analysis of spatial alternance with PCA. A reconstruction from the most relevant eigenvector allows to identify two different regions A and B with alternating activities.

of a calcium signal with a frequency different from the frequency imposed by external pacing. Fig. 4b shows an example of period-halving of the calcium signal with respect to the stimulation pulses, whereas Fig. 4c depicts a case in which every other stimulation pulse is blocked and evokes no calcium transient.

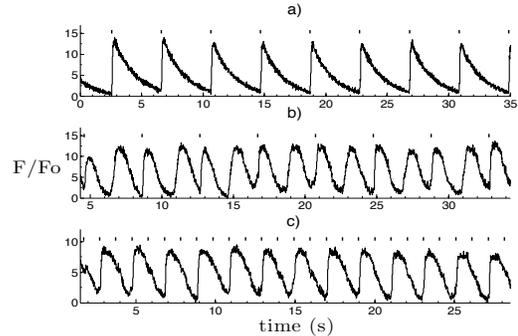


Fig. 4. Normal cell response and phase-locking at pacing frequency 0.25 Hz. a) Normal dynamics b) Example of phase-locking 2:1 (period halving): The cell responds with two  $\text{Ca}^{2+}$  transients every stimulation pulse. Note the correspondence between stimulation marks and signal peaks. c) Example of phase-locking 1:2 (period doubling): The cell responds with one  $\text{Ca}^{2+}$  transient every two stimulation pulses (blocking).

5) *Irregular dynamics*: Irregular dynamics occur when either inter-peak intervals present significant variability (i.e., non-periodic behavior) or when peak amplitudes are highly heterogeneous presenting no alternance. In such cases, we observe dynamical regimes as the ones shown in Fig. 5.

#### B. Performance evaluation

To quantify the performance of the method, we analyzed the 101 experimental sequences and compared the classification results to those obtained by an expert. True and false positive rates (TPR, FPR) were computed for each of the four classification groups (Normal, phase-locking, Alternans -both concordant and discordant- and Irregular) as FPR =

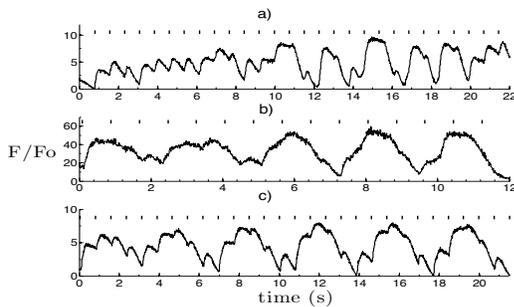


Fig. 5. Examples of irregular  $\text{Ca}^{2+}$  transients at stimulation frequency of 1.33 Hz.

TABLE I  
PERFORMANCE OF THE IDENTIFICATION METHOD

Index	Normal	Phase-Locking	Alternans	Irregular
TPR	92%	100%	80%	88%
FPR	6%	14%	0%	16%
Sample size	51	6	10	34

ratio of false positives over number of negatives and TPR = ratio of true positives over number of positives. Within the alternans group, the technique correctly distinguished all the cases presenting spatially discordant activity.

#### IV. CONCLUSIONS AND FUTURE WORK

##### A. Conclusions

We have developed an automatic method for the identification of spatiotemporal regimes in a sequence of calcium fluorescence images in stimulated cardiomyocytes. The method distinguishes between spatially concordant and discordant alternating patterns and is able to identify phase-locking dynamics such as period doubling or halving as well as the presence of an irregular behavior.

The technique can be used to obtain quantitative information about the dynamical response of the stimulated myocyte. In particular, it might be useful to characterize the sequence of bifurcations that the system undergoes as the pacing frequency is increased. Although the proposed method has been successfully applied to real experimental sequences, it would be necessary to quantify its performance and robustness under different signal-to-noise conditions. One of the straightforward improvements of the method is to substitute the PCA technique used for the identification of spatial alternans by an approach based on the use of Independent Components Analysis (ICA) [16]. This method would allow the decomposition of an experimental sequence in a set of statistically independent source signals associated

with the alternating spatial modes. This might improve the overall method since PCA only identifies uncorrelated modes which are not necessarily statistically independent. Moreover, further dynamical information about the sequence may be obtained by using temporal and spatial phase synchronization techniques [5], [17], [18].

#### V. ACKNOWLEDGMENTS

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#### REFERENCES

- [1] J. Rittscher, R. Machiraju, and S. Wong, Eds., *Microscopic Image analysis for life science applications*, ser. Bioinformatics and Biomedical imaging. Artech House, 2008.
- [2] M. J. Berridge, M. D. Bootman, and H. L. Roderick, "Calcium signalling: dynamics, homeostasis and remodelling," *Nat Rev Mol Cell Biol*, vol. 4, no. 7, pp. 517–29, Jul 2003.
- [3] J. P. Keener and J. Sneyd, *Mathematical physiology*, 2nd ed., ser. Interdisciplinary applied mathematics. New York, NY: Springer, 2009, vol. 8.
- [4] D. Bers, "Cardiac excitation-contraction coupling," *Nature*, vol. 415, no. 6868, pp. 198–205, 2002.
- [5] S. H. Strogatz, *Nonlinear Dynamics And Chaos: With Applications To Physics, Biology, Chemistry, And Engineering*, 1st ed. Westview Press, 2001.
- [6] A. Karma and F. G. Jr, "Nonlinear dynamics of heart rhythm disorders," *Physics Today*, vol. 60, no. 3, pp. 51–57, 2007.
- [7] E. Chudin, J. Goldhaber, A. Garfinkel, J. Weiss, and B. Kogan, "Intracellular  $\text{Ca}^{2+}$  dynamics and the stability of ventricular tachycardia," *Biophys J*, vol. 77, no. 6, pp. 2930–41, Dec 1999.
- [8] M. A. Watanabe, F. H. Fenton, S. J. Evans, H. M. Hastings, and A. Karma, "Mechanisms for discordant alternans," *J Cardiovasc Electrophysiol*, vol. 12, no. 2, pp. 196–206, Feb 2001.
- [9] D. Sato, Y. Shiferaw, A. Garfinkel, J. N. Weiss, Z. Qu, and A. Karma, "Spatially discordant alternans in cardiac tissue: role of calcium cycling," *Circ Res*, vol. 99, no. 5, pp. 520–7, Sep 2006.
- [10] J. G. Restrepo and A. Karma, "Spatiotemporal intracellular calcium dynamics during cardiac alternans," *Chaos*, vol. 19, no. 3, p. 037115, Sep 2009.
- [11] Z. Jia, H. Bien, and E. Entcheva, "Detecting space-time alternating biological signals close to the bifurcation point," *IEEE Trans Biomed Eng*, vol. 57, no. 2, pp. 316–24, Feb 2010.
- [12] L. Hove-Madsen, C. Prat-Vidal, A. Llach, F. Ciruela, V. Casadó, C. Lluis, A. Bayes-Genis, J. Cinca, and R. Franco, "Adenosine  $\text{a}_2\text{a}$  receptors are expressed in human atrial myocytes and modulate spontaneous sarcoplasmic reticulum calcium release," *Cardiovasc Res*, vol. 72, no. 2, pp. 292–302, Nov 2006.
- [13] R. O. Duda, P. E. Hart, and D. G. Stork, *Pattern Classification*, 2nd ed. John Wiley and Sons, Inc., 2001.
- [14] B. Ghanem and N. Ahuja, "Phase PCA for dynamic texture video compression," in *IEEE International Conference on Image Processing*, 2007.
- [15] G. Blanchet and M. Charbit, *Digital signal and image processing using MATLAB*. ISTE-Wiley, 2006.
- [16] J. V. Stone, *Independent Component Analysis: A tutorial introduction*. The MIT Press, 2004.
- [17] M. Palus, "Detecting phase synchronization in noisy systems," *Physics Letters A*, vol. 235, no. 4, 1997.
- [18] M. G. Rosenblum, A. S. Pikovsky, and J. Kurths, "Phase synchronization of chaotic oscillators," *Phys. Rev. Lett.*, vol. 76, no. 11, pp. 1804–1807, 1996.