Influence of Electrical Stimulation on the Contraction of Myotubes *in Vitro*

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Abstract— **Study of skeletal muscle cells in culture stimulated by externally-applied electical pulses with different characteristics, constitutes an interesting and potentially useful field still open to further investigation, in order to identify an electrical stimulation protocol that could be used for an optimal stimulation of single cells. On the other hand, previous researchers have demonstrated the usefulness of computer vision application to obtain important quantitative information on the deformation, disruption and development of biological tissues.**

In this paper, a method to study the influence that different parameters of electrical stimulation have on the contraction of single skeletal muscle cells stimulated *in vitro* is described. The investigation was carried out by analyzing image sequences of skeletal myotubes *in vitro*, stimulated through pulses at different frequencies, intensities and widths. The effects of these three pulse characteristics on the cell contraction responses were examined with the aim of providing a tool that can facilitate the search for an appropriate stimulation protocol that can be used also in clinical applications where prevention of muscle atrophy due to prolonged lack of exercise is desired. The results show that using single electrical stimulation pulses with a long time duration (*e.g.* 10 ms), as well as a high stimulus amplitude, allows a complete relaxation of the myotubes. In contrast, using a frequency of stimulation higher than 1 Hz prevents complete relaxation leading to sustained contractions.

Keywords— **Skeletal muscle cells, myotubes, contraction, electrical stimulation, computer vision.**

I. Introduction

In people with muscle weakness due to aging or various diseases where, for different reasons, they are unable to exercise physical activity, electrical stimulation of skeletal muscle can be a useful support to the improvement of life quality, reducing the muscle atrophy commonly associated with insufficient motor activity. In order to avoid muscle fatigue and other undesirable symptoms which often occur when current protocols of electrical stimulation are clinically applied, it is valuable to find new and more efficient methods of stimulation [1]. In the laboratory, the use of video microscopy combined with standard methods of computer vision constitutes a valuable tool to monitor the contraction of skeletal muscle cells that occurs during electrical stimulation *in vitro*, thereby facilitating the research of the most efficient stimulation protocol.

Various studies ([2, 3, 4]) have demonstrated that algorithms of computer vision can be applied to monitor and characterize the deformation of biological tissues. Some of these algorithms are able to extract visual characteristics like textured areas and track them from one frame to the next. Other methods are capable of estimating the motion of each pixel using differential techniques based on spatial and temporal image brightness variations (optical flow). In this way it is possible to evaluate motion characteristics (like direction, velocity, displacement) of objects in observed scenes.

In this paper a method based on standard tools of visual computation for the estimation of the motion field is proposed. This field is a 2-D vector of image point velocities induced by the relative motion between the viewing camera and the observed scene. Our method was developed for studying the influences that different patterns of electrical stimulation have on skeletal muscle cell contraction. This method was applied to the analysis of image sequences of myotubes (*i.e.* skeletal muscle fibers, with a tubular appearance, formed during a developmental stage) whose contraction is evoked *in vitro* using different parameters of electrical stimulation.

II. Methods

A. Myotube cultures

The experiments were performed on 6-8 day old cultured myotubes. Briefly, myotubes were obtained starting from primary myoblast cultures of i28 skeletal muscle cells, which were established from satellite cells derived from the hind-leg muscles of a 7-day-old male Balb/c mice. Muscle cells were seeded at 70,000 cells/35 mm on matrigel-coated Petri dishes and maintained at 37 °C and 5 % CO_2.

B. Electrical stimulation

Extracellular electrical stimulation of skeletal myotubes in culture was performed by giving a train of pulses at various frequencies (0.5, 1, 3 and 5 Hz), intensities (1, 2, 3 and 6 V)

E. Kyriacou et al. (eds.), *XIV Mediterranean Conference on Medical and Biological Engineering and Computing 2016*,
IFMBE Proceedings 57,
DOI: 10.1007/978-3-319-32703-7_83

and widths (1 and 10 ms) delivered by an S88 square pulse stimulator (Grass Technologies®).

C. Image acquisition

The Petri dish of myotubes in culture was placed on the stage of a Zeiss IM35 inverted phase microscope with a 16 x objective. Cell contraction was recorded using a Canon digital camera in Full HD 1080p format with frame rate of 24 fps. The acquisition of frames from the video stream was made using MATLAB®. All the images of myotubes have a resolution of 1920 x 1080 pixels.

D. Image processing and analysis

The evaluation of the motion field of myotubes during contraction was carried out by using a feature-matching technique. These techniques estimate motion field at special image points, called feature points, the motion of which is expected to be estimated reliably [5].

In our case, feature points were extracted from the first image of the sequence using the Canny Edge Detector [6], which identifies edges by looking for local maxima of the gradient of an image. In order to ensure short computation time, a subset of points obtained from the intersection of feature points with a grid of 3×3 pixels was considered.

In the following, a brief description of the feature-matching algorithm used for approximate the motion field at each feature point, will be provided.

Let $I(x,y,t)$ be the image brightness at the point (x,y) in the image plane at time t. Since the apparent brightness of the observed scene can be assumed constant, its variation is zero:

$$\frac{dI}{dt} = 0. \tag{1}$$

Using the chain rule for differentiation, the total temporal derivative is:

$$\frac{dI(x(t),y(t),t)}{dt} = \frac{\partial I}{\partial x}\frac{dx}{dt} + \frac{\partial I}{\partial y}\frac{dy}{dt} + \frac{\partial I}{\partial t}. \tag{2}$$

Letting I_x, I_y and I_t be the abbreviation for the partial derivatives of the image brightness in x, y, and t, respectively, the equation 2 can be rewritten as the image brightness constancy equation:

$$I_x u + I_y v + I_t = 0, \tag{3}$$

where u and v are the temporal derivatives dx/dt and dy/dt representing the components of the motion field (u,v).

Since equation 3 is in two unknowns, it is necessary to introduce additional constraints. The choice of these constraints diversifies one algorithm for motion field estimation from the others.

In our application we used the Lucas-Kanade method [7], which assumes that the motion field (u,v) is approximately constant in a small window of size $n \times n$ centered at the point (x,y).

Therefore,

$$\begin{bmatrix} I_x(\mathbf{p}_1) & I_y(\mathbf{p}_1) \\ \vdots & \vdots \\ I_x(\mathbf{p}_{n\times n}) & I_y(\mathbf{p}_{n\times n}) \end{bmatrix} \begin{bmatrix} u \\ v \end{bmatrix} = - \begin{bmatrix} I_t(\mathbf{p}_1) \\ \vdots \\ I_t(\mathbf{p}_{n\times n}) \end{bmatrix}, \tag{4}$$

where $\mathbf{p}_1, \ldots, \mathbf{p}_{n\times n}$ are the points inside the window.

This can be rewritten in matrix form as:

$$A\mathbf{x} = -\mathbf{b}, \tag{5}$$

and solved using the least squares method:

$$\mathbf{x} = (A^T A)^{-1} A^T (-\mathbf{b}). \tag{6}$$

Once the motion field (u,v) between each frame and the first one of the image sequence was estimated, the displacement of each feature point, *i.e.* the magnitude of the vector assigned at each point by the motion field, was determined. Finally, the average of the displacements on all the feature points greater than a fixed threshold, for each frame, was calculated. The threshold was determined by analyzing frame by frame the histogram of the displacements and was fixed to 1.5 pixels. The proposed method was implemented using MATLAB®.

III. Results

Fig. 1 illustrates an example of a motion field obtained during myotube contractions electrically evoked by a pulse of 1 V for 1 ms.

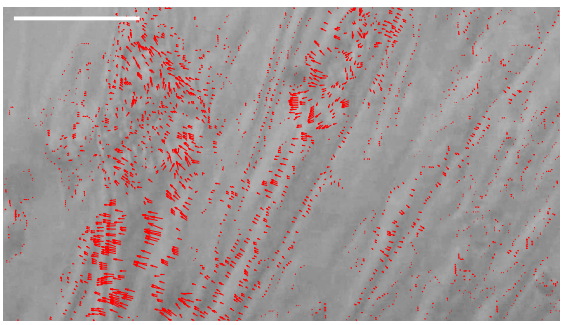

Fig. 1: Estimated motion field during myotube contractions. Each arrow indicates the direction of the motion field and its length represents the displacement. The arrows were amplified 5 times in order to make vectors more visible. The white bar is 50 μm wide.

Each arrow indicates the movement direction and its length represents the displacement.

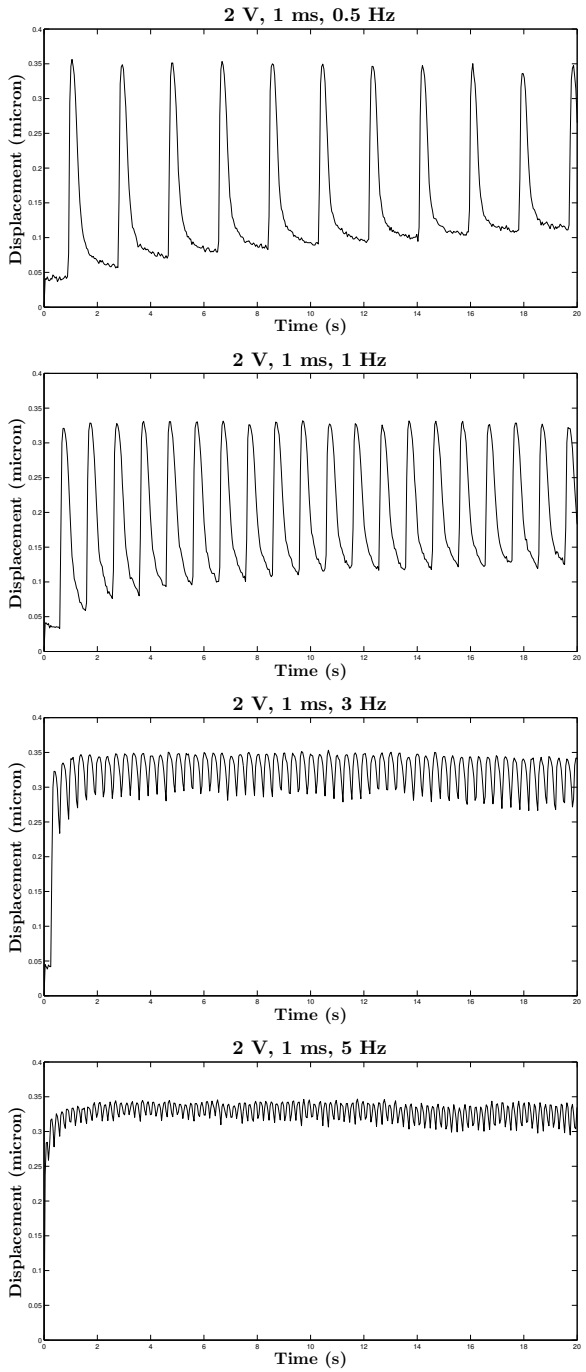

Fig. 2: Examples of contractions electrically evoked in myotubes by a train of pulses at 0.5, 1, 3 and 5 Hz (from top to bottom respectively) using an intensity of 2 V. The pulse width is 1 ms.

Fig. 2 shows the average displacement obtained by stimulating a representative myotube with a train of pulses with the same intensity (2 V) and the same width (1 ms) but with different frequencies (0.5, 1, 3 and 5 Hz). Through the displacement estimates, a quantitative analysis of the contraction induced in myotubes by stimulation pulses at various frequencies can be realized. The effect of frequency increase on myotube contraction is a gradual reduction in the return to the resting state with a progressive raising of the minimum value of contraction which saturates at a value and in an interval time depending on the frequency: the higher the frequency the lower the time to reach saturation and the higher its saturation value.

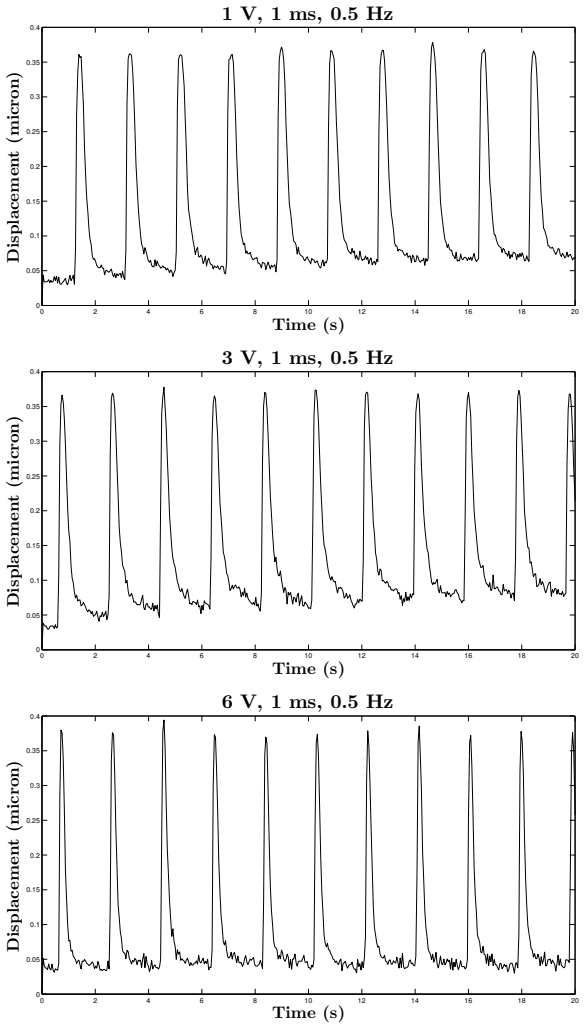

Fig. 3: Examples of contractions electrically evoked in myotubes by a train of pulses at 0.5 Hz using intensities of 1, 3 and 6 V (from top to bottom respectively). The pulse width is 1 ms.

At 0.5 Hz, myotubes can almost fully relax at the end of the pulse stimulation. At 1 Hz, they are forced to contract again before they reach the resting state and at higher frequencies (3 and 5 Hz) they show a fusion of successive contraction up to a tetanic one. On the contrary, maximal contraction values remain unchanged at different frequencies.

Fig. 3 shows the time course of the average displacement using different intensities (voltage) of stimulation (1, 3 and 6 V). The frequencies and the pulse widths are maintained constant (0.5 Hz and 1 ms, respectively). The effect of the stimulus intensity increase is a reduction in the relaxation time of the cells that allows the myotubes to reach the resting state at the higher voltages. For lower intensities, in which the relaxation is not completely obtained, the saturation at a minimum level of contraction occurs, like that showed in Fig. 2. The amplitude of the evoked contraction remains almost unchanged with the variation of the stimulus intensity.

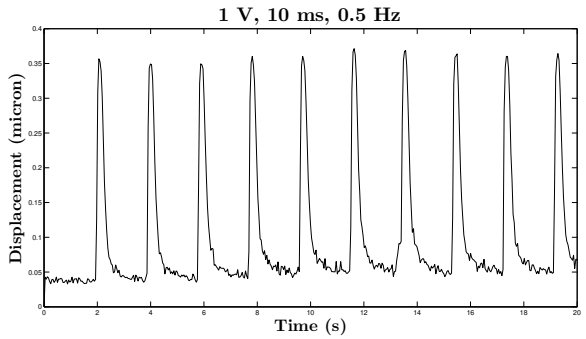

Fig. 4: Example of contractions electrically evoked in myotubes with pulses width of 10 ms at 0.5 Hz. The pulse intensity is 1 V.

Comparing the graph of Fig. 4, showing the behavior of the average contraction evoked by an amplitude of 1 V at 0.5 Hz with a duration of 10 ms, with that of Fig. 3 of the same intensity and frequency but with a duration of 1 ms, a different time constant of relaxation is evident in the two situations: the greater the pulse duration, the shorter the time constant. The duration of 10 ms allows to obtain the complete relaxation of the myotubes that is not the case of 1 ms. The amplitude of the contraction is maintained almost constant even varying the pulse duration.

IV. Discussion

The computer vision technique described in this paper allowed us to evaluate the effects of the different stimulation parameters examined on single myofibers in culture. In particular, the estimation of both the contraction amplitude and its time course was possible.

From the analysis of the frequency effects on myotube contraction, it is evident that a stimulation which ensures cycle by cycle cell relaxation without production of sustained contraction, is necessarily based on low values of stimulation frequency (for example 2 V, 1 ms under 0.5 Hz like Fig. 2). On the other hand, the increase of the stimulus intensity (Fig. 3) permits the complete relaxation of cells also with short pulse widths but with low frequencies. Furthermore, using a pulse width sufficiently long (for example of 10 ms like in Fig. 4) allows myotubes to fully relax. Consequently, the main factor which determines the increase in relaxation velocity is the amount of electrical charge that is given to cells: higher intensities or higher pulse widths both produce an increase of stimulus charge. Therefore, it appears that myotubes exhibit a better contraction response in terms of relaxation when they are stimulated using either high intensities, low frequencies and low pulse widths or low intensities, low frequencies and high pulse widths. Even if the amplitude of contraction is largely independent of the stimulation intensity, frequency and duration, on the other side, the frequency is a critical element to select in order to realize an optimal contraction-relaxation cycle.

In conclusion, the proposed method allowed us to evaluate the influence of the three stimulation parameters considered on myotubes contraction and to quantify the contraction itself. Furthermore, the study also allowed us to identify stimulus parameter combinations that were able to produce, in the case of myotubes, an appropriate contraction-relaxation sequence.

Acknowledgements

This work was partially supported by a Masters in Clinical Engineering at the University of Trieste, Italy.

Conflict of interest

The authors declare that they have no conflict of interest.

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