A Non-Invasive Method for Measuring Contractility in Cardiocytes

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Abstract: We are developing a non-invasive procedure for measuring contractile responses of both adult and neonatal mammalian cardiocytes. This method can be employed to quantify pharmacological effects of drugs on myocytes. Methods for quantifying neonatal cell contraction reported in the literature have required the use of elaborate equipment such as a proximity detector or an atomic force microscope (to measure the increase in cell elevation as a cardiocyte contracts), and typically interfere with simultaneous optical recording of cell signals. Such contraction quantification methods are expensive due to the equipment needed. Our approach to contractile measurement is being developed with the goal to be a practical and relatively inexpensive method.

Our approach uses two different image processing procedures that allow us to produce contractility records of both adult and neonatal cardiocytes. In our first procedure we analyze neonatal myocytes. Contraction curves generated from neonatal cells with our new method exhibit a very similar profile and time course to those produced by more sophisticated and expensive methods [11, 15].

Our second image processing procedure is an application that allows measurement of the adult cardiocytes area in each frame. Application of our two dimensional quantification technique to the study of adult cardiocytes produces contraction vs. time records virtually identical in time course and shape to records obtained from traditional one dimensional, cell boundary tracking procedures [12].

The contractility graphs created by the two image processing procedures are consistent with the expected results and graphs resulting from past studies performed on myocytes [10]. This new non-invasive approach to contractile quantification will be useful in the analysis of the myocyte contractile dynamics in the presence of drugs.

Keywords: Contractility, Myocyte, Image Analysis

1 Introduction

Isolated adult and neonatal cardiocytes from mammalian heart are widely used in cardiovascular cell biology research. The study of cardiocyte contractility can help unveil fundamental processes underlying heart function in health and disease. Many positive and negative inotropic factors modulate the contractile behavior of cardiocytes. Due to the high number of studies and the unlimited research possibilities with cardiocytes, we decided it would be useful to develop a non-invasive working protocol that could allow for the quantification of the contractility exhibited by both neonatal and adult cardiocytes. The image processing method for neonatal myocytes could be applied to the study of the effects of the extracellular matrix (ECM) on developing myocytes. The ECM has been shown to play a crucial role in the sarcomere development in neonatal myocytes [1, 7]. Currently our laboratory has one protocol for measuring adult cell contractility a one dimensional cell boundary video tracking method but no equivalent method for quantifying neonatal cell contractility [12].

We were previously able to use only the intracellular calcium transient signal amplitude or integral to measure neonatal cell contractile potential. The application of the cell boundary video tracking method and the use of cellular force measurements using single-spaced polymeric microstructures to detect amplitude of contraction and beating rate in neonatal cardiocytes have been reported in the literature [2, 9, 14, 17], but have been
found to be erratic and unreliable in our laboratory studies. The availability of a protocol for measuring both neonatal and adult cardiocyte contractions would allow a consistent method to characterize inotropic effects of drugs on both cell types, and could open the door to a new line of research in the world of cardiovascular biology.

2 Contractility and Myocyte Mechanics

Contractility is the intrinsic ability of heart muscle to generate force and to shorten. At the molecular level the contractile process originates from the changing concentrations of Ca$^{2+}$ ions in the myocardial cytosol. Calcium ions enter through the calcium channel that opens in response to the wave of depolarization that travels along the sarcolemma. These Ca$^{2+}$ ions trigger the release of more calcium from the sarcoplasmic reticulum (SR) and thereby initiate a contraction-relaxation cycle \[6,5,3\]. Figure 1 shows a schematic of the cytosol and extracellular space, depicting the exchange of Ca$^{2+}$, Na$^+$, K$^+$, and Cl$^-$ ions that leads to the contraction of the myocyte.

Fig. 1: Parts of the ultra-structure of a myocyte: The A-band, the I-band, the Z-lines, the sarcoplasmic reticulum, the transverse tubular system (T-tubule), and the mitochondria. (Original image from [4].)

3 Previous Work

Given the need that exists in cardiovascular research to analyze the aspect of contractility and force development in myocytes, several methods have been developed. All these methods require the use of expensive equipment and sophisticated techniques to perform the desired analysis.

One of the first efforts to measure contraction or shortening was performed with the assistance of a video based device the was able to capture the extent and rate of length shortening of isolated cardiac myocytes \[16\]. Even though several methods were published prior to this method, this was the first technique to specialize on contracting cardiac cells. The video based method uses two tracking points at each end of the myocyte to track edge displacement as the myocyte contracts. The distance between the two edges is measured using edge detection while a record of the data is stored in a file. The method produces satisfactory results and has been an approved and widely used method for measuring contractile responses of adult myocytes for almost twenty years.

The need for measuring different aspects of a myocyte has led some researchers to explore techniques such as scanning ion conductance microscopy \[15\]. This technique uses a distance modulated protocol for scanning ion conductance microscopy to provide a distance control mechanisms to image contracting myocytes. This technique combined
with laser confocal microscopy measures myocyte height and local calcium concentration during contractility.

In the last two decades soft materials have been explored by researchers in cell biology as a means to measure force development and tracking movement of cells. Myocytes have not been an exception in the application of this emerging science. Materials such as polydimethylsiloxane (PDMS) and polyacrylamide (PAM), have been widely explored in an effort to measure different aspects of cell mechanics during contraction. In 1980, Harris et al used soft silicone rubber to qualitatively measure cellular force [9]. In the last two decades, methods such as this one have evolved into robust and complex techniques that offer extensive cellular force development analysis. Despite the significant advances in such approaches, they are limited by extensive computation for force calibration due to a continuous substrate deformation [17].

In 2005, Zhao et al, created a PDMS microchip to measure cellular force and study subcellular mechanics [12, 17]. The chip consisted of micrometer scale polymeric pillars on a plain substrate. This chip was fabricated using a flexible polymer microfabrication technique to create polymeric microstructures of various aspect ratios, which allows for the measurement of a range of forces at the subcellular level. This was an attempt to avoid the common problems that result from the application of polymeric pillars, as often they are too far apart or close to one another and force measurement accuracy is compromised.

Similar studies have been done with micropatterned substrates to measure force development, cell motion and the creation of focal adhesion points. Such approach was developed with the intent to be a real-time, high resolution measurement of force applied by cells at single adhesion points by combining micropatterned substrates and fluorescence imaging. This method poses the same problems exhibited by the other methods by using expensive equipment and requiring robust and complex methodologies.

4 Materials and Methods

4.1 Cell Preparation

Following isolation using enzyme retrograde perfusion in the isolated heart, cardiocytes were plated to trap fibroblasts and avoid their presence in microscope images. Cells remained in the incubator in this state for approximately two hours. Cardiocytes were re-plated to trap any extra fibroblasts remaining in the cell suspension. Cells remained in this state for another hour. Cells were finally transferred to plates with 10% fetal bovine serum (FBS) solution in which they were to remain for the rest of the procedure. The 10% FBS solution was changed as necessary throughout the analysis in order to keep cells properly oxygenated and images free of unwanted objects such as death cells. Forty-eight hours later the cardiocyte’s contractile machinery is developed enough to be ready for video recording. Plated cells were electrically paced at 0.33 Hz for 3 msec duration.

4.2 Microscopy and Data Acquisition

The analysis in this study assumed that the volume of any given cardiocyte remains constant as it contracts. The magnitude of volume changes accompanying muscle cell contraction are known to be only a few millionths of the resting volume [4]. The cardiocytes were observed using a Nikon phase contrast microscope (Model ELWD). Output of a Cohu CCD camera (Model 2122-1000) mounted on the microscope was digitized by a frame grabber card. Images were recorded using Windows Movie Maker software at a frequency of 30 frames per second. Video frames were fed into Matlab for batch processing using various Image Processing Toolbox functions.

5 Image Analysis

5.1 Image Processing of Adult Myocyte Sequence

The analysis of the adult myocyte video sequence was done via the application of Matlabs Image Analysis Toolbox and its built-in functions. The analysis required the creation of a script that allowed an automatic
application of a series of image enhancement and quantification functions for each frame in the contraction sequence. The script loaded the image sequence into Matlab and processed the images one by one with each of the Matlab built-in functions listed in the script.

The first Matlab Image Analysis tool applied by the script was the *imclose* function. This function prepared the image for the rest of the analysis by performing a morphological closing on the grayscale image. The operation performed a dilation followed by an erosion, which tends to smooth the contours of objects and generally joins narrow breaks, fills long thin gulfs and fills the holes smaller than the structuring element \(8\). The resulting image was subtracted from the original image depicting the adult myocyte as a way to increase contrast between myocyte and background and to aid in the edge detection process. Figure 2 shows the morphologically closed image containing an adult myocyte after being processed by the *imclose* function (top-left) and the resulting image when the morphologically closed image was subtracted from the original image (top-right).

The next step in the adult image processing sequence, was the creation of binary images. In order to create a binary image, the Matlab command *im2bw* was applied; this command creates a binary (black and white) image by applying a threshold value. The threshold value was used as a break point to depict pixels with values higher than the threshold as white pixels and pixels with values lower than the threshold as black pixels, thus creating a binary image. In figure 2 we can see the image after (bottom-left) the *im2bw* command was applied.

The next step in the process was the removal of spurious optical background, leaving only a binary outline of the myocyte. Then the adult myocyte image was itself cleared of any trace of cell inclusions, leaving nothing but the cell outline in the image; ready for area measurements. In figure 2 we can see the binary image containing the adult myocyte and optical background (bottom-left), and finally the cell outline ready for area measurements (bottom-right).

The script proceeded to repeat the image processing with all the images in the contraction sequence. The area of the adult myocyte was determined for each individual frame. Finally, a graph depicting myocyte area vs. time was created. Figure 3 shows the final output of the script applied in Matlab with a sequence of 30 images.

5.2 Image Processing of Neonatal Myocyte Sequence

The analysis of the neonatal myocyte contraction was also performed via the application of the functions in the Matlab Image Analysis Toolbox. The analysis required the creation of a script that asked the user the name of the sequence of images to be analyzed and the number of images in the sequence. The script loaded the images into Matlab and processed the images one by one with each of the Matlab built-in functions listed in the script.

The first built-in Matlab Image Analysis function used was the *imadjust* function. This function prepared the image for the rest of the analysis by decreasing the dynamic range possible for the pixels in the image. The Matlab function *imadjust* is an intensity transformation function for gray scale images. The function maps the intensity values in the image in question to new values in an output image. The function takes values between \(low\_in\) and \(high\_in\) and maps them to values between \(low\_out\) and \(high\_out\). All the values below \(low\_in\) and above \(high\_in\) are removed. There exists a gamma parameter which determines the shape of the curve that depicts the way in which intensity values are mapped. In the case of the application to this project the gamma parameter was set to a value of 1, such that intensity values are mapped linearly \(8\).

The next step in the analysis process was the creation of a binary image that will uncover the inclusions resulting from the contractile mechanisms in the center of the myocytes. In order to create this binary image, the Matlab command *im2bw* was used. This command creates binary images using a threshold value as explained in the adult
myocyte processing section of this report.

The script then commanded the user to select a region of interest (ROI) where the inclusion area changes were to be quantified. Changes in these objects (corresponding to dark inclusions within the myocyte) with time were interpreted as an expression of contractile response of the neonatal cardiocyte. The script proceeded to repeat the process with all the images in the sequence. The total area of the inclusions was determined for each individual image. The individual values are stored in a data array. Finally, a graph depicting inclusion area vs. time was created. Figure 4 shows a sample of an image enhanced for processing, a binary image showing the region of interest and the corresponding contraction record. In figure 5 we can see the final output of the script run in Matlab of two different sequences of neonatal contractions.

Repeatability was an issue as soon as our first non-invasive procedure for measuring contractile responses was developed. In order to see whether we could produce consistent results on the same cell, we recorded a cell throughout multiple contractions. The image sequences were analyzed using the neonatal image processing procedure. The results showed satisfactory results, since we were able obtain a contraction record of the five consecutive contractions (see figure 6). Figure 7, shows the five consecutive contractions overlaid in the same graph, including an average of the contractile behavior of the myocyte.
6 Discussion

Neonatal myocytes represent a challenge when trying to obtain a functional response that can be compared with measurements of cell shortening vs. time (or cell force vs. time) from adult myocytes. Adult cardiac cells have a well defined cylindrical morphology, and contractility can be measured by measuring the cells shortening (or the shortening of individual sarcomeres within the cell, the engines of shortening and force development). Our laboratory measures adult cardiac myocyte responses with a one dimensional video-based edge detection circuit processing video images of the shortening cell [12].

Cell perimeter and cell outline changes recorded in adult cardiocytes using the new technique consistently decreased during contraction with a pattern similar to shortening vs. time curves recorded by tracking cell boundaries, and appeared to provide a reliable measure of contraction. The advantage offered by the new method is that it is a more inclusive method that takes into account the entire cell geometry rather than just the overall edge-to-edge length.

We originally hypothesized that a change in a neonatal cells perimeter and area could be detected by image analysis, with the understanding that contraction was likely accompanied by a retraction of the cells boundaries and a slight increase in its elevation. Such changes in elevation were recorded by Shevchuk in 2006, using the atomic force microscope, or AFM [15]. The signal generated by the new neonatal method produces contraction vs. time graphs are very similar in behavior to those of the adult cardiocytes. Our study has been successful in proving that a usable contractility signal can be obtained from neonatal myocytes based on the application of image analysis. We have great expectations for the research capabilities that the creation of these two procedures can provide.

References


changes accompanying stretch of frog muscle, Nature 204 (1964).


Fig. 5: Neonatal Myocyte Images after being processed. (A,C) Contraction record of neonatal myocyte. (B,D) Image of Neonatal Myocyte taken using inverted phase contrast microscopy.

Fig. 6: Multiple Contraction Record of Neonatal Myocyte. Record of 5 Consecutive Contractions Exhibited by a Neonatal Myocyte.


Fig. 7: Record of 5 Contractions and Average. All Contractions were exhibited by the Same Neonatal Myocyte in a Consecutive Fashion.