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A non-invasive method for measuring contractility in cardiocytes

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Abstract:

We have developed a convenient method 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 of quantifying neonatal cell contraction reported in the literature have required the use of elaborate methods such as a proximity detector or an atomic force microscope (measure the increase in cell elevation as cardiocyte contracts), and typically interfere with simultaneous optical recording of cell signals such as the calcium transient that can be recorded using calcium-sensitive dyes like Fluo-3. Such contraction quantification methods are expensive due to the equipment needed. Our new approach to contractile measurement was developed with the intention to be a practical and relatively inexpensive method. In this method, digital video images are obtained using a CCD camera mounted on an inverted phase contrast microscope. The video images are later analyzed frame by frame using functions available in the Matlab's Image Processing Toolbox. Image optimization and object recognition techniques are applied to the sequence of frames depicting a contracting myocyte. Our method uses two different image processing sequences that allow us to record the contractility of both adult and neonatal cardiocytes. In the case of the neonatal myocytes the analysis focuses on intracellular fine structure details, specifically by monitoring the area of small inclusions within the cell thought to be a result of protomyofibrils. Contraction curves generated from neonatal cells with the new method exhibit a very similar profile and time course to those produced by the elaborate methods (Shevchhuck et al, 2001, Kliche et al, 2006). Neonatal cardiocyte analysis utilizes phase contrast images showing inclusions that change their phase contrast image appearance (including inclusion size). Our other application using Matlab's Image Processing Toolbox allows measurement of the adult cardiocyte's area in each frame. Adult cardiac cells have a well defined cylindrical morphology, and contractility can be measured by tracking the cell's shortening, or the shortening of individual sarcomeres, the 'engines' of shortening and force development within the cell (Rieser et al., 1979). Application of this two dimensional quantification technique to the study of adult cardiocytes produces contraction vs. time records virtually identical in time course and shape of records obtained by traditional one dimensional, cell boundary tracking procedures (Rieser et al., 1979). The contractility graphs created by the two methods are consistent with the expected results and graphs resulting from the more complicated existing methods (Katz, 2001). This new practical approach to contractile quantification will be helpful in the analysis of the myocyte contractile dynamics in the presence of drugs.

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 practical working protocol that could allow for the quantification of the contractility exhibited by either neonatal or adult cardiocytes. The application of the neonatal contractility 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 (Bray et al, 2008). The 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 (Reuben et al., 1998). 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 has been reported in literature (Zhao et al, 2005), but have been found to be erratic and unreliable in our laboratory studies. The availability of a single protocol for measuring both neonatal and adult cardiocyte's 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.

Materials and Methods:

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% 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.

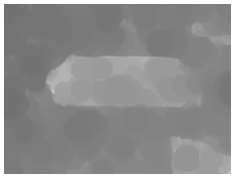
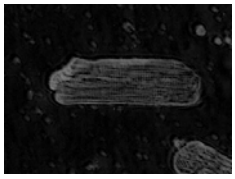
Analysis: The analysis in this study assumed that the volume of any given cardiocyte remains constant as it contracts. The magnitude of volume changes accompanying muscle cell contraction are known to be only a few millionths of the resting volume (Paolini et al., 1966). The cardiocytes were observed 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.

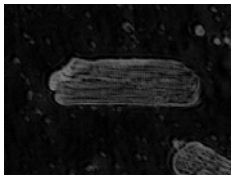
Image Processing

Image Processing of Adult Myocyte Sequence: The analysis of the adult myocyte video sequence was done via the application of Matlab's Image Analysis Toolbox and its built-in functions. The analysis required the creation of a script that allowed for the automatic application of a series of image enhancement and quantification functions per each frame in the contraction sequence.

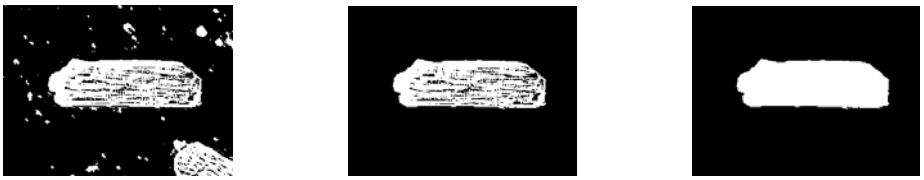
The script loaded the image sequence into Matlab and processed the images one by one with each of the Matlab built-in functions listed in the script. The first Matlab Image Analysis tool applied by the script was the imclose function. This function prepared the image for the rest of the analysis by performing 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 that the structuring element (Gonzalez and Woods, 2002). 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. The figures below show the morphologically closed image containing an adult myocyte after being processed by the imclose function (left) and the resulting image when the morphologically closed image was subtracted from the original image (right).

The next step in the adult image processing sequence, was the creation of binary images. In order to create this binary image, the Matlab command "im2bw" was applied; this command created a binary image by applying a threshold value. The threshold value was used as a breakpoint to depict pixels with values higher that the threshold as white pixels and pixels with values lower than the threshold as black pixels, thus creating a binary (black and white) image. In the figures below one can see the image before (left) and after (right) 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 was itself cleared of any trace of cell inclusions, leaving nothing but the cell outline in the image; ready for area measurements. Below one can see the binary image containing the adult myocyte and optical background (left), adult myocyte with cell inclusions (center), and finally the cell outline ready for area measurements (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. Below one can see the final output of the script applied in Matlab with a sequence of 30 images.

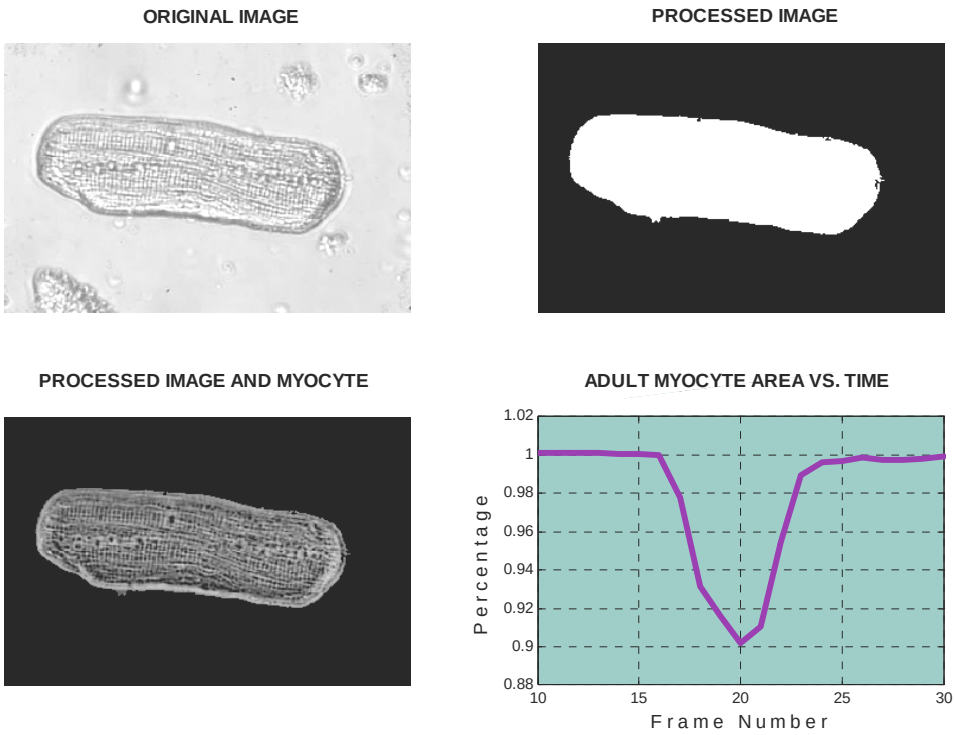
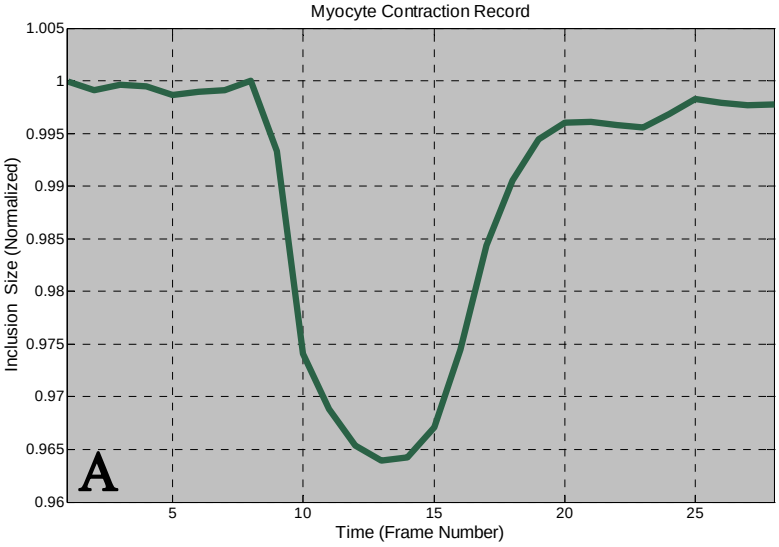


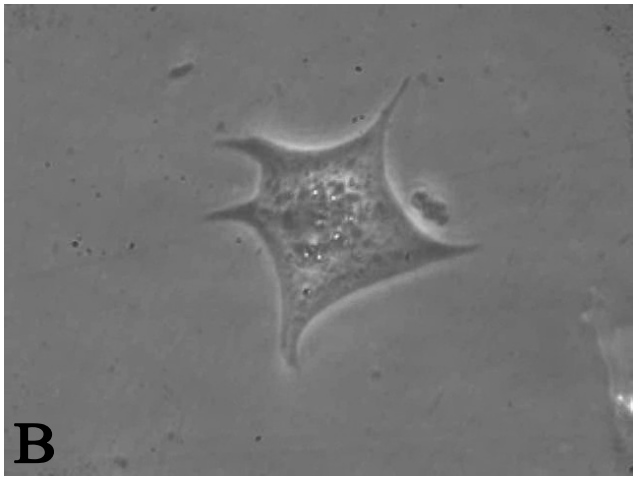
FIGURE 1. The figure demonstrates the Matlab Image Processing Algorithm applied to an adult cardiocyte. Figure 1a shows the original image containing the adult myocyte. Figure 1b shows a processed image that resulted in a binary image containing the outline of the myocyte. Figure 1c shows the result of the addition of Figure 1a and 1b. Figure 1d shows the resulting adult myocyte area vs time graph.

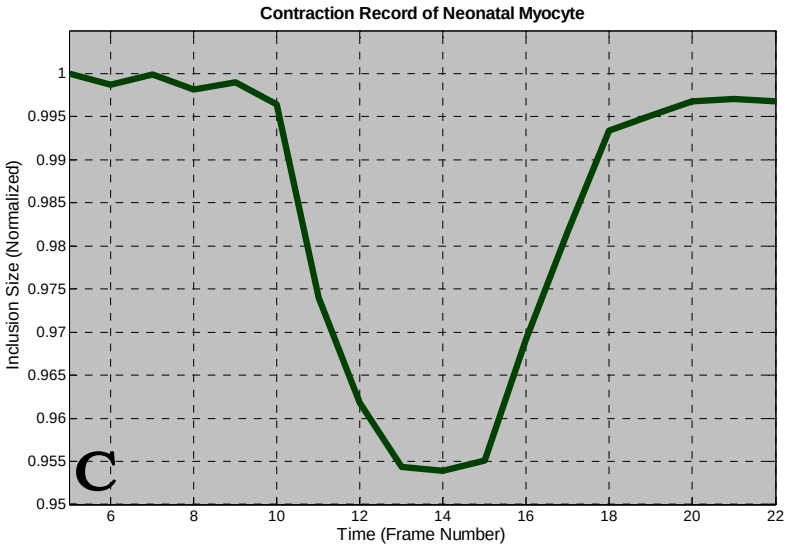
Image Processing of Neonatal Myocyte Sequence: The analysis of the neonatal myocyte was also performed via the application of the functions in the Matlab's Image Analysis Toolbox. The analysis required the creation of a script that asked the user the name of the sequence of images to be analyzed and 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 value array possible for the pixels in the image. The Matlab function imadjust is an intensity transformation function for gray scale images. The function mapped the intensity values in the image at question to new values in an output image. The function took values between low_in and high_in and mapped them to values between low_out and high_out. All the values below low_in and above high_in were 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 (Gonzalez and Woods, 2002). The next step in the analysis process was the creation of a binary image that will uncover the inclusions containing the contractile mechanisms in the center of the myocytes. In order to create this binary image, the Matlab command "im2bw" was used. This command created binary images using a threshold value as explained in the adult myocyte processing section of this report.

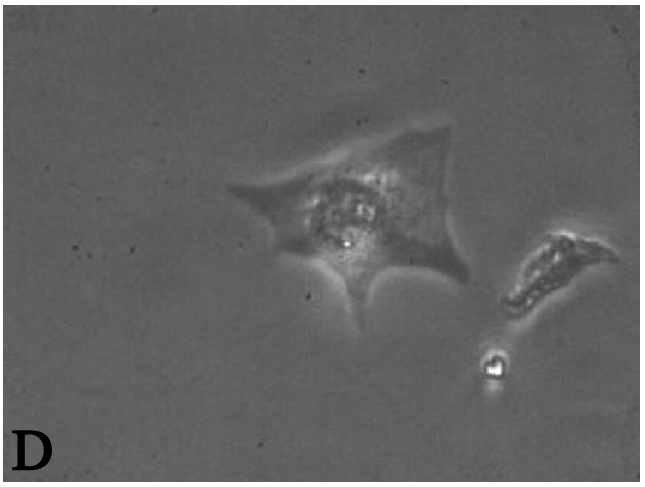
The script then commanded the user to select a region of interest (ROI) where the area changes were to be quantified. Changes in these objects (corresponding to dark inclusions within the myocyte) with time were interpreted as an expression of contractile response of the neonatal cardiocyte. The script proceeded to repeat the process with all the images in the sequence. The 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. Below one can see the final output of the script ran in Matlab with three different sequences of neonatal contractions.

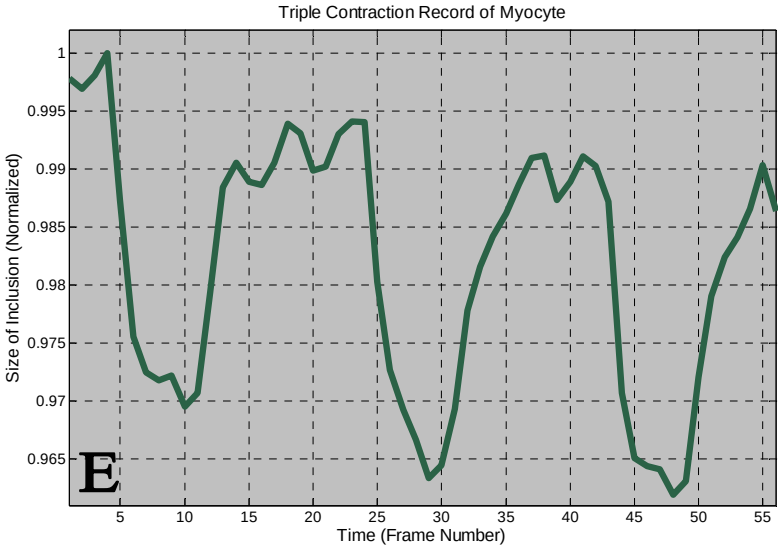
Fluorescence Studies: In order to validate our theory regarding the source of inclusions in the neonatal myocyte images after the application of image analysis, we decided to implement fluorescence tools. Fluorescence has been used in the study of the developing myofibrils to gain insight into what mechanisms play important roles in myofibrillar development (Schultheiss et al, 1990 and Lin et al, 1989). Fluorescence allowed us to visualize the protomyofibrilles and contractile machinery in the myocyte. We used a plasmid that targets myosin in the myocyte and allowed the myosin present in the protomyofibrilles to glow in the presence of fluorescent light (See Figure 3). The well developed myofibrilles may be a direct result of the myocyte to myocyte contact and interaction (Atherton et all, 1986).











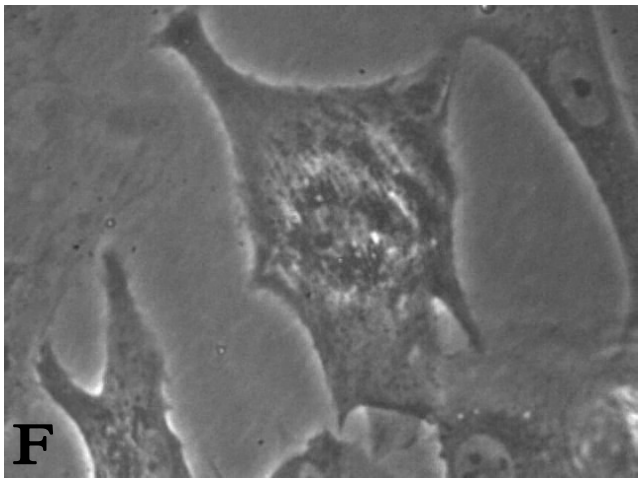


FIGURE 2. The figure above shows the contractility record and phase images of three different neonatal myocytes. Images were taken 72 – 84 hrs after the myocytes were platted. **A** and **C** depict the contractility record of a single contraction. **B** and **D** show the phase contrast images of the myocytes platted at low density. **E** shows the contractility record of a myocyte exhibiting three consecutive contractions. **F** shows the phase image of the myocyte that was platted at mid-density allowing interactions with other myocytes.

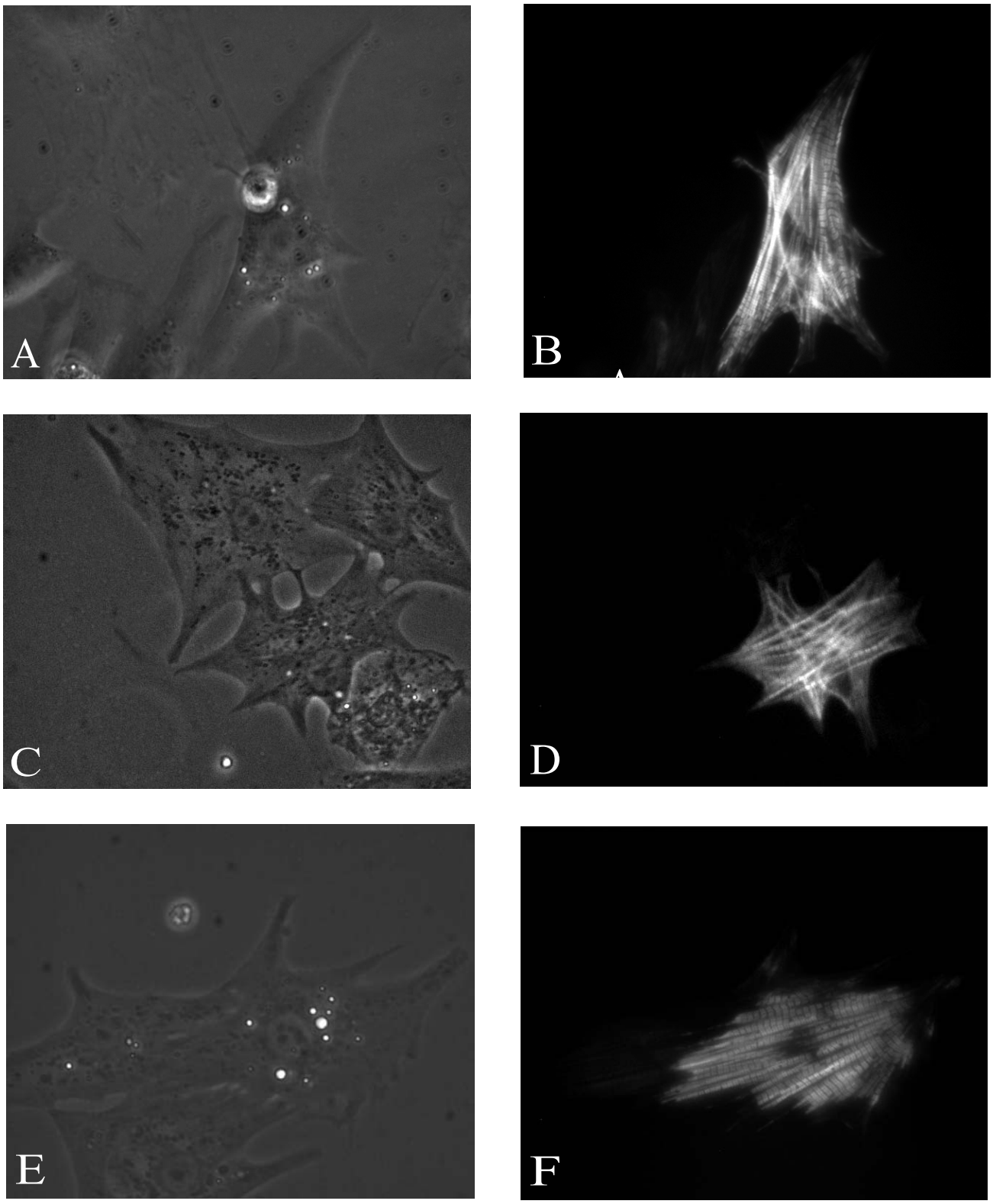


FIGURE 3. The figure above shows the phase and fluorescence images of three different neonatal myocytes. Images were taken 72 – 84 hrs after the myocytes were exposed to a plasmid that targets myosin and platted. **A**, **C**, and **E** show the phase images for three different myocytes that were platted at mid to high density to allow interaction with other myocytes. **B**, **D** and **F** show three different myocytes that absorved the plasmid during electroporation, making the myosin in thier

protomyofibrils visible under fluorescent light. Notice the unorganized, multidirectional protomyofibrils in **B** and **D**, which contrast with the well organized protomyofibrils in **F**.

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 cell's 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 (Reuben et al., 1998). The new method for analyzing the adult cardiocytes produced results equivalent to those obtained by tracking myocyte boundaries. 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 cell's perimeter and area could be detected by image analysis, with the understanding that contraction was likely accompanied by a retraction of the cell's boundaries and a slight increase in its elevation. Such changes in elevation have been recorded using the atomic force microscope, or AFM (Kliche et al, 2006). Cell perimeter and cell outline changes recorded during neonatal cell contraction proved to be inconsistent and erratic, and usually showed increases, not decreases, in area and perimeter. Such measurements in adult cardiocytes, on the other hand, 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 signal generated by the new neonatal method produces contraction vs. time graphs that are very similar in behavior to those of the adult cardiocytes.

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