



Short communication

A software tool for studying the size and shape of human cardiomyocytes



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ABSTRACT

Background and objectives: Due to development of imaging systems the amount of digital images obtained in the biological field has been growing in recent years. These images contain information that is not directly measurable, e.g. the area covered by a single cell. In most of the current imaging programs the regions of interest (ROI), e.g. individual cells, need to be manually outlined. Automation of processing and analyzing the images would ease researchers' workload and provide results that are more reliable. In this work our goal was to write software that automatically segments human cardiomyocytes from images, calculates their areas and variations in the direction of the largest and smallest spread.

Results: We developed software that eased the workload of biomedical laboratory personnel such that they do not have to do manual image segmentation or learn to use software that requires programming skills. The software made a correct segmentation in most of the cases and outperformed the intensity oriented baseline method written in ImageJ in 95% of comparisons. The baseline method estimated cell- and background areas by averaging dark background and bright foreground areas.

Conclusions: Our software can be used in the calculation of cell areas and extents in the case where immunolabeled cells are imaged with a fluorescent microscope. In the future the functionality of the program could be extended with machine learning methods that use the user actions as teaching material in the cases where automatic segmentation fails.

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1. Introduction

Human induced pluripotent stem cells (hiPSCs) offer a powerful tool to study diseases, especially genetic cardiac diseases, which have mostly been studied with animal models or genetically modified cell lines. Sets of hiPSCs have been used to study various cardiac diseases including dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) [1–4]. In all these publications the size of hiPSC-derived DCM or HCM cardiomyocytes has been larger than the size of hiPSC-derived control cardiomyocytes. This reflects the situation in the heart tissue of HCM patients [5].

The study of living differentiated cells is largely based on light microscope imaging augmented with fluorescent labeling techniques [6]. Single images can further be combined into a video

stream. For instance, Ahola et al. have presented video based methods for studying beating dynamics and segmentation of cardiomyocytes [7]. Large amounts of high quality images or video streams can provide such information about cell characteristics that has not been considered before. Processing the growing number of images would require an entirely automated program. Such a program could be used as a full time analyzer that captures the responses of cardiomyocyte samples exposed to different treatments and environments in real time. Furthermore, it could be used in different pattern recognition [8] and data mining contexts [9]. However, the implementation of such is very difficult. The difficulties arise from varying noise content in the images, artefacts and overlapping cells in a sample.

The aim of this research was to take a step towards an automated system and develop easy and user-friendly software for analyzing the cell sizes. One of the main features was that the cells would be outlined or segmented automatically without any manual region of interest (ROI) selection. Automatic segmentation of images is

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a widely studied area and good solutions exist [10,11]. However, many applications have their own distinct nature that has to be taken into account in the implementation. As a starting point the multipurpose ImageJ program [12] can be applied. ImageJ provides tools for manual ROI selection and has a property that allows users to create their own macros that can be used in automatic segmentation. However, we wanted to develop an automated software tool that allows the user to calculate the cell areas without any programming skills.

2. Materials and methods

2.1. Cell culture, immunocytochemistry and imaging

This study was conducted in accordance with the Ethics Committee of Pirkanmaa Hospital District (R08070). UTA.07801.HCMM hiPSC line, carrying HCM mutation in myosin-binding protein C (*MYBPC3-Gln1061X*), was differentiated into cardiomyocytes with END-2 co-culture and dissociated into single cells by Collagenase A treatment prior to immunocytochemical staining [13]. Cardiomyocytes were fixed with 4% paraformaldehyde and stained with primary antibodies identifying sarcomeric proteins: 1:200 Troponin T (Abcam), 1:400 myosin-binding protein C (MYBPC, Santa Cruz Biotechnology), 1:1500 α -actinin (Sigma Aldrich), 1:200 myosin light chain 2a (MLC2a Synaptic Systems), 1:200 MLC2v (Protein Tech), and 1:200 α -tropomyosin (Santa Cruz Biotechnology). Next, the cells were probed with Alexa Fluor 488 or 568 secondary antibodies (Invitrogen) and mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) containing 40, 6-diamidino-2-phenylindole (DAPI) for nuclear staining. A detailed staining protocol has been published before [14]. Cells were imaged by Zeiss AxioScope A1 fluorescent microscope and Zeiss AxioCam MRC5 camera (Carl Zeiss AG, Oberkochen, Germany) using 20 \times and 40 \times air objectives. The pixel size of the image sensor was 3.4 $\mu\text{m} \times 3.4 \mu\text{m}$ and 2 \times 2 binning was used.

2.2. Images

Our data consisted of 228 tiff images with the resolution of 968 \times 1292 pixels. Of these images, 95 had the magnification of 20 and their mean intensity value was 41 and the standard deviation was 41. The other 133 images had the magnification of 40 and their mean intensity value was 27 and the standard deviation 49. The main difficulties in segmenting cells from the images were differences in the intensities of cell areas within an image and slow changes in the intensity in the border of a cell and image background. In this study, the cardiomyocytes were stained with antibodies recognizing sarcomere proteins. Fluctuations in image intensity caused by the striated pattern of the sarcomere structure makes the segmentation difficult due to the merging of low-intensity cellular regions with the background. This can lead to the failure of segmentation methods based on intensity thresholding. Slow intensity changes can also originate from the imaging process. An example of slow intensity change is presented in Fig. 1. The left side image is processed with an intensity thresholding method implemented with ImageJ and the right side is the result of our method.

2.3. Image preprocessing

The images were processed using the OpenCV [15] library in two successive phases. First the areas of significant change in intensity were searched in x - and y -directions for all pixels in an image. As a significant change we considered values that were greater than 1.2 times the average change in the image. In order to suppress the effect of single noise spots, but at the same time, to spread the

change to a larger area we used a 7 \times 7 window where a single row was $[-1-1-10111]$ when searching for a change in y -direction. In the case of x -direction a single row was $[-1-1-10111]^T$ where T is the transpose operator. The changes were finally calculated by summing the squares of changes in individual directions and taking the square root from the sum. From this change image we formed a black & white image that resembles the area of the studied cell. Parts c and d in Fig. 2 present the intermediate result of the first phase using a randomly selected example image. Frequency response images are transposed in order to get the image sizes of both rows equal. The result of a Sobel filter is presented for comparison because it is a corner stone of any edge detection algorithm. Use of Sobel filter leaves a significant amount of noise spots in the image while average intensity change filter suppress them. Finally, in the first phase, we removed such connected components of the images that had less than 500 pixels.

In the second phase we made a morphological dilation to the black&white image that accounts for the locations of significant change. The structuring element used in the dilation was a 3 \times 3 matrix of ones. This procedure spread the effective cell candidate area from the first phase. Next, we sampled the original image only from the spread candidate area. The black&white mask image that represents the cell areas was created by assigning the value one to such points where the intensity of the sampled image exceeds half of the mean value in the original image. This covers also such cell areas that are darker than the cell area on average. Finally, the potential holes in the mask were filled with selecting small connected components from the complement image and taking the bitwise OR between sampled original images. The selection of thresholds and sizes used in the preprocessing phase is addressed in the discussion section of this paper.

2.4. Cardiomyocyte measures

In our study of cardiomyocytes we were primarily interested in the cell size and shape. In our program the cell size was calculated by counting the number of pixels that covered the segmented area. The shape of a single mature human cardiomyocyte is elongated. In other words, the extent of a mature cardiomyocyte along its longitudinal axis is much larger than the extent of the cell along its transverse axis. This measure is naturally captured in the eigenvalues of vectors that span the segmented area. Eigenvectors are calculated using principal component analysis (PCA).

2.5. User interface

The user interface of our program was implemented using the QT library [16] and was made as simple as possible. A screenshot of our program is presented in Fig. 3. In the file menu the user can select a folder that contains the images to be processed and close the program. When an image is loaded into the software, cells are automatically segmented and the result is presented on the screen. If the user accepts the segmentation, the areas and the eigenvalues of segmented areas are calculated. When the measures for segmented areas have been calculated, the user can select the next image in the folder by choosing 'Next' in the user interface. If there are errors in the segmentation, e.g. the software has segmented some unwanted cells or artefacts, the user can cut out the faulty areas. In addition, the user can manually cut apart areas that touch each other, e.g. two cells that are attached to each other. When the segmentation fails due to the threshold value used, the user can adjust the threshold with a slide bar. When the user closes the program, all calculated values are automatically saved to a text file that can be used later in statistical analysis.

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