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An automated approach to analyze microstructural remodeling from confocal microscopies of ventricular myocytes from diseased hearts

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Abstract

Various types of heart disease are associated with structural remodeling of cardiac cells. In this work, we present a software framework for automated analyses of structures and protein distributions involved in excitation-contraction coupling in cardiac muscle cells (myocytes). The software framework was designed for processing sets of three-dimensional image stacks, which were created by fluorescent labeling and scanning confocal microscopy of ventricular myocytes from a rabbit infarction model. Design of the software framework reflected the large data volume of image stacks and their large number by selection of efficient and automated methods of digital image processing. Specifically, we selected methods with small user interaction and automated parameter identification by analysis of image stacks. We applied the software framework to exemplary data yielding quantitative information on the arrangement of cell membrane (sarcolemma), the density of ryanodine receptor clusters and their distance to the sarcolemma. We suggest that the presented software framework can be used to automatically quantify various aspects of cellular remodeling, which will provide insights in basic mechanisms of heart diseases and their modeling using computational approaches. Further applications of the developed approaches include clinical cardiological diagnosis and therapy planning.

1 Introduction

According to the annual survey of the Statistisches Bundesamt, chronic ischemic heart disease (IHD) and heart failure (HF) were the most and third-most common cause of death in Germany in 2010 [1]. IHD and HF combined accounted for 14.1% of deaths. In developed countries, the most common cause of HF is ventricular dysfunction after myocardial infarction [2]. Infarction is associated with structural remodeling, for instance, morphological changes of ventricles [3].

This work aims at improving our understanding of microstructural remodeling of cardiac myocytes associated with infarction. Analyzing the microstructure of myocytes requires an

imaging modality that allows us to identify cell compartments and protein distributions. For this purpose, scanning confocal microscopy is a well established imaging modality in various fields of biological and biomedical research. Scanning confocal microscopy yields three-dimensional (3D) image stacks of sub-micrometer resolution [4]. This imaging method is based on labeling of cell components and proteins with fluorochromes.

Here we investigate structures and proteins involved in excitation contraction coupling (ECC). ECC in cardiac myocytes comprises processes initiated by electric signals at the sarcolemma [5]. These signals ultimately lead to contraction. After sarcolemmal depolarization L-type Ca channels (LCCs) open and allow Ca^{2+} into a junction between the sarcolemma and the terminal cisternae of the sarcoplasmic reticulum (SR). Ryanodine receptors (RyRs) are found in the membrane of the SR. LCCs and RyRs are closely apposed at these junctional sites, which are mainly found in transverse tubules (t-tubules). Ca^{2+} ions that enter junctions through LCCs gate RyRs, which then release Ca^{2+} from the SR into the cytosol where it activates contraction. A number of studies have demonstrated that these processes and the underlying structures are affected by cardiac disease.

In this work, we introduce a software framework for identification of sarcolemma and RyRs as well as characterizing their spatial relationship. The framework was applied to large volume 3D image stacks from fluorescent labeling and confocal microscopy of rabbit ventricular cells from an infarction model. Methods of digital image processing were selected and integrated into the software framework to automate processing and data analysis.

2 Methods

2.1 Animal Model of Infarction

Infarction was induced in male New Zealand white rabbits (n=5) by ligating the circumflex coronary artery as previously described [6]. After 21 days, the heart was extracted and cells were isolated from the right ventricle and 4 regions of the left ventricle. The regions were located in increasing distance to the infarct.

2.2 Fluorescent Labeling

Wheat germ agglutinin (WGA) conjugated to Alexa Fluor 555 (Molecular Probes, Eugene, OR, USA) was used to label the glycocalyx surrounding the sarcolemma. Cells were incubated with WGA and fixed as described in [7]. RyRs were labeled using monoclonal anti-RyR2 (Pierce Biotechnology, Rockford, IL, USA) and a goat anti mouse IgG (H+L) antibody conjugated to Alexa Fluor 488 (Molecular Probes). Incubation followed the procedure described in [8] with two exceptions: Permeabilization was done using phosphate buffered saline (PBS) with 0.1% Triton X-100, and the primary antibody was prepared in PBS.

2.3 Confocal Imaging

3D image stacks of WGA and RyR signals were acquired using a LSM 5 Live Duo confocal microscope (Carl Zeiss, Jena, Germany). The objective lens was a 63× oil immersion lens

with numerical aperture of 1.4. The RyR associated Alexa Fluor 488 was excited with light at a wavelength of 488 nm. Emitted light was band-pass filtered with wavelengths from 505 to 530 nm. The WGA conjugated Alexa Fluor 555 was excited at a wavelength of 543 nm. Emitted light was long-pass filtered with a wavelength of 560 nm. The pinhole diameter was set to 1 Airy unit. A two-track protocol was used, where two-dimensional WGA and RyR images were acquired immediately after each other. Thus, acquisition was quasi-simultaneously for corresponding images. In previous work we established that crosstalk was negligible using this protocol.

In these images, the y-axis was aligned with the long axis of a cell. Images were sampled with a resolution of 100 nm in x-, y- and z-direction. Each stack comprised 512×128 voxels in x- and y-direction, respectively. Up to 200 images were acquired in z-direction.

2.4 Image Processing

2.4.1 Noise Removal—Images were filtered with spatial low-pass filters to reduce Poisson-distributed shot noise [9]. A Gaussian filter ($5 \times 5 \times 5$ voxels, $\sigma = 1$) was applied to the WGA images. RyR images were filtered with a 6-neighbor-average filter.

2.4.2 Removal of Background Signals—Image data exhibited background signal intensities, which were detected and removed. To detect the background signal, intensity profiles along the z-axis were extracted from regions outside of the cell. The lines with the least mean intensity (containing only the background signal outside the cells) are averaged slice-wise. A depth-dependent exponential fit is applied to the average background intensities. For each slice, the corresponding intensity from the exponential fit for its depth is subtracted from all voxels.

2.4.3 Correction of Depth-Dependent Attenuation—The image stacks exhibited depth dependent attenuation of signal intensity, which is caused mainly by absorption of excitation and emitted light. We measured the attenuation for each stack and fitted it to a mono-exponential function. Image stacks were corrected for attenuation by slice-wise multiplication of intensities with the inverse of corresponding values of the exponential fit.

2.4.4 Deconvolution—Impulse responses of a confocal microscope are characterized by the so-called point spread function (PSF). Images from the confocal microscope are the result of a convolution of the original fluorescent distribution with the PSF. Intensity g is therefore described as

$$g(\vec{x}) = (f \star h)(\vec{x}) = \int \int \int_{-\infty}^{\infty} f(\vec{x}') h(\vec{x} - \vec{x}') d\vec{x}'$$

with the original intensity distribution f and the PSF h .

We partially reversed this convolution by using the iterative Richardson-Lucy deconvolution algorithm [10]. Based on Bayes' theorem, a maximum likelihood estimate of f is determined for a measured g and known h [11]. The algorithm is iterative and each iteration is calculated as

$$g_{n+1} = g_n \left(\frac{g_0}{g_n \star h} \otimes h \right)$$

with the cross-correlation operator $\otimes$ and $g_0 = g$.

PSFs for different wavelengths and lenses were generated by averaging images of small fluorescent beads stabilized in agar. Cross-sections through an exemplary PSF are presented in fig. 1.

2.4.5 Analysis of WGA Images—WGA stacks were segmented by thresholding after closing by dilation, median filtering and erosion, repeated 3 times [9]. The threshold was set to mode + 1.3 standard deviations (SD). A 3D Euclidean distance map was created from the segmented WGA data. We calculated the intracellular volume of a cell segment after performing a closing operator and median filtering to the WGA channel. The intracellular volume was then determined by region growing [9].

The spatial distribution was characterized using 3D discrete Fourier analysis [12]. Fourier transform mathematically maps a function to a frequency spectrum. Using the fast Fourier transform (FFT) algorithm, discrete 3D frequency spectra of the intensities were calculated from the image stacks.

2.4.6 Analysis of RyR Images—Local maxima in the RyR signals identified RyR clusters. The corresponding values in the WGA distance map determined the RyR–sarcolemma distance. Dividing the number of detected RyR clusters by volume of the cell segment yielded the RyR density. Frequency spectra were calculated as described above for the WGA images.

2.4.7 Data Conversion and Software Framework—Image data were converted to the kaLattice data format [13]. The processing methods described above were implemented in MATLAB (Version 7.11.0.584; The MathWorks, Inc., Natick, MA, United States) using the Signal Processing Toolbox, the Statistics Toolbox and the Image Processing Toolbox. Some processing tasks were accomplished using efficient tools from the kaTools distribution, a framework of image processing and visualization tools around the kaLattice data format implemented in C++ [13]. All tasks – including calls to external tools – are automated within the MATLAB programs. A graphical user interface was designed to simplify the process. After selecting the microscope images, no further user interaction was required for image processing and data analysis. However, various parameters can be adjusted interactively, either for an image or a set of images.

3 Results

3.1 Spatial Organization

We evaluated the developed software framework using data from one exemplary animal after 21 days of infarction. Fig. 2 shows a cross-section from a 3D stack (a) before processing, (b) after noise reduction and attenuation correction, and (c) after deconvolution.

The intensity distribution of the Fourier transform was analyzed for spatial frequencies corresponding to sarcomere spacing ($2 \pm 0.5 \mu\text{m}$). The intensity histogram in fig. 3 reveals a predominant orientation in x (0°) and y (90°) direction. The reciprocals of dominant spatial frequencies in y-direction were used as measure for the spacing of the t-tubules. Table 1 shows the t-tubule spacing in different regions. The spacings of t-tubules in normal cells and cells closer to the infarction were similar with the exception of region 2. The intensity ratio as a measure of the directionality were similar as well with the same exception.

3.2 RyR–Sarcolemma Distance

Fig. 4a shows RyR clusters in a slice of the WGA distance map. In the animals studied, most RyR clusters were in close proximity to the sarcolemma. However, fig. 4b shows that some clusters were not associated with the sarcolemma. To characterize the spatial relationship, we measured the distance between RyR clusters and the sarcolemma. Fig. 5 indicates that this distance increases for cells closer to the infarct.

3.3 RyR Cluster Density

Remodeling of RyR clusters was assessed by measuring their volume density and spatial relationship. Table 2 indicates that the density decreases while the Euclidean nearest neighbor distance increases for cells close to the infarction.

4 Conclusions

We presented an automated software framework for analysis of microstructural remodeling in cardiac myocytes. The software framework allowed us to detect remodeling of cellular micro-structure associated with infarction.

We suggest that findings from this work can be used to adapt electrophysiological cell models to pathological cases. Modeling and simulation of cardiac electrophysiology helps understanding mechanisms in the heart that lead to pathologies like HF.

The presented software framework can be applied to tissue sections, e.g. from clinical cardiac biopsies. Potential clinical employment of the approach include cardiological diagnosis and therapy planning.

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Figure 1.

Cross-sections through averaged PSF for 63 $\times$ oil immersion lens and excitation at a wavelength of 488 nm. The PSF was averaged from 1544 images of fluorescent beads with a diameter of 100 nm. Images were sampled at 100 nm $\times$ 100 nm $\times$ 100 nm.

Figure 2. Overlay of WGA (red) and RyR signals (green) from a cross-section through a 3D image stack from a rabbit ventricular myocyte. (a) Unprocessed image. (b) Image after noise filtering and attenuation correction. (c) Deconvolved image. Scale bar: 2 μ m.

Figure 3. Mean intensity distribution in the Fourier transform of image stacks (n=36) for spatial frequencies corresponding to distances of $2 \pm 0.5 \mu\text{m}$. Both channels show maxima for 0° – 10° and 90° – 100° .

Figure 4.

(a) Overlay of sections from the WGA distance map (higher red intensity $\equiv$ greater distance) and thresholded RyR clusters (green). (b) 3D reconstruction of the WGA (red) distribution and markers for RyR cluster centers (green).

Figure 5.
Distance of RyR clusters to the sarcolemma in different regions. Number of samples per region as in table 1. Error bars show the SD.

Table 1

Reciprocal of the dominant spatial frequencies in y-direction of the WGA channel from cells. Intensity ratio is the ratio of intensities within $\pm 22.5^\circ$ of the y axis to all intensities in the considered frequency region. Regions 1–4 are from the left ventricle, 1 being closest to infarction, 4 being farthest. RV is right ventricle. n is the number of analyzed cells. Data are presented as mean $\pm$ SD.

Region	n	y-spacing	intensity ratio
1	9	1.84 $\pm$ 0.09 μ m	8.4 $\pm$ 1.2 %
2	4	1.71 $\pm$ 0.18 μ m	6.4 $\pm$ 0.8 %
3	8	1.80 $\pm$ 0.11 μ m	8.5 $\pm$ 2.1 %
4	8	1.79 $\pm$ 0.13 μ m	8.8 $\pm$ 0.7 %
RV	7	1.80 $\pm$ 0.14 μ m	8.1 $\pm$ 1.1 %

Table 2

Densities and nearest neighbor distances of RyR clusters in cells from one animal.

Region	n	RyR cluster density	cluster distance
1	9	$0.37 \pm 0.05 \mu\text{m}^{-3}$	$0.76 \pm 0.08 \mu\text{m}$
2	4	$0.44 \pm 0.05 \mu\text{m}^{-3}$	$0.77 \pm 0.05 \mu\text{m}$
3	8	$0.37 \pm 0.07 \mu\text{m}^{-3}$	$0.75 \pm 0.06 \mu\text{m}$
4	8	$0.43 \pm 0.09 \mu\text{m}^{-3}$	$0.73 \pm 0.07 \mu\text{m}$
RV	7	$0.46 \pm 0.10 \mu\text{m}^{-3}$	$0.70 \pm 0.10 \mu\text{m}$