Automated segmentation analysis of gap junctions using confocal fluorescent microscopy

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Introduction

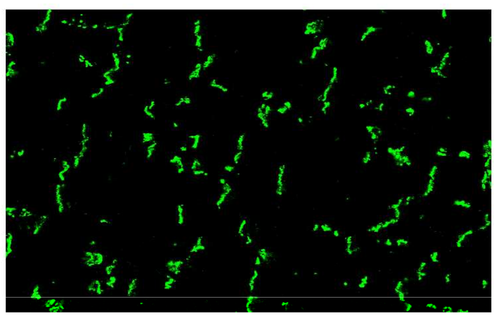
Fluorescent microscope techniques are frequently used to measure the intensity, the cellular distribution of the immunofluorescent labeled cardiac gap junctions (GJ).

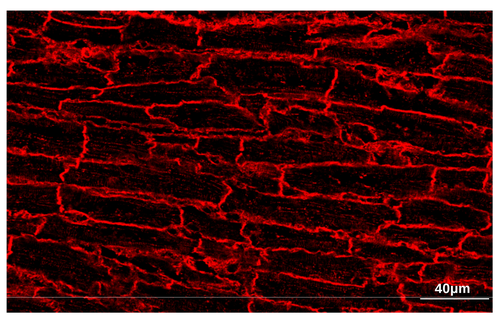
Detection of GJs on the fluorescent images is time-consuming and usually manually performed with the available image analyzers, because accurate and reproducible object identification is necessary for quantification.

The aim of this study was to develop a fully automated algorithm for segmentation and quantification of connexin 43 (Cx43) the main gap junction forming protein in the heart.

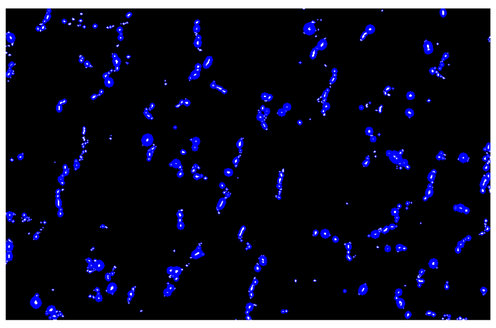
Results

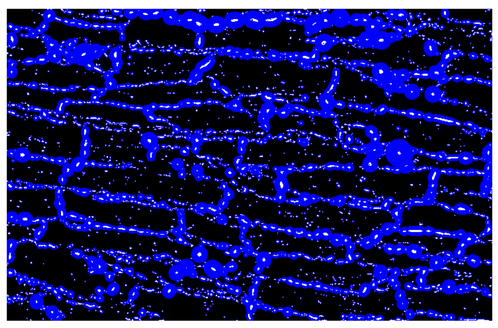
Automatic segmentation algorithm



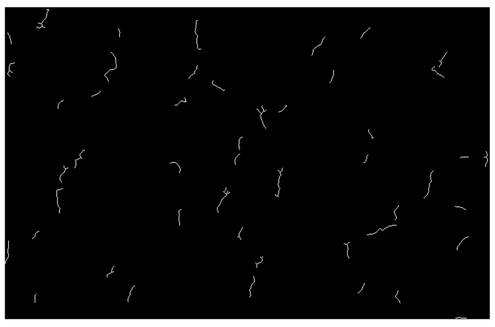


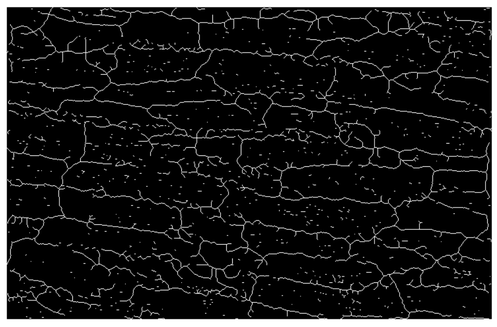
1. Microscopy images from myocardial sections, left panel shows FITC labeled Cx43 (green) located at the intercalated discs or on the lateral membrane part and the right panel shows the myocardial sarcolemma labeled with WGA - TexasRed (red)



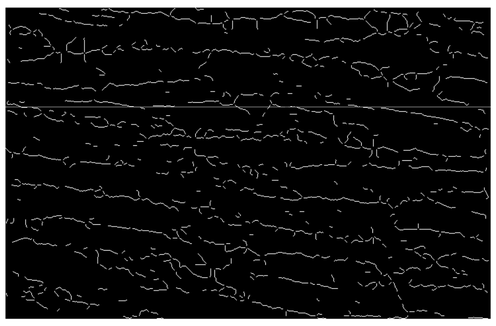


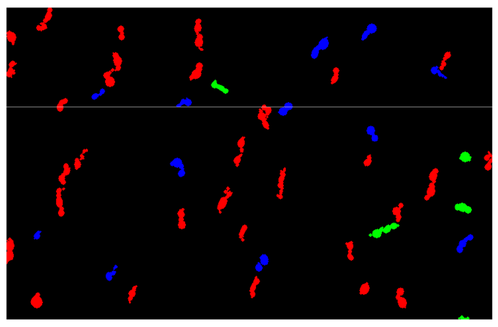
2. Finding discs with maximum radius to locate the possible center of cell edges on both the Cx43 and membrane labeled images





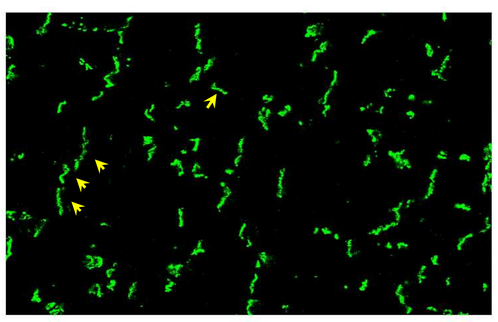
3. Standard 2D skeletonization using the union of discs from the previous step

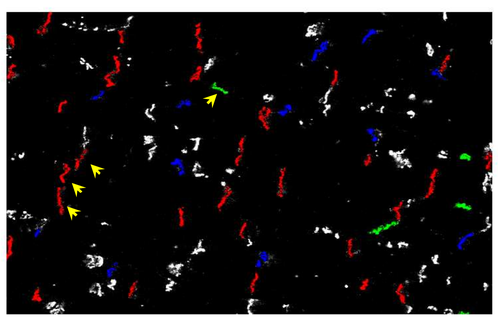




4. After adaptive noise suppression the skeleton obtained from the Cx43 labeled image was subtracted from the skeleton of the membrane image to determine the orientation of the cells.

5. Clusterization of the "Cx43 mask" from the enhanced skeleton was carried out using the orientation angle obtained from the previous step. The three clusters are, the frontal (red) the lateral (green) and unknown (blue) Cx43 signals.





6. Lateral and frontal Cx43 signals can be distinguished using the clustered "Cx43 mask"
   - red objects: frontal Cx43 (Cx43 intensities located at the intercalated discs)
   - green objects: lateral Cx43 (Cx43 intensities located at the lateral membrane parts)
   - blue objects: unknown Cx43 signals
   - white objects: unspecific staining mainly caused by membrane ruptures

7. The average intensities of the previously determined Cx43 clusters were exported automatically to a csv. file

Materials and Methods

1. Experimental groups:

   - To develop and test the segmentation algorithm images obtained from a previous study were used:

     Under light pentobarbital anaesthesia, in 24 dogs a pacing electrode was introduced into the right ventricle. Twelve out of these animals were paced (**P** group) four times for 5 min at a rate of 240 beats min^{-1}, whereas the other 12 dogs served as controls (**SP**). Three-three dogs form each group were euthanized at various time intervals; i.e. immediately (**P-0h**), six (**P-6h**), twelve (**P-12h**) or twenty-four (**P-24h**) hours after the pacing stimulus and myocardial tissue samples were taken for immunofluorescent staining.

2. Immunofluorescent technique:

   - transmural tissue blocks were taken from the regions supplied by the LAD (left anterior descending coronary artery) then the samples were immersed in OCT compound, immediately frozen in liquid nitrogen, then stored at -80℃ unt il processing
   - longitudinal sections (8µm) were cut from the mid-myocardium in a Cryostat (Leica)
   - tissue sections were labeled with polyclonal rabbit anti-Cx43 antibody at 4℃ overnight, then incubat ed with FITC (fluorescein isothiocyanate) conjugated goat, anti-rabbit secondary antibody for 1h at room temperature WGA Texas Red (Wheat Germ Agglutinin conjugated to Texas Red) was applied to visualize the myocardial sarcolemma.

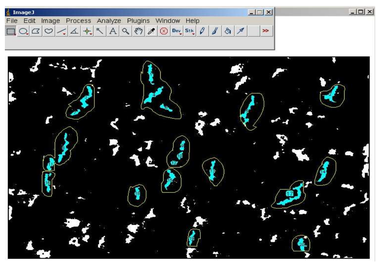
2. Digital image capture:

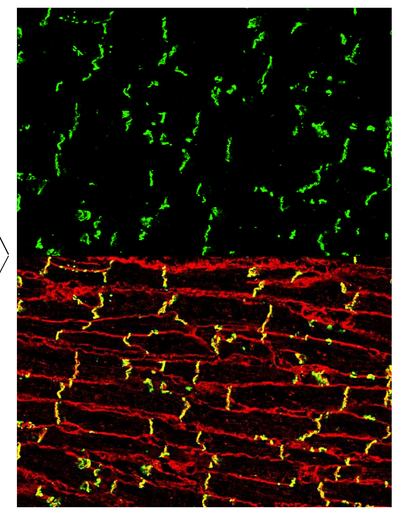
   - Images were acquired by a confocal microscope (Olympus FV1000). Parameters controlling image properties, including zoom, pinhole dimensions, and objective and laser power, were kept constant for image acquisitions. The red emission of WGA-TexasRed was collected at 605±25 nm, and the green emission of FITC was recorded at 525±25 nm. The images of the two emission channels were collected simultaneously.

3. Method for manual quantification:

ImageJ:

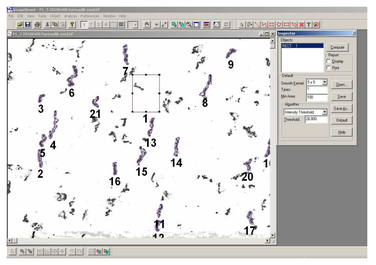
- creating a binary mask from the original image (using 'threshold' and 'close-in' function in ImageJ)
- identification of the intercalated discs with particle analyzer on the binary mask
- calculation of pixel intensities (area) on the original image
- results exported to excel
- data analysis pixel intensities were normalized to the sample with the highest value on each slide





ImageQuant 5.2:

- identification of the intercalated discs with "spot finder" on the grayscale image created from the original
- calculation of pixel intensities
- results exported to excel
- data analysis pixel intensities were normalized to the sample with the highest value on each slide



Comparison of the segmentation algorithm with other image analyzers (ImageJ and ImageQuant5.2)

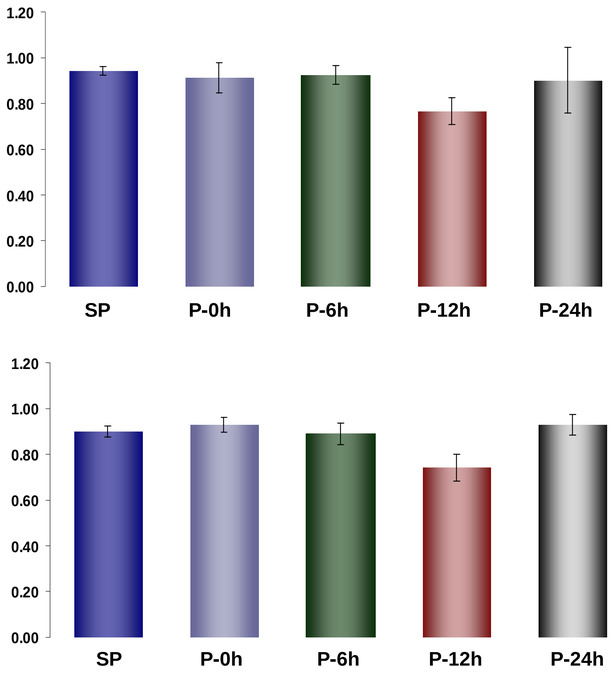


Figure1: Quantification of Cx43 by using ImageQuant5.2

The pixel intensities of each group were normalized to the highest on each slide then the normalized values of the parallel slides were averaged.
All the experiments were performed in triplicate. All data were expressed as mean ± SD

When compared to the sham paced hearts, no alterations were found in the Cx43 content immediately and 6 hours after rapid cardiac pacing. However there was a marked reduction of the Cx43 labeled area 12 hours after the rapid cardiac pacing that was elevated to the control level at 24 hours.

Figure2: Quantification of Cx43 by using the segmentation algorithm

The pixel intensities of each group were calculated as described in Figure 1.

After using the new segmentation algorithm, the same alterations were found in the Cx43 content when compared to other image analyzers (ImageQuant5.2)

Conclusion

This new algorithm provides a fully automatic segmentation method for identification of both the lateral and frontal Cx43 signals. The final clusters highly represents their corresponding original data.

The quantification process is user independent and accurate. The same alterations in Cx43 signal intensities were found in response to rapid cardiac pacing when compared to other image analyzers (ImageJ and ImageQuant5.2).

This segmentation method is not iterative such as the region growing or snake algorithms, therefore can be adapted easily for real time operations. An average runtime required to process one image is about 6.5 second on a single core PC.