Automated segmentation of gap junctions using confocal fluorescent microscopy

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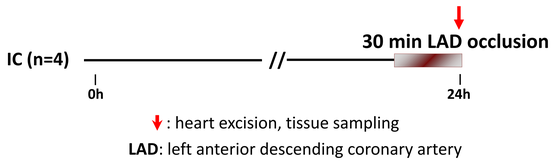
Introduction

Fluorescent microscope techniques are frequently used to measure the intensity and the cellular distribution of connexin 43 (Cx43), which is the main gap junction (GJ) forming protein in the heart. In our previous study, a fully automated algorithm was developed for the segmentation and quantification of GJs at the longitudinally oriented myocardial tissue sections. This segmentation method detects separately the sarcolemma and the intercalated discs (IDs) therefore, the 'polar' GJs (intensities at the IDs) and 'lateral' GJs (signals at the lateral membrane) can be distinguished.

Purpose: We have now developed new algorithms for GJs identification in the transversely oriented myocardial tissue sections and for the segmentation of 'polar' GJs without the need of the sarcolemma staining.

Experimental protocols and Methods



The ischaemic control group (**IC**) from a pervious study was used to obtain images for developing and testing the segmentation algorithms. Myocardial tissue samples were taken from the area supplied by the LAD and the left circumflex (LCX) coronary arteries; samples obtained from the LCX region of the IC controls were considered as non-ischaemic samples.

1. Immunofluorescence analysis
- longitudinal and transversal sections (10µm) from transmural tissue blocks (Cryostat, Leica)
- dual immunofluorescence protocol: **anti-Cx43-Ab** - **anti-ZO1-Ab**

2. Digital image capture
- confocal microscope (Olympus FV1000)
- constants parameters controlling image properties: zoom, pinhole dimensions, objective and laser power
- A_{em}**(Alexa594)=605±25 nm** and A_{em}**(FITC)=525±25 nm**

3. ImageJ
- identification of the IDs with object detection on the confocal images from tissue sections labeled with ZO1
- generation of a binary mask (e.g. ZO1-mask) using imageJ macro
- **Cx43 immunofluorescence**: average pixel intensity of Cx43 segmented with the ZO1 mask
- **Cx43 area fraction**: the area fraction occupied by Cx43 fluorescence within the IDs segmented with ZO1 mask
- the data obtained were expressed as percentages of the non-ischaemic samples (**% of IC-LCX**)
- all the experiments were performed in triplicate
- data are expressed as mean ± S.E.M; *P<0.05 vs. IC-LCX

Conclusion

Detection of GJs on the fluorescent images is time-consuming and usually manually performed, because accurate object identification is often not possible with the implementation of traditional segmentation methods (such as edge detection, region growing or histogram-based methods, watershed transformation and model based segmentation etc.).

These new algorithms provide fully automatic segmentation methods for identification of differently positioned IDs and the final clusters highly represent their corresponding original objects e.g. GJ plaques.

The quantification process is user independent and accurate. The same alterations in Cx43 signal intensities were found in response to acute myocardial ischaemia compared to other image analyzers (ImageJ).

We conclude that these new algorithms provide an automatic, accurate, fast and objective segmentation method for identification of variously oriented GJs at the intercalated discs.

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Results I.

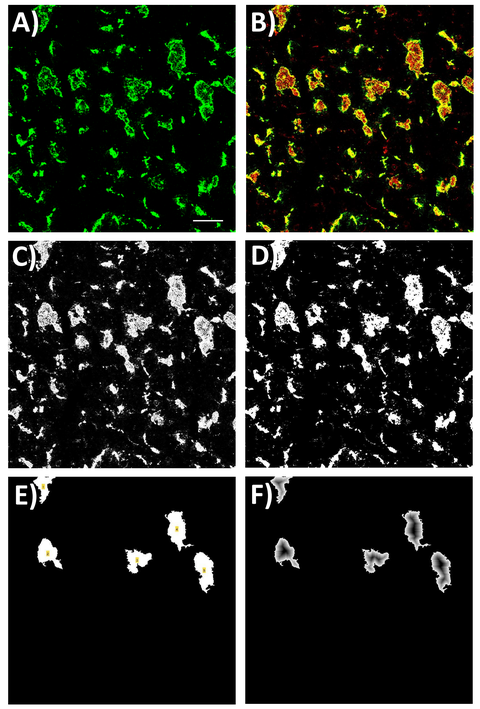

Fig.1. Identification of GJs with automatic segmentation on the transversally oriented myocardial sections
The left panel shows FITC labeled GJs (green) and the right panel shows the composite image of Cx43 co-labeled with anti-ZO1 antibody (red) (Fig1.A-B). After image enhancement (Fig1.C), Otsu algorithm was applied for black and white conversion (Fig1.D). Following morphological closing operation, connected components were labeled and sorted based on their size and the larger objects were selected for quantification (Fig1.E). Incremental peeling was applied on these regions which measures the intensity changes on each layer. All these individual layers are shown on Fig1.F and labeled with grayscale; the lighter the color the outer the layer. Scale bar = 30 µm.

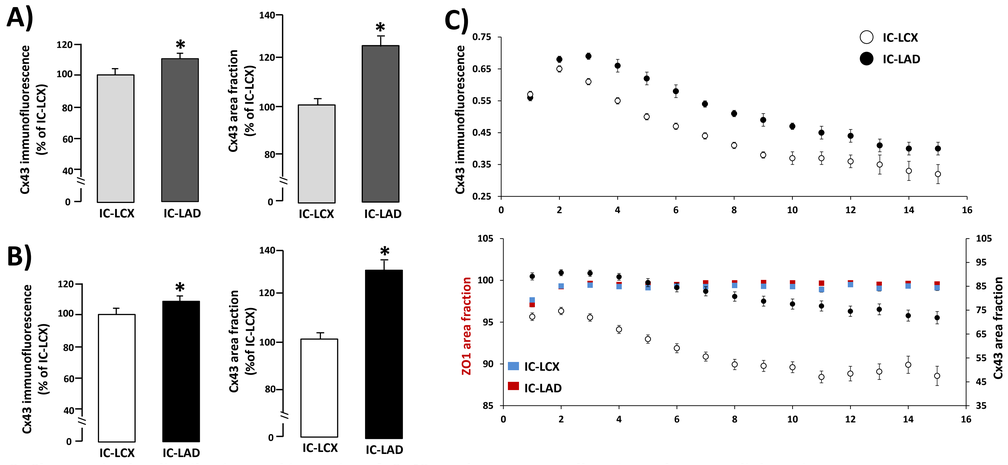

Fig.2. Changes in the distribution and intensity of Cx43 on the transversally oriented myocardial sections
Within the ischaemic region (IC-LAD), the distribution pattern of Cx43 is altered; i.e. an enhanced intensity and area fraction of Cx43 was found on the ZO1 labelled IDs in the IC-LAD samples (Fig2.A).
The same intensity and area fraction changes were found both with the manual (Fig2.A) and automatic (Fig2.B) segmentation method. Furthermore, a more pronounced fluctuation was found in the measured parameters between these two groups when the incremental peeling was applied combined with automatic segmentation (Fig2.C).

Results II.

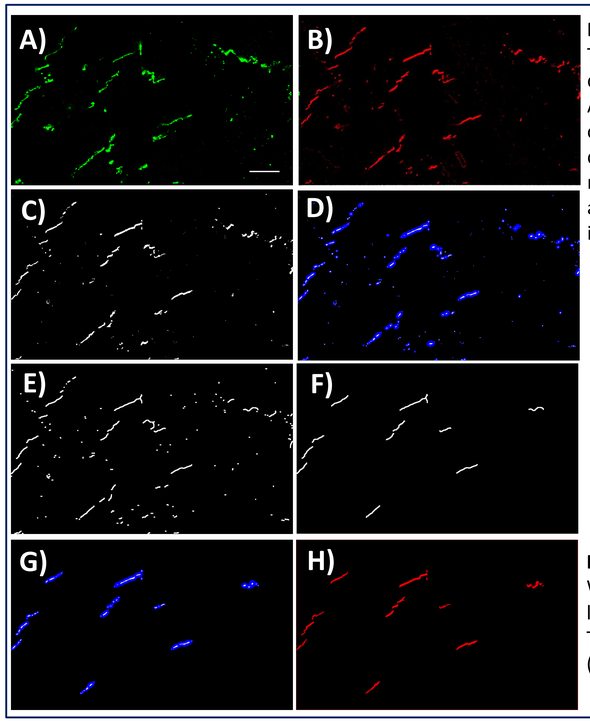

Fig.3. Identification of GJs with automatic segmentation on the longitudinally oriented myocardial sections
The left panel shows GJs labeled with anti-Cx43 antibody (green) and the right panel shows the intercalated discs labeled with anti-ZO1 antibody (red) (Fig3.A-B).
After image enhancement (Fig3.C), discs with maximum radii were applied to locate the possible center of cell edges on the ZO1 labeled images (Fig3.D). Standard 2D skeletonization was used on the union of the discs from the previous step (Fig3.E). The connected components were sorted and selected based on their major axis length and the difference from the mean orientation (Fig3.F). The discs from Fig3.D were selected again to generate ZO1 mask based on the locations of the skeletons from the previous step (Fig3.F). The intensity and area fraction values of Cx43 were measured using this mask. Scale bar = 30 µm.

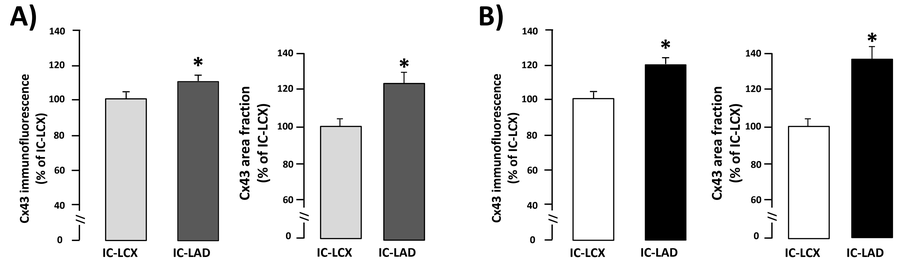

Fig.4. Changes in the distribution and intensity of Cx43 on the longitudinally oriented myocardial sections
Within the ischaemic region (IC-LAD), both the intensity and area fraction of Cx43 was increased on the ZO1 labelled IDs compared to the non-ischaemic controls (IC-LCX) (Fig2.A).
The same intensity and area fraction alterations were found both with the manual (Fig4.A) and automatic (Fig.4B) segmentation method.