Method for Adult Cardiomyocyte Long-Term Viability Monitoring using Confocal Microscopy Techniques

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Abstract

Freshly isolated myocytes lose their viability and functionality very early, from single hours to single days. So their viability is usually tested before the experiments or monitored continuously in periodic time intervals. But simple observations using viability kits (e. g. LIVE / DEAD Cell Imaging Kit, Life Technologies) for this purpose are not sufficient because of Calcein photobleaching and furthermore repeated testing is not possible. In this paper we tested advanced methods based on Calcium degradation and fluorescence lifetime measurement or cardiomyocyte shape properties calculations.

1. Introduction

Enzymatically isolated cardiac cells are widely used in cardiovascular research as single models due to their electrical and mechanical properties. Cardiomyocytes viability and functionality is often necessary to be monitored in long-term experiments. This variable can be monitored through many techniques.

In LIVE/DEAD kit, Calcein as a membrane-permeable live-cell labeling dye is used, mainly in flow cytometry [2,4] or fluorescent microscopy [3]. Upon entering the cell, intracellular esterases cleave the acetoxymethyl (AM) ester group, yielding the membrane-impermeable Calcein fluorescent dye. The polyanionic dye calcein AM is well-retained within live cells, producing an intense uniform green fluorescence in live cells) [6].:

For long-term monitoring simple methods using membrane permeability tests with Calcein AM are not available, e.g. because of photobleaching [5]. Then, once cells are tested Calcein stays inside even while their viability is decreasing rapidly. So another methods - connections of cells shape parameters, Calcein fluorescence intensity and lifetime changes with cardiomyocytes viability were tested for this purpose using Calcein AM (Sigma-Aldrich) and confocal microscope (Leica TCS SP8X) supported by fluorescence lifetime imaging technique.

2. Data acquisition and evaluation

2.1. Data Acquisition

Ensymatically isolated adult rat cardiomyocytes were used in many experiments and isolation protocol is well described [1]. The cardiomyocytes were stained with 1 µM concentration of Calcein AM (Sigma-Aldrich) in about 2-3 hours after the isolation in K-tyrode solution with low level calcium (0.9 mM) and incubated for 40 minutes at 37°C. After the wash-out with the same K-tyrode the final solution was placed to 35 mm confocal dish. Final cardiomyocytes concentration was optimized by checking microscopy images to minimize the overlapped cardiomyocytes and to place enough number of cells useful for statistical analyses on the other hand. Sample image with optimal cell concentration is in Figure 2.

Experiments were performed on the confocal laser scanning microscope Leica TCS SP8 X (Figure 1) equipped with gateable hybrid (HyD) detectors as internal spectral detectors. The picosecond White Light Laser (WLL) Leica Microsystems freely tunable in the spectral range 470-670 nm with 80 MHz repetition rate was used for excitation. The measurement setup was equipped with transparent incubation chamber with temperature and CO_2 level control.
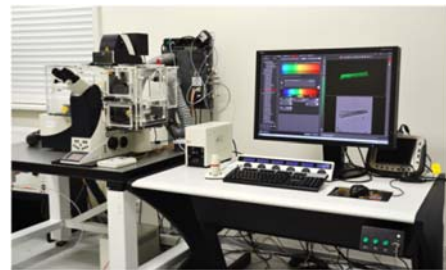

Figure 1. Measurement setup. Scientific laser scanning confocal fluorescent microscope Leica TCS SP8X equipped with heating chamber with CO2 regulation.

Combination of gateable HyD detectors and WLL enables both spectrally and time resolved study of cardiomyocytes. Time resolved settings of HyD detectors in Photon Counting Mode was adapted by TimeGate function that allows arbitrary setting of time window /band in the range of 0-12 ns with the minimum opening of the time window 3.5 ns.

Tile Scan function used for mosaic image acquisition managed to get large scans in multiple FOVs in high resolution.

Overall we hold a set of 8 long-term experiments. Every experiment lasted for 48 cycles. 1 mosaic of brightfield images and stack of 18 mosaics of fluorescent images were acquired in each cycle.
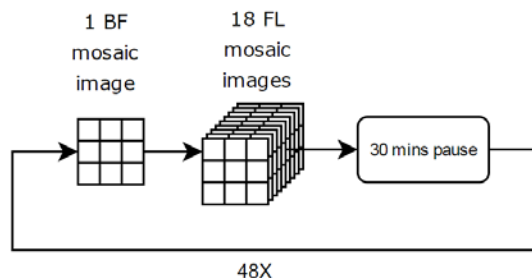

Figure 2. Data acquisition process repeated in cycles.

Every long-term experiment was set to 30 hour. One bright-field (BF) image and stack of eighteen scans using TimeGate function are acquired in a sequence. The 30-minute pause follows. To reduce the photobleaching effect of Calcein each image is scanned in resolution of 512x512 px. For Calcein photobleaching elimination excitation laser power was lowered and high sensitive HyD were used in image scanning process. This approach reflects to image resolution and quality which are sufficient for fluorescence measurement and cell shape evaluations.
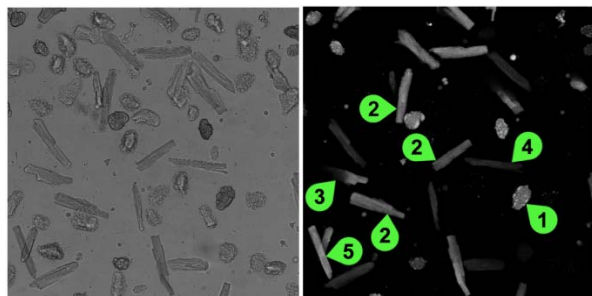
2.2. Data classification

Figure 3. Images with optimal cardiomyocyte concentration sample. Bright-field microscope image (20X) (left) and fluorescent image with Calcein stained cardiomyocytes (right).

Tested cardiomyocytes can be devided into several groups depending on fluorescence intensity, their viability or position in the image.

Five groups of image objects are marked with numbers in Figure 2. Dead cardiomyocytes (1) are later eliminated from analysis together with overlapped two or more cardiomyocytes (5). Cardiomyocytes (2-4) are suitable for analysis. Cardiomyocytes (2) are ideal for long-term analysis due to their high fluorescence level, on the other hand cardiomyocytes (4) easy lose their sufficient fluorescence level in early stages of experiment. Some cardiomyocytes (3) are partly out of the confocal plane and are not suitable for shape evaluations.

In time scale there are several types of cardiomyocytes concerning their viability time changes:

A) Cardiomycytes already dead before the experiment starts.
B) Cardiomyocytes that start decreasing their viability before the experiment starts and die soon.
C) Cardiomyocytes that live and die during the experiment process.
D) Cardiomyocytes that start decreasing their viability at the end of experiment but do not dye before the finish completely.

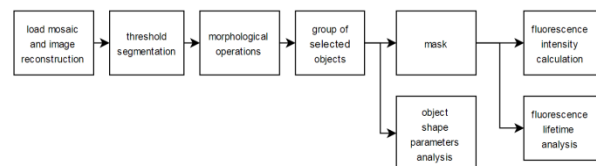## 2.3. Image processing and analysis

Figure 4. Scheme showing stacks of images processing and cardiomyocytes properties evaluation.

Image analysis was fully automated using developer algorithm in Matlab (Figure 4). Each mosaic of FOVs was merged into one complete image that was then used for analysis. The first fluorescent image was used for image objects detection after its segmentation to BW using a simple thresholding. Morphological operations helped to eliminate small objects and objects rounding. Cylindrical viable cardiomyocytes were then kept and circular dead cardiomyocytes were eliminated depending on the level of object eccentricity. The selected objects were used both for cardiomyocytes shape evaluations and concurrently as masks in fluorescence images analysis.

3. Viability evaluation

18 TimeGate bands (Fig. 6, right) with the 0.5 ns step size and minimum opening of 3.5 ns in every 30-minute cycle were measured. Acquired image stacks were

processed in Matlab using another developed algorithm [7]. The result is a pseudocolor image representing different fluorescence lifetimes.
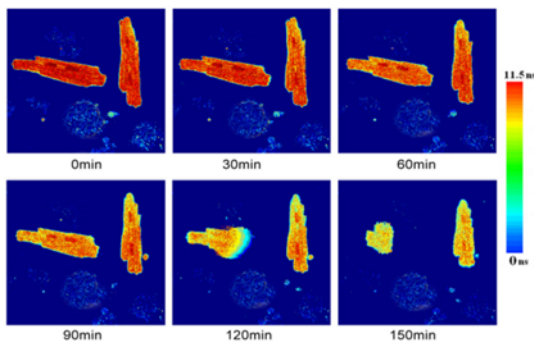

Figure 5. Comparison of flurescence intensity image with resulted pseudo-coloured time resolved image from 0 ns to 11.5 ns. Images are in resolution of 512x512 pixels and 133x133 μm in real size.
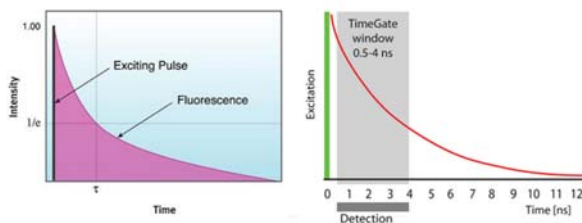

Figure 6. Fluorescence lifetime [8] (left) and illustration of TimeGate time window.

Proposed simplified time resolved observation is similar to Fluorescence Lifetime Imaging (FLIM) but is not comparable in the time resolution. These time resolved images were used for further lifetime analysis by calculating the total lifetime values in analysed image objects.

Overall three different cardiomyocyte parameters were tested:

- Total Calcein fluorescence intensity.
- Cardiomyocyte shape changes.
- Calcein fluorescence lifetime (Quasi-FLIM image analysis).

Classified cardiomyocyte shape parameters include major axis length, object perimeter, object eccentricity, total object area and equivalent diameter. The calculations were made with a help of Matlab functions for measuring properties of image regions.

4.   Results

The graphs show average values calculated from total 67 cardiomyocytes. Cardiomyocytes (28%) were selected from 8 experiments corresponding to 2B and 4B types of cardiomyocytes described in chapt. 2.2. The time range in

graphs was normalized to 150 minutes – to the approximated average time during which cardiomyocytes viability is decreasing from high viability level to necrosis.
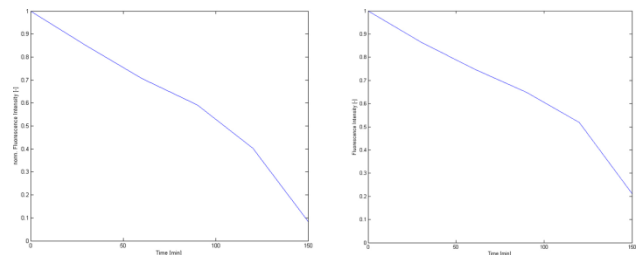

Figure 7. Normalized Calcein fluorescence intensity in 150 min (left) and the same Calcein fluorescence intensity divided by cardiomyocyte object size (right).
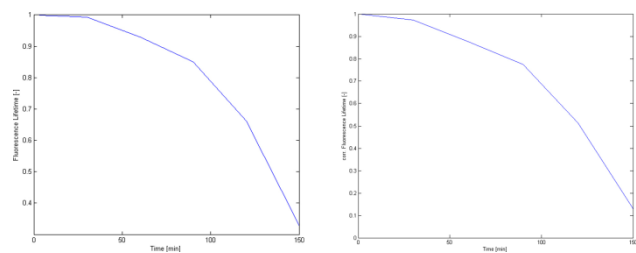

Figure 8. Normalized calculated total fluorescence lifetime in 150 min (left) and the same calculated total fluorescence lifetime divided by cardiomyocyte object size.
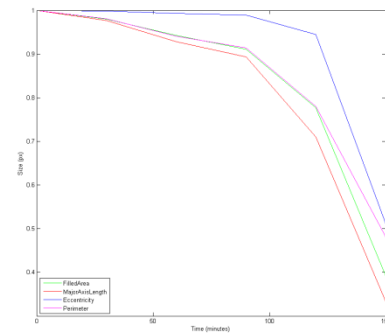

Figure 9. Calculated objects shape properties using Matlab functions for measuring properties of image regions.

MajorAxisLength	Perimeter	Eccentricity	FilledArea	Diameter
34%	49%	54%	40%	63%

Table 1. Lower level in percents where each shape parameter decreased.

In conclusion, the results show a markable connection of both cardiomyocyte shape properties, Calcein fluorescence intensity and quasi-FLIM lifetime with their viability. Calcein fluorescence intensity decrease occurs early and is more strong, however it is affected by its photobleaching. Shape changes (Figure 9) and quasi-

FLIM lifetime changes (Figure 8) occur in late stage with coming necrosis and are independent to environment conditions. Both Calcein fluorescence intensity decrease and total fluorescence lifetime decrease are caused by Calcein degradation.

As shown on the Fig. 7 and Fig. 8, the measured parameters were divided by cardiomyocyte size in each cycle, on which both measured parameters may be dependent, because the cardiomyocyte shape decreases too. But the result charts show no outstanding change in curves character.

Acknowledgements

References

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