

Stereologic analysis of isolated rat ventricular myocytes using the method of vertical sections

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Abstract. Morphology of cardiac muscle cells is difficult to characterize quantitatively for several reasons. In search for a practical approach, we tested applicability of the method of vertical sections, suitable for anisotropic structures, to estimation of surface and volume densities in enzymatically isolated rat ventricular myocytes processed for transmission electron microscopy. Electron micrographs of longitudinally sectioned myocytes were scanned and analysed using generally available digital technologies. We found that the method of vertical sections is a method of choice for cardiac myocytes. Moreover, using of isolated myocytes instead of excised samples brought in advantages of a truly random sampling of the tissue and of improved processing of samples for electron microscopic observation. The new approaches exploited in this study provided reliable data on surface and volume densities and might be considered in future stereological studies of cardiac muscle.

Introduction

Ultrastructure of cardiac muscle cells is profoundly remodelled during development, adaptation, and disease. Quantification of these changes, which are of utmost importance to cardiology, has three difficult aspects: preparation of the tissue without distortions for observation by electron microscopy [1]; selection of a representative sample of the tissue [2]; and statistically correct sectioning and quantification of the image [3]. These are not trivial tasks. Particularly cardiac muscle, as a highly diversified tissue, needs special considerations for fixation and sampling of the tissue [4]. Cardiac myocytes themselves are highly organized (anisotropic) structures and therefore represent difficult objects for stereological analysis, as are striated muscle cells in general. This might be the reason why most studies confined themselves to estimation of volume fractions only, which in contrast to the surface area are less prone to errors [5]. On the other hand, understanding function of cardiac muscle cells needs reliable quantitative data on the surface area of their cellular constituents. Such data are needed for more exact characterization of muscle remodelling, but also to create virtual computer models usable for testing specific hypothesis and verifying, comparing or presenting various theories. To untangle these problems we tested two methodical approaches. First, the applicability of the method of vertical sections [3], developed specially for estimating surface areas in anisotropic structures, and second, the use of enzymatically isolated myocytes. We show by comparison with the published values that the combination of these approaches provided reliable data on both the surface and volume densities of major constituents of cardiac myocytes.

Methods

Isolation of myocytes. Myocytes from the left ventricle and septum of adult male Wistar rats (228 ± 5 g, mean \pm SE, $n = 5$) were isolated with the use of the enzymatic digestion method [6]. Isolation provided at least 50% of brick shaped cells tolerant to calcium. In parallel experiments, functionality of the isolated myocytes was assessed in whole cell patch-clamp experiments [6]. All preparations provided myocytes that had well-developed ionic currents, including the calcium current, and that were responding to stimulation by brief contractions.

Fixation. Isolated myocytes were resuspended in osmotically balanced cacodylate buffer (in mmol/l: 150 NaCacodylate, 2.0 CaCl_2 , pH 7.3) with 2% glutaraldehyde, and fixed for 30 minutes. A loose pellet of freely deposited cells was then post-fixed with 1% OsO_4 (in cacodylate buffer), and contrasted with 1% uranyl acetate (in aqueous solution). After gradual dehydration in ethanol, the pellet was embedded through propylene oxide in Durcupan (Fluka AG, Switzerland). As was shown by Gerdes et al. [1], this procedure provides preparations with almost no changes in cell volume.

Sample preparation. Pellets of fixed isolated myocytes were prepared from 5 animals. Each pellet was divided into 3-5 parts, each of which was embedded separately into Durcupan blocks. From a randomly selected block three ultrathin serial sections of silver interference colour (60 - 80 nm thick) were cut and placed on a Formvar-coated copper grid. The sections were contrasted with lead citrate and studied under JEM 1200 electron microscope (JEOL, Japan). In the inspected sections, longitudinally sectioned myocytes were photographed (Scientia EM Film, 6.5×9 cm, Agfa, Belgium). No myocyte was included more than once in the analysis. Total of 21 myocytes, 7 per animal, were evaluated.

Stereology

The surface and volume densities of the intracellular constituents were estimated from micrographs of the central parts of 23 myocytes photographed at $7500\times$ magnification, so that whole diameter of the myocyte seen in the longitudinal section was captured on the micrograph. The micrographs were digitised at 800 dpi resolution. Stored images were displayed as a positive on the monitor, divided into 6 - 9 fields (total of 174) at $60,000\times$ magnification, and overlaid with the testing grid composed of 143 test points and cycloids. The length of the cycloid corresponded to $0.23 \mu\text{m}$. The cycloid testing grid was generated and properly overlaid with the testing image using the program STESYS [7].

Morphometric analysis was performed using a Pentium computer (133 MHz, 32 MB RAM, 2Mb Matrix Millennium graphic card, 15 inch monitor display screen) equipped with ImageTool v. 1.27 (University of Texas Health Science Centre at San Antonio, TX, USA) and Adobe Photoshop v. 4.0 (Adobe Systems Inc., USA) software.

The volume density, V_v (volume fraction, [2]) of the cell component was estimated as:

$$V_v = p / P, \quad (1)$$

where p is the number of the test points hitting the image of the evaluated component, and P is the number of all points falling on the cell image.

The surface density S_v (surface area per unit volume of the cell) of cellular components was estimated using the method of vertical sections [3], according to which:

$$S_v = 2Ni/LP, \quad (2)$$

where N is the number of all test points in the grid, i is the number of intersections between the cycloid curves and the surface of the structure, L is the total length of all cycloids in the grid, and P is the number of test points that hit the cell image.

Points and intersections were counted manually. Statistical analysis was performed using Origin v. 5.0 (Microcal, USA).

Results

The structural quality of analysed myocytes was assured by selecting only preparations that were satisfactory by the following criteria: high yield of calcium tolerant myocytes in Tyrode solution (> 50% of rod shaped cells); appearance of cells under light microscope (sharp edges, no granules, no blebs, relaxed); and performance in patch-clamp experiments (about 10 pA/pF peak calcium current, brief contractions on stimulation). Nevertheless, special care was still given to selection of myocytes with no signs of ultrastructural damage identifiable under electron microscope (relaxed sarcomeres, no swelling of membranous organelles). It should be mentioned, however, that in samples of isolated myocytes this task was very simple, as most cells showed well-preserved morphology when viewed at any sectioning direction. A large number of good cells in the section turned out to be very convenient since every section contained a large number of exactly longitudinally sectioned myocytes, necessary for the method of vertical sections (see below).

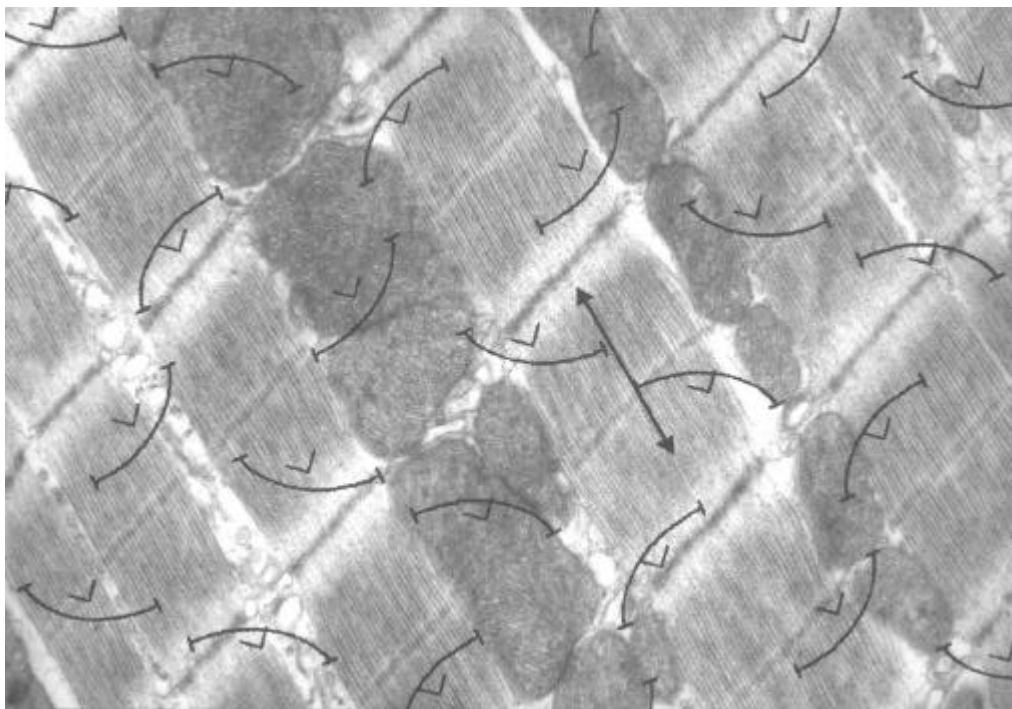


Figure 1: An example of the longitudinally sectioned cardiac myocyte overlaid with the cycloid testing grid. Cusps of perpendicular angles represent the test points. Cycloids delimited with short stripes represent the test lines. Please, note, that the short axis of the cycloids follows the vertical direction of the myocyte (double arrow) parallel to the long axis of the myofibrils. Magnification 24750 \times .

Stereological evaluation of micrographs was done on the computer screen where the image was superimposed with the testing grid (Figure 1) as described in Methods. For illustration of the principle of the method of vertical sections, the test grid shown in Fig. 1 is magnified about 5 \times relative to the image of the myocyte, because the real view cannot be reproduced in print at an acceptable resolution. On the monitor screen, the authentic grid looked much denser with very thin and short lines. The parameters of the grid used for measurements were set to minimize uncertainties in identification of the coincidence of the grid and image features, and to optimise the frequency of intersecting the evaluated structures with a cycloid.

An important requirement of the method of vertical sections [3] is the clear identification of the vertical direction. As shown in Fig. 1, the vertical direction (double arrow) could be unanimously identified in every image, simply as the longitudinal axis of the myofibrils.

Statistics of the volume and the surface density data are given in Tables 1 and 2, respectively. In this study, emphasis was given to organelles participating in the contractile function, as is the common practice in most studies. Therefore we have evaluated the central parts of the myocytes, which were free of nuclei. Counts for other minor constituents were combined with counts of the cytoplasm.

Evaluation of the t-tubules represents a serious problem for difficulty with their identification. As we

Table 1: The mean volume densities [%] of major structures in rat ventricular myocytes.

R	Myofibrils	Mitochond	M+M	Cytoplasm	SR	T-tubule	C+SR+T	Ä%
i	46.3/0.5	30.8/1.2	77.1	12.0/0.9	10.4/0.8	0.4/0.06 ^a	22.8	-0.1
8	46.7/3.5	36.6/0.7	83.3	-	-	-	(16.7)	-16.7
9	48.1	34.0	82.1	(13.2)	3.5	1.2	(17.9)	-13.2
10	47.6/0.7	35.8/0.6	83.4	12.1 ^b	3.5 ^c	1.0/0.1	16.6	0
11	46.7	36.0	82.7	2.0	3.5	-	(17.3)	-11.8
12	55.0/0.1	29.0/1.0	84.0	(12.9)	2.2/0.1	0.9/0.1	(16.0)	-12.9
4	57.8/1.7	28.3/1.3	86.1	-	-	-	14.3 ^d	+0.4
13	61.1/1.0	32.0/1.8	93.1	6.9/0.9	-	-	(6.9)	0
14	54.7/0.9	29.6/1.2	84.3	15.1/0.9 ^e	-	-	(15.7)	-0.6
15	58.5/1.0	35.8/1.0	94.3	3.5/0.2	-	1.4/0.1	(4.9)	-0.8

Data, estimated by the point counting method [2], are rounded to the first decimal and given as mean/SE. Some of the missing values were estimated from the published data by us and are given in parentheses. M+M stands for myofibrils + mitochondria. C+SR+T stands for cytoplasm + sarcoplasmic reticulum + t-tubules. Ä% stands for the disparity of the sum of reported volume densities from 100%. ^a in dyads only; ^b includes nuclei and other structures; ^c sum of the sarcotubules, terminal cisterns, and the rest of the sarcotubular network; ^d all other organelles; ^e includes glycogen, ribosomes, and endoplasmic reticulum. R stands for references.

[i]: This work: 5 male Wistar rats, 230 g; enzymatically isolated myocytes, left ventricle and septum; 300 mOsm all solutions; central parts of myocytes; longitudinal sections; method of vertical sections. [8]: 5 male Wistar rats, 210 g. [9]: rat ventricle. [10]: 5 female rats, 200 - 220 g; left ventricles; transversal sections. [11]: rat ventricles. [12]: 4 male Sprague-Dawley rats, 200 - 350 g; samples from the inner third of the left ventricular free wall; images with nucleus not evaluated; transversal sections. [4]: 3 adult rats, 300 g; 3x3 samples; 400 mOsm Tyrode; fixation by whole heart perfusion. [13]: 3 male Wistar rats, 250 g. [14]: 4 young female Wistar rats, 213.5 ± 10.2 g at the beginning of the study +(63+14) days; 360-380 mOsm fixative; hearts arrested by KCl; left ventricle inner zone between anterior and posterior papillary muscle between the base and the apex; longitudinal sections. [15]: 5 female adult CEHR (Wistar) rats. have not used any labelling, we decided to evaluate t-tubules only when they were located in dyads together with the terminal cisterns of the sarcoplasmic reticulum. Free running t-tubules were usually included to the counts of the sarcoplasmic reticulum, which resulted in about 1% overestimation of the SR volume fraction.

Dispersion of the volume density data among animals was relatively low (see Table 1). With the exception of the t-tubules, the standard errors were inversely proportional to their volume density (correlation coefficient of 0.99). This correlation confirms proper setting of the grid parameters. The large error in the volume density of the very small and scanty t-tubules indicates that the test grid should be set

substantially denser or the images should be evaluated at much higher magnification to increase substantially the probability of finding test points within the t-tubule and reduce dispersion of data.

The surface density data varied slightly more (Table 2), with relative errors between 4 and 10%. In this case dispersion reflects real differences among animals, as frequencies of finding intersections with the evaluated structures were sufficiently high. Again, standard errors were inversely proportional to their surface density (correlation coefficient of 0.86). Although evaluation of t-tubules in dyads does not provide full information on t-tubule membranes, which might be of interest especially to those looking for the surface area of the plasma membrane, it has still its meaning as a comparative measure of the excitation-contraction coupling apparatus.

Table 2: Overview of the mean surface densities [μm^{-1}] of major structures in rat ventricular myocytes.

R	Myofibrils	Mitochondria	SR	T-tubules
				0.14 ±
i	2.36 ± 0.14	1.93 ± 0.07	1.99 ± 0.16 1.46	0.07
10	-	-	1.22 ± 0.05	0.09 ± 0.02
11	-	-	1.19	0.15
12	-	-	1.09 ± 0.05	-
15	-	-	0.71 ± 0.02	0.10 ± 0.01 ^a

The references (R) correspond to those of Table 1. Data are rounded to two decimals and given as mean ± SE. Except of our work [i], all other studies used the line intersection method [2]. ^a T-tubules in dyads only.

The size and tortuousness of the structures is reflected by the surface to volume density ratio. For the myofibrils, mitochondria, sarcoplasmic reticulum, and t-tubules we found values of [$\text{in } \mu\text{m}^{-1}$] 5.06 ± 0.26 , 6.28 ± 0.18 , 19.46 ± 1.28 and 36.73 ± 3.23 , respectively. As expected, standard errors were proportional to the value of the surface to volume density ratio (correlation coefficient of 0.99). These results correspond to the known fact that the cylindrical myofibrils and oval mitochondria have a much smaller S_v/V_v ratio as the highly branched membranes of the sarcoplasmic reticulum or the very thin and long t-tubules.

Discussion

The aim of this work was to develop an alternative approach to quantitative analysis of morphology of cardiac myocytes, which would improve comparability of data among laboratories. We tested the method of vertical sections developed by Baddeley et al. [3] especially for anisotropic structures. This method, although widely used and accepted [16], was never applied to striated muscle cells. A part of the reason could be in the difficulty with generation of the testing grid with cycloid curves. This difficulty was recently overcome by the computer program of Karen et al. [7], which allows convenient optimisation of the grid parameters for the given magnification and analysed structure. In addition to requirements for statistically correct sampling of the tissue and the images, the method of vertical sections requires correct estimation of

the vertical direction in the image of the cell [3]. In the case of striated muscle cells, imminent solution for defining the vertical direction is its identification with the longitudinal axis of the myofibrils, which always form bundles parallel to the longitudinal direction of the muscle cell. This condition of the methods of vertical sections is in perfect agreement with the recommended use of longitudinal sections for reliable stereologic analysis of muscle cells [5]. The very high degree of preferred orientation and periodicity of muscle cell structures in the longitudinal direction makes the use of transversal sections problematic. On the other hand, longitudinal section of a muscle cell can be reliably identified from the appearance of the sarcomere, that is, the contractile filaments between z-lines should be parallel to the plane of the ultrathin section. In our preparations, images of longitudinally sectioned myocytes were found in every section. This is not the case in samples of the whole tissue preparations, in which the longitudinal direction has to be found by a proper reorientation of the sample.

Isolated cardiac myocytes, used in our study, are superior to whole tissue preparations also from the statistical point of view. The cells in the sediment are dispersed at random, regarding both their origin in the tissue and their orientation in the sample. In other words, the specimen of deposited cells is isotropic, and any sectioning plane through the sample is truly random with respect to myocytes as required by the theory [2]. When for some reasons the cells from a specific region of the heart are to be studied, they can also be obtained with enzymatic isolation. In the case of small animals, enzymatic digestion is first applied to the whole heart and then the region of interest is dissected from partially digested tissue and digested to completion. In the case of large animals, a chunk digestion technique may be applied.

Isolated myocytes are preferable to perfused heart or tissue samples also from the viewpoint of fixation. In suspension, a fixative can access every cell directly, which results in very fast and homogenous fixation. Gerdes et al. [1] have shown that with isolated myocytes, application of osmotically balanced fixation solutions may entirely prevent changes in the cell volume during sample preparation. This is not the case with larger tissue samples, where the fixation may not be homogeneous.

Overview of the available literature data in Table 1 demonstrates large differences in the volume density estimates of major cell constituents, in spite of the small values of the reported standard errors. Our data differ from the average of published data by a smaller content of myofibrils and a higher content of sarcoplasmic reticulum. This might point to possible changes in the cell volume either due to swelling in our study, or due to shrinking in most other studies. It should be kept in mind that the average of published data does not represent the true value, which has not been standardized yet. We consider substantial swelling of myocytes in our study improbable, as we kept solutions isoosmotic and as was shown previously [1], under these conditions fixation should not result in changes of the cell volume. Note also that the amount of cytoplasm and mitochondria found in our study is similar to values reported in other studies. We cannot exclude the possibility that the volume of SR in our study was increased due to calcium overload, which could happen during isolation of myocytes, but in parallel patch-clamp experiments we found the function of cells normal. Therefore we believe that our data are not far from the physiological situation. For a brief overview, we have listed experimental conditions reported by others in the legend of Table 1. The reported differences among laboratories are so substantial that we could conclude that the reasons for differences in reported volume densities of different cell constituents are probably complex and include: fixation of the tissue; sample size; selection of myocytes; direction of sectioning; osmolarity of solutions; gender, strain, age, activity, diet and weight differences, etc.

We did not find any data about surface densities of myofibrils and outer mitochondrial membrane in the literature. It is interesting to see that both these values obtained in our study are comparable to the surface density of the sarcoplasmic reticulum. This finding underlines the role of the surface area in the function of these organelles, which occur near each other but differ largely in their volume densities. Comparison of our and published surface density data was possible only for the sarcoplasmic reticulum and the t-tubules, see Table 2. The value for the SR is slightly higher than the average of all reported data obtained by different stereological technique, but it corresponds to the higher volume density of SR observed in our study. The

value for the t-tubules is in the higher range of the reported values. It should be noted, however, that we have counted t-tubule membranes only in dyads, similar as in [15]. Due to the lack of labelling, most reported values seem to underestimate the real extent of the tubular system. Recently, Soeller and Cannell [18] determined in isolated ventricular myocytes of the rat the volume and the surface density of t-tubules of 3.6 % and $0.44 \text{ } \mu\text{m}^{-1}$, respectively, using fluorescent labelling and two-photon confocal microscopy. Both values are considerably higher than those reported before in electron microscopic studies. They also found that only about 60% of the tubules contiguous with the external space occur near Z-lines where they may form dyads. This allows us to estimate the total t-tubule surface density by multiplying our data by a factor of 1.7, resulting in a value of $0.23 \text{ } \mu\text{m}^{-1}$. Considering that only 48 % of t-tubule membranes near the Z-line create dyads [17], we can come to the final estimate of about $0.48 \text{ } \mu\text{m}^{-1}$, a value very close to the result of Soeller and Cannell [18].

Taken together, we show that the combination of the stereological method of vertical sections and the use of isolated myocytes provided reliable data on both the surface and the volume densities of cellular components in a complex tissue, as is the cardiac muscle. Using suspension of myocytes was found very practical for even fixation of the cells, for a statistically representative selection of myocytes from the tissue, for truly random sectioning of myocytes, and for the ease of finding longitudinal sections. The method of vertical sections was found to be a practical tool for straightforward analysis of highly anisotropic structures represented by striated muscle cells.

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