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Original article

Sub-microscopic analysis of t-tubule geometry in living cardiac ventricular myocytes using a shape-based analysis method

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ABSTRACT

Transverse-axial tubules (TTs) are key structures involved in cardiac excitation-contraction coupling and can become deranged in disease. Although optical measurement of TTs is frequently employed to assess TT abundance and regularity, TT dimensions are generally below the diffraction limit of optical microscopy so determination of tubule size is problematic. TT diameter was measured by labeling both local surface membrane area and volume with fluorescent probes (FM4-64 and calcein, respectively), correcting image asymmetry by image processing and using the relationship between surface area and volume for a geometric primitive. This method shows that TTs have a mean (±SEM) diameter of 356 ± 18 nm in rabbit and 169 ± 15 nm in mouse ($p < 0.001$). Rabbit TT diameters were more variable than those of mouse ($p < 0.01$) and the smallest TT detected was 41 nm in mouse and the largest 695 nm in rabbit. These estimates are consistent with TT diameters derived from the more limited sampling of high-pressure frozen samples by electron tomography (which examines only a small fraction of the cell volume). Other measures of TT abundance and geometry (such as volume, membrane fractions and direction) were also derived. On the physiological time scale of E-C coupling (milliseconds), the average TT electrical space constant is ~175 μm in rabbit and ~120 μm in mouse and is ~50% of the steady-state space constant. This is sufficient to ensure reasonable electrical uniformity across normal cells. The image processing strategy and shape-based 3D approach to feature quantification is also generally applicable to other problems in quantification of sub-cellular anatomy.

1. Introduction

In cardiac muscle, the transverse-axial tubules (TTs) are invaginations of the surface sarcolemma (SS) that form a complex network throughout the cell [1,2]. TTs enable rapid propagation of the action potential throughout the cell, and permit near synchronous excitation-contraction (E-C) coupling in ventricular [3] and atrial cardiomyocytes [4] via activation of "calcium release units" [5]. Many disease states are associated with disruption of TT structures and, in heart failure and this correlates strongly with reduced contractile performance (e.g. [6,7]). Among pathological changes in the TT system are local dilations, loss of t-tubules and their malformation [7–10] that accompany the progression to heart failure. It has also been proposed that altered diffusion between the TT lumen and the extracellular space may take place [11].

Although previous studies have already quantified some features of TT organization in living cells, for example TT regularity (e.g. using Fourier analysis [7]), density [12,13] and orientation [6,8], few have measured TT diameter in living myocytes. The primary obstacle to direct optical measurement of TT diameter is the limitation imposed by the conventional optical diffraction limit. Electron micrographs suggest that mouse TT diameters are likely to be 40–200 nm [14,15] which will be unresolved in confocal microscopes with a diffraction limit of ~200 nm. Super-resolution microscopy (such as Stimulated Emission Depletion, or STED microscopy) can reveal the lumen of some tubules [9], but such methods are technically demanding, have a more limited axial resolution and may not always be applicable to living cells (the latter being desirable to avoid fixation artifacts and to allow correlation with function). As a more accessible alternative, the limited optical resolution of a (confocal) microscope can be circumvented by immersing cells in a fluorescent medium and using signal intensity as an indicator for TT size [16,17]. The latter methods rely on accurate segmentation to produce a binary skeleton of the TT network, which is needed to control for the complexity of TT branching, and this becomes more difficult at smaller TT diameters (due to decreasing signal-to-noise ratio).

Abbreviations: E-C, excitation-contraction; EM, electron microscopy; ET, electron microscopic tomography; FWHM, full-width at half maximum; SS, surface sarcolemma; TTs, transverse-axial tubules; D_{TT}, t-tubule diameter; *V:SA*, volume to surface area ratio.

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The mouse is a widely used model for studying cardiac function. However, existing data suggests that murine cardiac TTs are likely to be narrower, and therefore harder to image, than those of human [8] and other laboratory animals, such as rat (~0.25 μm diameter [16]) or rabbit (~0.45 μm diameter [17]) (see also Discussion). We therefore developed a new dual wavelength, intensity based 3D analysis method to quantify local TT geometry. We have applied this method to confocal images of mice and rabbit ventricular cardiomyocytes (two commonly used small animal models). Simultaneous imaging of two dyes (calcein and FM4-64) provides measures of local TT volume and surface area from which we can then derive, using a cylindrical model, local TT diameter and geometry. This improves the reliability of TT detection/characterization, as well as providing a cell-wide measure of TT abundance. The utility of the method for deriving a quantitative measure of TT width was confirmed using ultra-rapid high pressure cryo-fixation and 3D electron tomography reconstructions after freeze substitution [18].

2. Materials and methods

2.1. Myocyte preparation

All procedures were performed in accordance with the Animals (Scientific Procedures) Act (UK). Ventricular myocytes were enzymatically isolated from the hearts of male mice (C57BL/6, 25 g) or rabbits (New Zealand White, 2.5 kg), as described previously [19,20]. Briefly, rabbit and mouse hearts were rapidly removed and washed in a standard physiological saline solution (see below) that contained 0.1 mM $CaCl_2$ and 10 IU/mL heparin. The aorta was mounted on a Langendorff system (37 °C) for perfusion with oxygenated standard solution for 5 min, followed by standard solution containing 0.1 mM $CaCl_2$, 0.8 mg/mL collagenase II (Worthington Corp., USA) and 0.6 mg/mL protease XIV (Sigma-Aldrich Co. Ltd., U.K.) for 15 min. The ventricles were then minced and filtered to isolate single cells. The cells were centrifuged and re-suspended in storage solution (see below).

2.2. Solutions

The standard solution used for mouse cell isolations contained (in mmol/L): 130 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 0.4 NaH_2PO_4, 10 D-glucose, 4.2 HEPES, 20 taurine and 10 creatine, pH = 7.4. For rabbit cells, the solution was similar, except that it contained 4.5 KCl, 3.5 $MgCl_2$ and 5 HEPES. The cell storage solution was a low Ca Kraftbrühe (KB) medium to relax cells and prevent possible TT compression by cell contraction, which contained: 100 L-glutamic acid, 30 KCl, 10 HEPES, 1 EGTA, 5 Na pyruvate, 20 taurine, 20 glucose, 5 $MgCl_2$, 5 succinic acid, 5 creatine, 2 Na_2ATP, 5 ß-OH butyric acid. All experiments were performed at room temperature in KB medium.

2.3. Confocal imaging

Cell membranes were labeled with 5 μM FM4-64 (Thermo Fisher, USA) for 5 min, washed, then bathed in 400 μM calcein as an extracellular marker (Sigma-Aldrich). This dye selection allowed good separation of fluorescence signals, although other dye pairs could be used with suitable correction of cross-talk between channels if necessary. Cells were imaged using a LSM 880 (Carl Zeiss, Germany) with an Airyscan detector set to super-resolution mode, and water-immersion objective with 40× magnification and 1.2 numerical aperture. Dyes were excited with the 488 nm Argon laser line, and fluorescence recorded at 495–550 (calcein) and >605 nm (FM4-64). Volume images were recorded at 12-bit resolution with voxel size set to 60 nm in the focal plane (x-y) and ~180 nm along the optical axis (z) to ensure oversampling.

2.4. Measurement of TT width in living myocytes

A novel method was developed to assess TT width in living myocytes. The algorithm was written in MATLAB R2015a (MathWorks, Inc., USA), and ImageJ (v1.50f, National Institutes of Health, USA) was used to aid the analysis. (Codes can be obtained by contacting the authors.)

The improved method relies on microscopic measurement of the local TT luminal volume (calcein signal) and membrane area (FM4-64 signal) to calculate the volume to surface area ratio, *V:SA*. For any given shape *V:SA* increases with size, so *V:SA* can be used to compare object sizes with no explicit assumptions beyond shape similarity. *V:SA* can be calibrated to physical dimensions using a geometric model, as described below.

The ratio of the calcein to FM4-64 signal intensities is a measure of *V:SA*. Since *V:SA* increases linearly with t-tubule diameter (D_{TT}), it can be used to monitor changes in average D_{TT}. As pointed out previously, use of confocal fluorescence intensity signals assumes a spatially invariant and symmetric probe point spread function (PSF) [16]. This can be realized by suitably blurring the data produced by an asymmetric or aberrated confocal PSF. Fig. S1 illustrates the *V:SA* method with a flow diagram showing the image processing steps. Briefly, calculation of the TT *V:SA* involved segmentation of cell interior and surface, background subtraction, conversion of the normal microscope asymmetric PSF to a spherical PSF, and data normalization to remove experimental variations in dye concentration and to permit calibration to size units.

A TT skeleton was produced by applying an Otsu threshold to produce a binary image mask, which was then skeletonized by thinning (via an ImageJ plugin based on [21]) until objects were reduced to single-pixel wide lines in 3D. A cell area mask was created by applying a threshold to the calcein signal and applying a morphological closing operator to remove any artifactual 'holes' in the mask. The cell border was refined from the local gradients of both signals, so that only in-focus and non-z-groove regions were used as the surface sarcolemma (SS skeleton). A TT skeleton was created by combining the cell area mask and membrane skeletons.

For both signals, background subtraction was achieved using pixels within the cytosol mask outside the TT skeleton. The background values for TT pixels were nearest-neighbor interpolated from surrounding pixels in the cytosol mask. This background image was then subtracted from the data.

To overcome the problem of an asymmetric confocal PSF, which would cause signal intensity to depend not only on TT width, but also TT orientation [16], the data was blurred to reduce the x-y resolution to that of the z-resolution, making the effective PSF spherical. This involved convolution with a 3D Gaussian function whose x-y dimensions were matched the z-resolution of the measured PSF (obtained from images of 0.17 μm Yellow-Green Fluorospheres, Thermo Fisher).

To control for variations in dye concentration and microscope efficiency, the calcein (volume) signal was normalized to that recorded from in the perfusion bath, and the FM4-64 (surface membrane) signal to that at the cell surface sarcolemma (SS). *V:SA* images were calculated by division of these normalized images. Thus, *V:SA* at the SS should be ~0.5, while the *V:SA* at the TT skeleton will be proportional to D_{TT} according to

$$V:SA = \frac{D_{TT}}{2FWHM} \cdot \sqrt{\frac{\ln(2)}{\pi}} \quad (1)$$

where FWHM is the full-width at half maximum of the modified microscope PSF (see Theory in Supplementary materials).

2.5. Electron microscopy/tomography and analysis

TT shape and dimensions were also measured with 3D electron tomography (ET) and electron microscopy (EM), for comparison with