Examination of the Transverse Tubular System in Living Cardiac Rat Myocytes by 2-Photon Microscopy and Digital Image–Processing Techniques

C. Soeller, M.B. Cannell

Abstract—The transverse tubular system (t-system) of cardiac muscle is a structure that allows rapid propagation of excitation into the cell interior. Using 2-photon molecular excitation microscopy and digital image–processing methods, we have obtained a comprehensive overview of the t-system of rat ventricular myocytes in living cells. We show that it is possible to quantify the morphology of the t-system in terms of average local tubule diameter, branching pattern, and local abundance of the t-system by immersing living myocytes in a dextran-linked fluorescein solution. Our data suggest that previous electron microscopic examinations of t-system structure have underestimated both the geometric complexity of the t-system morphology and the fraction of cell volume occupied by the t-system (3.6% in this species). About 40% of tubules occur between Z-lines, and the t-tubule diameter is 255±0.85 nm (mean±SEM). The t-tubules leave the outer surface of the cell in an approximately rectangular array; however, at some points junctions between the t-tubules and the surface membrane are missing. In view of the complexity of the t-system apparent from our images, we propose that the t-system be renamed the “sarcolemmal Z rete.” The methods presented here are generally applicable to the quantification of the sarcolemmal Z rete and other structures within cells by fluorescence microscopy in a variety of cell types. (Circ Res. 1999;84:266-275.)

Key Words: heart structure ■ t-system ■ imaging ■ myocyte ■ rat

Mammalian ventricular heart muscle cells have a fine sarcotubular system that was first identified by electron microscopy (EM).1 Subsequent EM studies revealed that the tubular system was continuous with the sarcolemma and located at the level of the Z-lines (eg, Reference 2). Since most of the tubules were associated with the Z-lines, they were assumed to run transversely and were named the transverse tubular system or “t-system” (for review, see Reference 3). However, subsequent studies have shown that considerable numbers of tubules run in the axial direction,4,5 leading to the descriptive term “transverse-axial tubular system”6 or TATS.7

Most studies have used EM to provide sufficient resolution to identify the tubular membranes. However, this method suffers from the disadvantage that the specimen must be fixed (or frozen), dehydrated, thin sectioned and heavy-metal stained. Obtaining structural information through the thickness of a cell with EM is problematic because of the poor penetration of the electron beam. Even “thick EM sections” are limited to a thickness of ≈5 μm,8 and because of this problem only an 11×24×2–μm section of the TATS in a mouse myocardial cell has been shown.6 EM of thin sections relies on absorption and so has no intrinsic 3-dimensional (3D) resolution. This leads to images of thicker sections becoming blurred and losing internal detail. Three-dimensional reconstruction from stacks of thin EM sections is complicated by the difficulty of obtaining the necessary registration between separate thin sections that are subject to distortion. Indeed, the TATS has only been reconstructed over a 5.5-μm-thick segment of a heart cell with manual section registration.7

The t-system in skeletal muscle provides a rapid propagation of electrical excitation into the cell interior.9–11 Evidence for such a role for the t-system in heart12,13 has been less clear, since there is no correlation between contraction speed and abundance of t-system.3 However, optical measurements of latencies for calcium release across rat ventricular cells show that the t-system provides both a rapid inward spread of electrical excitation and the calcium influx that triggers calcium release from the sarcoplasmic reticulum.14 The microarchitecture of the t-system has been suggested to be important in determining the levels of ionic accumulation/depletion during excitation.7,15,16 The t-system may not be a fixed structure within the cell, since short-term cell culture has been reported to be associated with a loss of t-tubules,17 and t-tubule proliferation occurs with hypertrophy.13 These observations suggest that a method for investigating t-tubular morphology in living myocytes may be useful for studying pathological changes in myocyte structure.

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The confocal microscope provides a means for optically sectioning living specimens, thereby overcoming problems associated with fixation and dehydration. As shown by Shacklock et al. and Cheng et al., it is possible to detect t-tubules by immersing cells in a soluble fluorescent marker that penetrates the tubular system. We have extended this approach by using 2-photon molecular excitation (TPME) microscopy and staining the extracellular compartment with a fluorescent probe that does not cross the cell membrane. Although the t-tubules themselves are generally below the optical limit of resolution, this does not preclude quantitative measurement of the fractional cell volume occupied by these structures, since the imaging properties of the microscope are known from its point-spread function (PSF). We have used this approach to obtain estimates of fractional volumes and tubule diameters in rat ventricular myocytes. A preliminary account of some of these methods has been published elsewhere.

**Materials and Methods**

**Cells**

Enzymatically isolated rat cardiac myocytes were prepared as described elsewhere. Briefly, 250-g Wistar rats were killed by cervical dislocation and the hearts quickly removed and mounted on a Langendorff perfusion system. The heart was initially perfused for 5 minutes at 37°C with a nominally calcium-free solution containing (in mmol/L) NaCl 120, KCl 5.4, MgCl₂ 1, HEPES 10, pyruvate 5, glucose 20, and taurine 20 (pH 7.05 with NaOH). This basic solution was then switched to one containing collagenase (Worthington type II, 1 mg/mL) in place of protease for a further 5 to 10 minutes. On completion of the enzyme treatment, the ventricles were cut free and dispersed the subresolution beads in the bath containing the myocytes in bathing medium without fluorescein. Beads that spontaneously stuck to the cell membrane were imaged at several heights above the coverslip, and from these images a resolution of 400 nm (full width at half maximum; FWHM) in x-y and 800 nm in z was measured. Figure 1 illustrates the TPME microscope PSF measured in this way. To exceed the Nyquist criterion for image sampling, the voxel size was set to 0.1×0.1×0.2 μm (x, y, and z, respectively). This fine voxel resolution also improved convergence of the deconvolution procedure (see below), but it limited the cell area that could be imaged to ≈102×102 μm. Since this encompassed most of the chosen myocytes, this limitation was considered acceptable.

**Data Processing**

To improve the resolution and signal-to-noise ratio of the volume data, digital deconvolution was applied to “stacks” of images. In a recent comparison of image-restoration methods for confocal microscopy, the Richardson-Lucy algorithm was judged superior to other algorithms. For this study, the deconvolution algorithm was implemented in the language IDL (Research Systems Inc) to produce a maximum likelihood reconstruction for the specimen in the presence of Poisson (phonon shot) noise. A Poisson noise model was used, because the number of photons collected was probably insufficient to justify using a gaussian noise model. The update step of the procedure can be written as follows:

\[ I_{n+1} = I_n + \frac{D}{I_n * H} \]

where \( D \) represents the raw data, \( I_n \) is the revised image, \( I \) the previous estimate of the image, and \( H \) the PSF, and * and \( \otimes \) are the convolution and cross-correlation operators, respectively. In general, about 15 iterations of Equation 1 were used to generate a deconvolved image data set. All calculations were performed with an Indigo workstation (Silicon Graphics).
Volume Visualization

To assist visualization of the t-system architecture, a topological “skeleton” was constructed from the deconvolved stack of images. In this 3D skeleton, all information about tubule signal intensity was removed by replacing tubule segments with a thin line of uniform thickness, which followed the direction of the t-tubule in space. Since the 3D connectivity of the skeleton is identical to that of the t-tubular system, this method of data presentation clarifies the organization of the t-tubular system. In any case, the calculated t-tubule diameters (see below) were generally less than the wavelength of light so the original light image contained no more spatial information than the topological skeleton. The first step in skeleton construction was generation of a binary mask from the enhanced image stack. Pixels with intensities greater than the mean background+5 SD were assumed to represent t-tubular segments and were set to 1.0; all other pixels were set to 0.0. From this 3D binary mask, a topological skeleton was generated by deleting all pixels that did not change the connectivity of the 3D structure.

The need to construct a topological skeleton forced us to adopt a lower-limit threshold for t-tubular cross-section. For the data sets presented here, tubules less than ~50 nm in diameter would be lost in the noise and therefore missed. Since the smallest t-tubule diameter reported in left ventricular myocytes of rat is ~70 nm, this limit was considered acceptable. However, more extensive signal averaging (to reduce photon noise) or a relaxation of the stringency of the binary mask would allow any desired lower limit to be achieved.

Estimation of Fractional t-Tubule Volume

Since we were unable to detect any entry of dextran-linked fluorescein into the intracellular compartment, and cell autofluorescence was negligible (not shown), the calculation of fractional t-tubular volume \( V_{tt} \) was straightforward and required no assumptions about t-tubular microarchitecture. The total intensity of fluorescence recorded within the boundary of the sarcolemma is directly proportional to the amount of dye contained within the t-system, as shown in the following:

\[
FV_{tt} = \frac{F_{average}}{F_{bath}}
\]

where \( F_{average} \) is the average fluorescence in a volume inside the cell, and \( F_{bath} \) is the fluorescence recorded from the surrounding bath (representing the limiting case, \( FV_{tt} = 1 \)).

Estimation of t-Tubule Diameters

The peak signal from a piece of t-tubule is a function of the tubule geometry and PSF of the microscope (see Figure 2A) as follows:

\[
I_i = \iint_{A} PSF(x,y,z) \times t(x,y,z) dx dy dz
\]

where \( t(x,y,z) \) describes the t-tubule geometry (idealized as a cylinder of radius \( R \) at an angle \( \varphi \) with respect to the optical axis) being 1 at points inside the segment and 0 elsewhere.

As shown in Figure 2B, even for a (typical) PSF that is symmetrical about the \( z \)-axis, Equation 3 remains a function of both radius \( R \) and angle \( \varphi \). However, if the PSF is completely symmetrical, then the signal does not depend on \( \varphi \) (see Figure 2C) and becomes only a function of the PSF and \( R \), as follows:

\[
I_R = 2\pi \int_{0}^{\infty} R \int_{0}^{\infty} PSF(\sqrt{r^2 + z^2}) rdrdz
\]

Therefore, the local radius of a t-tubule can be calculated from the unique relationship between \( R \) and the normalized signal intensity \( I_R/I_{R,t=0} \), if the tubules are adequately described by cylinders and the PSF is symmetrical. Although the microscope does not have a symmetrical PSF, the effective PSF applied to the data (cell structure) can be made symmetrical by convolving each of the images of the 3D data set in the \( x-y \) plane with a gaussian kernel so that the in-plane FWHM equals the axial FWHM (0.8 μm). Blurring the images to create identical resolutions in \( x, y, \) and \( z \) results in the image data losing the directional dependence of the local fluorescence signal. For segments of t-tubules of which the architecture is more complicated than simple nonbranching cylindrical segments, the binary topological skeleton contains the information needed to correct the relationship between \( I_R \) and \( R \) for any branching that may occur in the t-system. The correction factor is obtained by convolving the binary skeleton with the PSF used to process the data to yield a weighting \( w \) that is applied to the data to allow for elements such as blind-ended tubules (\( w<1.0 \)) and branches (\( w>1.0 \)) (see Figure 2D). Figure 2E shows the relationship between \( I_R/w \) and \( wR \).

We also used the calculated distribution of tubule diameters to estimate the total surface area of t-system within the sampled cell volume. In the calculation, pieces of t-tubule are approximated by cylinders of varying diameter so that a piece of tubule of length \( s_i \) and radius \( R_i \) contributes a surface area \( S_i = 2\pi R_i s_i \). The total t-system area \( S_{tt} \) in a given volume can then be obtained by summing all the contributions of pieces of t-tubule within the sample volume, as follows:

\[
S_{tt} = \sum_i 2\pi R_i
\]
After division by the size of the sampled cell volume, we finally obtain an estimate of surface area of t-system per unit cell volume. This should give a lower limit to the surface area, as the circular cross section assumed in Equation 5 provides the smallest boundary for the enclosed volume.

To visualize the distribution of tubule diameters, the skeleton was intensity coded according to the calculated t-tubule diameter and displayed by isosurface rendering using IrisExplorer software (Silicon Graphics).

**Results**

Figure 3A shows a typical fluorescence micrograph from a stack of images that were acquired from a quiescent rat ventricular myocyte. In this image, t-tubules and the exterior of the cell are bright because of the presence of dextran-linked fluorescein in the bathing medium. An enlarged part of the image is shown in Figure 3B, and some fine structure is visible, although there is photon noise in the image (which could have been reduced by further signal averaging). The lower panel (Figure 3C) shows the results of applying digital deconvolution to the stack of images (see Materials and Methods). The 3D skeleton of a rat ventricular myocyte t-system is shown in Figure 4. Visual inspection of such skeletons suggested that the overall macroarchitecture of the cell t-tubular system resembles a complicated mesh with some limited regularity at the level of the Z-line (at a regular axial spacing of ~1.8 μm). There are also a few regions relatively devoid of t-tubules that probably coincide with the locations of cell nuclei. To quantify the preponderance of t-tubules at the Z-lines, Figure 5A shows a graph of tubule density as a function of the longitudinal position inside the cell. Although 60% of the tubule volume occurs at the Z-line (as defined by their presence in a region 0.55 μm around Z-lines), the remaining 40% of the tubules are found at other locations along the myofibrils. It is apparent from Figures 4 and 5A that a considerable amount of the t-tubular system forms longitudinal connections between t-tubules, although most of the t-tubules are organized in transverse planes. However, the t-tubules do not run across the cell at the level of the Z-lines for great distances but instead run at the level of 1 Z-line for a short distance and then change direction to join other tubules at other Z-lines. In some places, no t-tubules ran in the transverse direction.

Figure 3. Fluorescence micrograph from a stack of images of a quiescent rat ventricular myocyte. In panel A, an original image from the stack of images is shown. The signal from the fluorescently labeled bathing medium clearly identifies pieces of the t-system in this “optical slice.” An enlarged section of this image shows that the data are noisy and blurred (B). After digital deconvolution, the noise level has decreased and details have sharpened (C).
(across the cell) but instead passed through the cell at an angle to its longitudinal axis (see also stereo views in Figure 6). Having reconstructed the skeleton of t-tubular architecture, it is straightforward to determine the distribution of the length of t-tubular segments between branching points. Figure 5B shows a graph of t-tubule lengths between branching points obtained from the skeleton data in Figure 4. The distribution of interbranch lengths is approximately exponential, with short segments being most prominent and a mean branch length of 6.87 μm. To help clarify the complex macroarchitecture of the tubular system, Figure 6A shows an enlarged stereo view of the tubular system from a randomly selected part of the cell. In the upper right portion of this view, it can be seen that t-tubules form ring-shaped connections, possibly wrapping around myofibrils. A region almost devoid of tubules is apparent in the center of this section of tubular network. The predominance of t-tubules in planes located at the Z-line can be identified most clearly in the region at the left of this view.

Electron micrographs suggest that tubules have nearly circular cross sections, so it is possible to calculate the apparent width of the tubule (see Materials and Methods). The calculated tubule diameters have been imposed on the skeletons by gray coding in Figure 6B. Although the majority of tubules appear to have diameters of ~200 to 300 nm, there are also local increases and decreases in tubule diameter at (apparently) random points in the cell. The data shown in Figure 6B are summarized in a histogram of tubule diameters shown in Figure 7. It is apparent that the distribution is approximately gaussian, and almost no tubules larger than 450 nm were found. The histogram has a maximum at a diameter of 240 nm, and most t-tubules (51%) are between 180 and 280 nm wide. Using the calculated distribution of tubule diameters to estimate the surface area of t-tubular membrane per unit of cell volume (see Materials and Methods), we obtained a value of $S_{TT}/V_{Cell} = 0.44 \, \mu m^2/\mu m^3$.

The positions at which the t-tubules leave the cell surface can be visualized from the same data set. In Figure 8, the...
lead to some tubular elements being missed, while distortion of thin sections without fiducial marks will lead to geometric errors.

Our data suggest that the fractional volume (\(VF_{tt}\)) of the t-tubular system is \(\approx 3.6\%\). Earlier measurements based on EM studies suggested a much lower \(VF_{tt}\) in the rat (typically 1% for adult rat; eg, References 12 and 32). This large difference is not easily explained by an error in our method of measurement, since the total fluorescence from the extracellular marker should depend only on the volume of the t-tubules and the concentration of marker (see Materials and Methods). Although a large contribution of autofluorescence to the recorded signal would lead to an overestimate of \(VF_{tt}\), inspection of the images shows that the autofluorescence background was barely detectable compared with the t-tubule signal. Similar arguments rule out the possibility that marker dye penetration into the cytoplasm could explain the larger \(VF_{tt}\) (since any entry of the dye into the cell would appear as generalized cytoplasmic staining). Although low levels of dextran binding to phospholipids have been reported and such an effect would cause an overestimate of \(VF_{tt}\), 4 pieces of evidence suggest that this explanation is unlikely. (1) The reported densities of dextran binding by phospholipids are too low (\(\approx 0.4\) mg D-40/1,2-dipalmitoyl-3-sn-phosphatidylcholine) to materially affect our results. (2) Our estimates of t-tubule diameters and EM data are in reasonable agreement (see below), which suggests that our method accurately measures t-tubules. (3) The level of membrane binding needed to explain the difference between our results and those of Stewart and Page would have led to an elevated level of fluorescence near the cell edge, but this was not observed. (4) Further support for the idea that EM studies have underestimated the extent of the t-tubular system is provided by electrophysiological studies. We estimate a surface-to-volume ratio \(S_{TT}/V_{Cell} = 0.44 \mu m^2/\mu m^3\) that is almost 3 times larger than previously reported stereological measurements from EM data (0.15 \(\mu m^2/\mu m^3\)). A recent study of capacity/volume ratios in cardiac myocytes from different species suggests a specific membrane capacitance \(C_{m}/V_{Cell} = 6.76\) pF/\(\mu m\) for myocytes from young (3-month-old) and 8.88 pF/\(\mu m\) from older (6-month-old) rats. Since the specific membrane capacitance is \(\approx 1\) \(\mu F/cm^2\) (see, eg, Reference 3), these values can be converted into a ratio of total sarcolemmal membrane area (outer sarcolemma + t-tubular system) that is \(S_{TT}/V_{Cell} = 0.68 \mu m^2/\mu m^3\) for the younger and 0.89 \(\mu m^2/\mu m^3\) for older rats. For a typical myocyte 100 \(\mu m\) long with a diameter of 20 \(\mu m\) (ie, a volume of \(\approx 3.1 \times 10^4 \mu m^3\)), we can estimate from our data that \(S_{TT}/V_{Cell}\) would be \(\approx 0.68 \mu m^2/\mu m^3\), which is in good agreement with the electrophysiological data.

The above agreement between the estimate of cell capacitance and our data suggests that the lower \(VF_{tt}\) and \(S_{TT}/V_{Cell}\) derived from previous EM studies must be in error. Since there can be problems in identifying t-tubules in electron micrographs (eg, Reference 38), as well as specimen shrinkage during section preparation, such an underestimate of \(VF_{tt}\) and \(S_{TT}/V_{Cell}\) might easily occur. It is also possible that the method of measurement used by Stewart and Page would have underestimated \(VF_{tt}\), since, as they noted, (1) average...
Figure 6. Stereographs of segments of the t-system skeleton from a rat myocyte. A, Small segment of a reconstructed t-system skeleton that demonstrates the complicated reticular structure. Pieces of tubule can be observed forming ringlike structures that probably wrap around myofibrils at the Z-line. B, Calculated local tubule diameter has been superimposed on the morphology skeleton by gray coding. The top pair in panel B shows small-diameter tubules (50 to 160 nm); the middle pair, tubules of intermediate diameter (160 to 300 nm); and the lower stereo pair, large tubule elements (300 to 500 nm).

Figure 7. Distribution of tubule diameters in the t-system of a rat myocyte. The histogram of tubule diameters calculated from the data shown in Figure 6B has an approximately gaussian distribution around a mean diameter of \(\approx 250\) nm. Almost no tubules larger than 450 nm were found.

The t-tubule diameter is much less than the EM section thickness, and (2) t-tubules were assumed to run at right angles to the long axis of the cell only, whereas we have detected large amounts of t-tubules running in other directions.

“t-System” Morphology

The volume data show that the term “transverse-tubular system” is something of a misnomer, since t-tubules run in all directions. The general impression of the t-tubular organization is that of a rete, although such a term does not fully describe the preponderance of elements near the Z-lines. We have been able to confirm the presence of large numbers of tubules running in axial directions, the presence of which led to the suggestions that the t-system be referred to as the TATS, or T-Ax.\(^5,6,38\) However, there are also large numbers of tubules that run in neither axial nor transverse directions, so a more accurate descriptive term might be the “sarcopla
al Z rete” (SZR). For convenience, this nomenclature will be adopted in the following discussion, although the possible general acceptance of such a term will depend on the findings of future studies on other species.

The SZR often formed complete loops around the (assumed) location of myofibrils. However, there are also regions within the cell that are relatively devoid of tubules. We suspect that some of these regions may be associated with parts of the cell that are not involved in excitation-contraction coupling (such as the nucleus), but further work will be required to confirm this view. Visual inspection of the SZR skeletons suggested that there was little or no correlation of tubule width with position along the sarcomere (see Figure 6B). However, quantitative analysis suggested that the average tubule diameter was \(\sim 15\%\) larger between the Z-lines than in longitudinal elements (not shown). Since the cells were relaxed, this increase in average diameter could be the result of the constant volume behavior of the cell. We suspect that if the cell were stretched to working lengths, this difference would largely disappear, and there would be almost no correlation between tubule diameter and longitudinal position.

Although the optical methods used here cannot resolve the fine microarchitecture of the SZR, they give good insight into the gross morphology of the SZR. For surveying the general organization of the SZR, our optical approach produces a comprehensive overview of it, which would not be possible (for any reasonable amount of effort) with EM. This is because with one image stack we are able to resolve the entire SZR within a selected myocyte. In addition, we have shown that it is straightforward to extract quantitative information about the fractional cell volume occupied by the SZR and equivalent tubule diameters. In contrast to the utility of our optical method, stereological analysis of electron micrographs is extremely time consuming. In addition, our fluorescent method does not require extensive geometrical correction factors to take account of the variations in the plane of section of t-tubules.\(^32\)

Surface Membrane Topology

Using the methods introduced here, we have been able to produce images that portray the organization of junction between the SZR and the outer sarcolemmal surface. These images are very similar to freeze-fracture images of myocyte surfaces.\(^33\) However, we have been able to examine a much larger area of the cell surface than has been achieved by freeze-fracture techniques. In addition, we can be certain that the organization of SZR “mouths” is not confused with caveolae. The SZR mouths are mostly arranged in linear arrays with a longitudinal spacing of \(\sim 1.8\) \(\mu\)m. However, these linear arrays are interrupted by missing mouths or by a misregistration with another region of linear arrays (Figure 8). It is possible that such an organization may reflect the nonuniform development of the SZR during maturation, since it is known that in some species neonatal myocytes have no SZR (eg, cats\(^36\)). It is likely that during hypertrophy the SZR will grow and form new connections with the surface membrane. Whether this occurs as a result of new invaginations of the surface membrane or by extension of the SZR with subsequent fusion with the surface of the cell is unknown. It may be possible to distinguish between these possibilities by applying our methods to myocytes that are actively undergoing hypertrophy.

The fluid contained within the SZR can only directly exchange with the extracellular fluid at the junction of the SZR with the outer cell surface. Since accumulation/depletion phenomena will be limited by the efficiency of this exchange, it is possible that a part of the propensity of hypertrophied hearts to develop arrhythmias (eg, Reference 41) may reflect a mismatch between SZR volume and the number of SZR mouths. Again, future work should be able to directly examine this possibility.
In conclusion, we have shown that it is possible to examine the gross morphology of the SZR using optical methods combined with digital image processing. The advantage of this approach is that most of the analysis and data acquisition can be automated. In addition, we can examine the cell microarchitecture in identified living myocytes that have not suffered from the extensive preprocessing required for EM.

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