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# SUB-MICROMETER ANATOMICAL MODELS OF THE SARCOLEMMA OF CARDIAC MYOCYTES BASED ON CONFOCAL IMAGING

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We describe an approach to develop anatomical models of cardiac cells. The approach is based on confocal imaging of living ventricular myocytes with sub-micrometer resolution, digital image processing of three-dimensional stacks with high data volume, and generation of dense triangular surface meshes representing the sarcolemma including the transverse tubular system. The image processing includes methods for deconvolution, filtering and segmentation. We introduce and visualize models of the sarcolemma of whole ventricular myocytes and single transversal tubules. These models can be applied for computational studies of cell and sub-cellular physical behavior and physiology, in particular cell signaling. Furthermore, the approach is applicable for studying effects of cardiac development, aging and diseases, which are associated with changes of cell anatomy and protein distributions.

## 1. Introduction

Computational simulations of physical behavior and physiology of biological tissues have given valuable scientific insights, which are applied in drug research, development of medical instrumentation and clinical medicine to improve diagnosis and therapy of patients. In the cardiac field, for example, computational simulations have been carried out to understand effects of drugs and mutations of ion channels on cellular electrophysiology,

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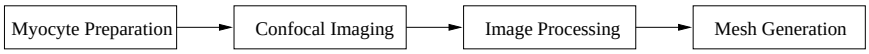


Figure 1. Pipeline for generating anatomical models of cardiac myocytes.

metabolism and mechanics. Furthermore, the simulations helped to improve pacemaker and defibrillator efficacy, and to understand and prevent arrhythmogenesis.

Frequently, detailed anatomical models are applied in these simulations[15]. These models describe geometry of tissues and their microscopic properties such as fiber orientation and lamination. Commonly, these anatomical models were created by digital image processing of computer tomographic and magnetic resonance imaging. Eventually, the computational models are generated by extending the anatomical models with descriptions of physical and physiological properties.

In this work, we will address first steps in the generation of realistic detailed anatomical models of heart cells (Fig. 1). Our focus is on describing the geometry of the sarcolemma of ventricular myocytes with sub-micrometer resolution. The sarcolemma represents a semi-permeable barrier delimiting the extracellular from the intracellular space. The sarcolemma is built up primarily by a phospholipid bilayer with a thickness of $3-5\ nm$. The bilayer contains peripheral proteins attached to the surface of the sarcolemma and transmembrane proteins spanning over the sarcolemma. The proteins are responsible e.g. for signaling and cell-adhesion. Important transmembrane proteins are ion channels, exchangers, and ion pumps as well as gap junctions and receptors. Control of intracellular ion concentrations and cellular signaling in myocytes is mostly governed by these proteins in the sarcolemma.

In mammalian ventricular myocytes, the sarcolemma invaginates into the cytosol forming the so-called transverse tubular system (t-system)[5,2]. The t-system is composed of transversal tubules (t-tubules), which enter the myocyte primarily adjacent to Z disks[3]. The t-system occupies a large area of the sarcolemma. The ratio of t-system to sarcolemma area is species specific[1]. For instance, 42% and 33% of the sarcolemma comprise the t-system in rabbit and rat ventricular myocytes, respectively[11]. The t-system supports fast propagation of electrical excitation into the cell interior. Various proteins are associated with the t-system[10,14]. Morphological changes of the t-system have been associated with cardiac development, hypertrophy and heart failure[7,23].

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Our modeling of the sarcolemma and t-system started by obtaining three-dimensional images of isolated cardiac myocytes and cell segments with scanning confocal microscopy. Usually, this technique is applied with fluorescent indicator dyes or antibodies tagged to a suitable fluophore, which permits specific labeling of compartments and proteins. For our modeling, we used a fluophore conjugated to membrane-impermeable dextran (excitation wave length: 488 nm, emission wave length: 524 nm, Invitrogen, Carlsbad, CA) to label the extracellular space.

Major processing steps in our modeling were image deconvolution and segmentation. We deconvolved the three-dimensional image datasets with the Richardson-Lucy algorithm using point spread functions (PSFs), which characterize the optical properties of our two confocal microscopic imaging systems. PSFs were extracted from images of fluorescent beads, which were suspended in agar to avoid Brownian-type motion. After deconvolution, the extra- and intracellular space were segmented in the images with methods of digital image processing. Furthermore, the t-system was decomposed into its components.

We identified the border between the extra- and intracellular segment with the sarcolemma and represented it by triangle meshes. Similarly, single t-tubules of various shapes and topologies were described with triangle meshes. This representation of the sarcolemma and t-tubules with triangle meshes permits application of standard tools for generation of computational models, such as volumetric mesh generators and automated annotation of mesh elements with protein density data. The resulting anatomical models provide a basis for computational studies of various physiological and pathophysiological processes at cellular level.

## 2. Methods

### 2.1. *Preparation and Imaging of Cardiomyocytes*

Our approach for preparation and imaging of alive cardiac cells was previously described in more detail[16,17]. In short, ventricular myocytes were isolated from adult rabbit hearts by retrograde Langendorff perfusion with a recirculating enzyme solution. After isolation, myocytes were stored at room temperature in a modified Tyrodes solution. Imaging of whole cells or segments of them was performed 4-8 h after isolation. Cells were superfused with membrane impermeant dextran conjugated to fluorescein and then transferred to a coverslip. Either a BioRad MRC-1024 laser-scanning confocal microscope (BioRad, Hercules, CA, USA) with a 63x oil

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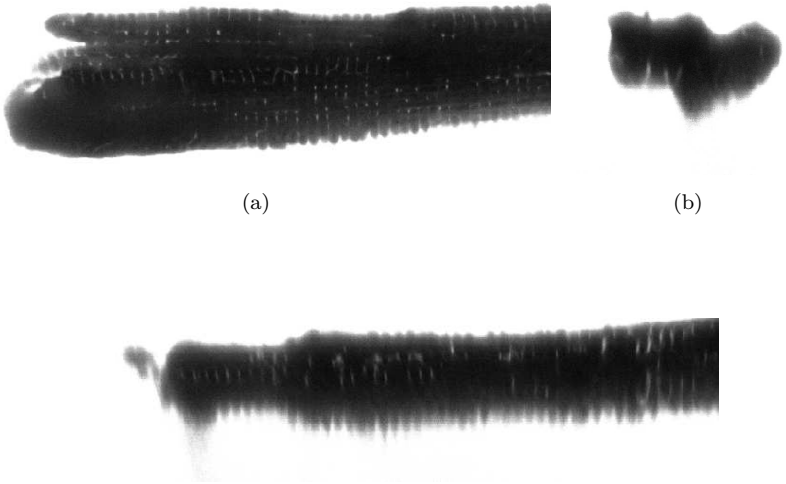

(a) (b)

(c)

Figure 2. Exemplary image of ventricular myocyte segment. The high intensity of the extracellular space results from staining with a fluophore conjugated to membrane impermeable dextran. Dots and lines of high intensity Inside of the myocyte label the t-system. The dataset describes a hexahedral region with a size of 102 $\mu m$ x 34 $\mu m$ x 26 $\mu m$ by a lattice of 768 x 256 x 193 cubic voxels. Intensity distributions are shown in the central (a) XY, (b) XZ and (c) YZ plane.

immersion objective lens (NA: 1.4, Nikon, Tokyo, Japan) or a Zeiss LSM 5 confocal microscope (Carl Zeiss, Jena, Germany) together with a 60x oil immersion objective lens (NA: 1.4) was used for imaging. It resulted in three-dimensional image stacks consisting of cubic voxels with a volume of $(133\ \text{nm})^3$ and $(100\ \text{nm})^3$, respectively (Fig. 2). The dimension of the stacks varied with size of the region of interest. The data volume of the stacks ranged from 20 to 250 million voxels.

### 2.2. *Image Processing*

The image processing was carried out in three dimensions and consisted of the following tasks:

- Correction of depth-dependent attenuation
- Image deconvolution
- Segmentation of intra- and extracellular space
- Decomposition of the t-system
- Surface extraction

- Visualization

Our approach for correction of depth-dependent intensity attenuation was a-posteriori using information from each individual image stack: Average intensities were slice-wise calculated in regions filled only with dye. A 3rd order polynomial $P$ was fitted to the averages by least squares. For each slice $z$ a scaling factor $s$ was determined by:

$$s(z) = \frac{Max_{i\in[0,\ldots,N-1]}P(i)}{P(z)} \tag{1}$$

with the average background intensity $P$ and the number of slice $N$. The scaling factor $s$ was used for correction of each slice.

We applied the iterative Richardson-Lucy algorithm to reconstruct the source image $f$ from the response $g$ of the confocal imaging system[13,4]:

$$g_{n+1} = g_n \left( \frac{g_0}{g_n \star h} \otimes h \right) \tag{2}$$

with the PSF $h$, cross-correlation operator $\otimes$, convolution operator $\star$, and $g_o \equiv g$. We determined the PSF $h$ by imaging fluorescent beads with a diameter of 100 nm in agar. 10 images of single beads were extracted in $\approx$ 10 nm distance to the coverslip, aligned and averaged yielding the PSF $h$. Specific care was given to detection and suppression of ringing artefacts, which are a common problem associated with this deconvolution method. We applied edge tapering methods to avoid intensity jumps at image borders. Furthermore, we cropped images manually to remove regions related to the coverslip and in excessive distance to the myocyte.

We segmented the extracellular space with morphological operators and the region-growing technique in the median filtered deconvolved image data[6,15]. Subsequently, the extracellular segment was applied as a mask to extract a segment containing the myocyte together with the t-system. Single t-tubules were segmented with the region-growing technique in the latter segment and with seed points determined by thresholding in a high-pass filtered image.

### 2.3. *Surface Mesh Generation and Visualization*

A modified marching-cube algorithm was applied to reconstruct the sarcolemma by creating surface meshes with sub-voxel resolution[9]. The algorithm generated meshes of triangular elements approximating iso-intensity surfaces in the three-dimensional image stacks. Modifications of the original

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algorithm assured closeness of the generated surfaces and permitted sub-voxel resolution by adjusting positions of mesh nodes based on edge-wise interpolation of intensities[8]. Meshes were visualized with software based on OpenInventor and can be exported in the VRML format[22].

We used the triangular meshes together with node-wise calculated surface normals for three-dimensional visualization of the sarcolemma. The normals were determined from gradients in averaged images stacks.

## 3. Results

We applied the foregoing methods to create and visualize anatomical models of 6 cells and 3064 t-tubules. The cells were from the left ventricle of rabbits and selected from an image library of more than 250 cells.

An exemplary model created from a living ventricular myocyte is shown in Fig. 3. The image dataset includes 1000 x 376 x 252 cubic voxels and describes a volume of 100 $\mu m$ x 37.6 $\mu m$ x 252 $\mu m$. The segmentation assigned 21 % of the voxels to the myocyte and the remainder to the extracellular space. The shape of the myocyte appears to be horizontally flattened and has sharp edges particularly at its endings. The sarcolemma exhibits a partly regular pattern of indentations, which refer to mouths of t-tubules.

An enlargement of an area at the cell bottom shows two rows of three mouths of t-tubules (Fig. 4a). Distances between the mouths are $\approx 1.5\mu m$ and $\approx 3.1$ $\mu m$ in row and column direction. Application of the marching cube algorithm led to a surface represented by a triangular mesh (Fig. 4b).

A single t-tubule is visualized in Fig. 5. The t-tubule has a length of $\approx$ 2.6 $\mu m$ and is of simple topology without branching and lateral connections, so-called anastomoses. Constrictions of the t-tubule diameter are visible close to the mouth and slightly above the middle. The triangular mesh representing the sarcolemma is shown in Figs. 5b and d.

In our set of 3064 t-tubule models extracted from 6 cells, lengths varied between 1 and 7 $\mu m$, with mean values of 2.8 $\mu m$. The occurrence of constrictions was correlated with t-tubule length. The t-tubule diameter was in average $\approx$400 nm.

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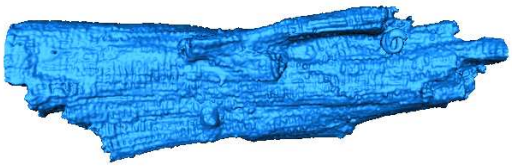
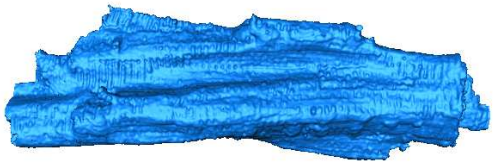


(a)



(b)

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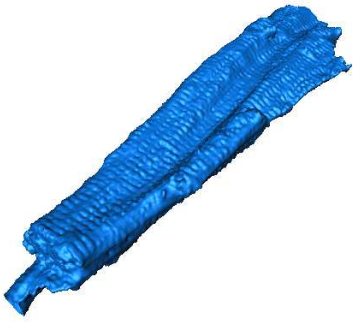

(d)

Figure 3. Three-dimensional visualization of single myocyte from different perspective. The myocyte is shown from (a) above, (b) below, (c) lateral and (d) lateral-below.

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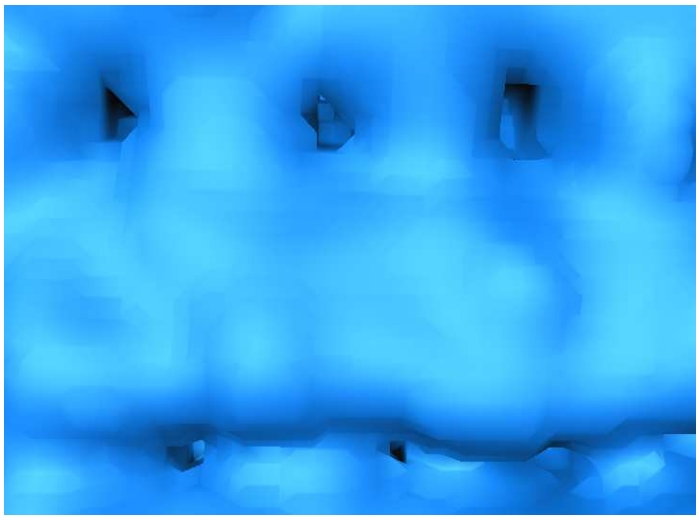
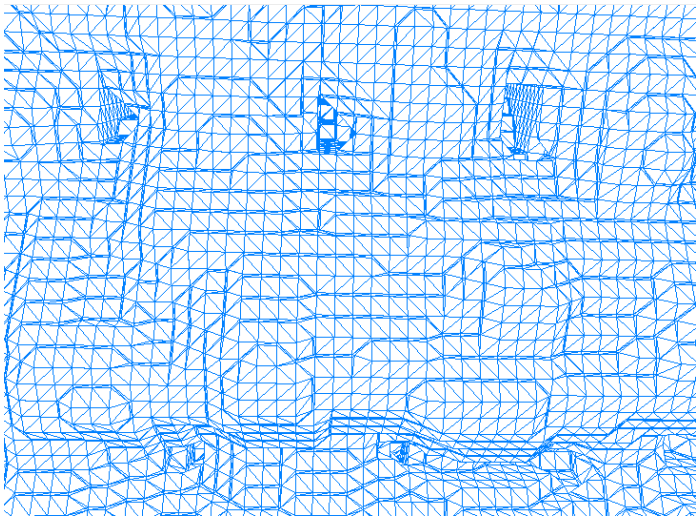


(a)



(b)

Figure 4. Visualization of sarcolemma segment with mouthes of t-tubules. The surface was generated with the marching cube algorithm and is shown with (a) filled triangles and (b) edges only.

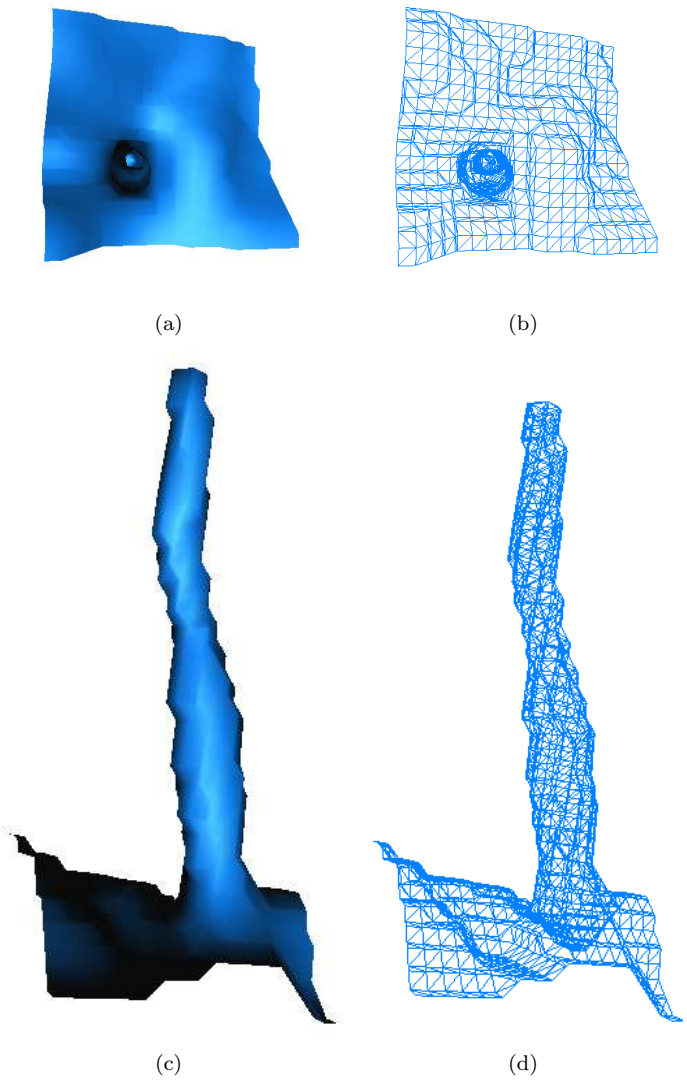


Figure 5. Visualization of single t-tubule (a,b) through mouth into cavity and (c,d) from lateral. The surface is shown with (a,c) filled triangles and (c,d) edges only.

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## 4. Discussion and Conclusions

We presented an approach to generate anatomical models of cardiac cells. The models describe with sub-micrometer resolution the sarcolemma including the t-system by processing of confocal images. Our approach complements analytical methods of cell surface modeling such similar as those introduced by Stinstra et al[20] and provides realistic geometrical data for their approach.

Our focus on modeling the sarcolemma is motivated by its central role as a border between the intra- and extracellular environment as well as for cell signaling. The sarcolemma comprises various proteins for cellular signaling such as controlling inward and outward flows of ions. Annotation of our anatomical models with published information of sarcolemmal protein density distributions is straightforward and will allow us to generate novel computational models of cellular physiology.

Our methodology is related to work of Soeller and Cannell[19], who used confocal microscopy and methods for digital image processing to characterize the topology of the transverse tubular system (t-system) in rat ventricular cardiac myocytes. In this work, we focused on generation of anatomical models, which are applicable in computational studies. The t-tubule diameter in our study on rabbit ventricular cells was in average $\approx$400 nm and thus mostly above the resolution of the confocal imaging system. The t-tubule diameter was much larger in rabbit than in rat, which corresponds to the reported differences of t-system surface area between the two species[11]. The large diameter allowed us to apply the surface meshing method not only for generation of models of the outer sarcolemma but also for modeling of the t-system.

Of particular interest for us is extending the models with information on distributions of ion channels, exchanger and pumps, which would permit to study electrophysiological processes at nanometer level. Resulting from recent advantages in confocal imaging technology, this information can be gained by using combinations of multiple fluorescent labels. In currently ongoing work, we are exploring dual labeling methods to relate proteins involved in excitation-contraction coupling to regions of the sarcolemma and t-system. Here, one label is associated with a specific type of ion channel and imaged together with another for labeling the extracellular space.

An application of our models can be found in studying ion diffusion in the t-system. In previous simulation studies of Shepherd and McDonough[18]

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and Swift et al[21], t-tubule geometry was simplified and diffusion approximated in one dimension. The presented models would allow us to gain insights into the significance of morphology and topology of the t-system for ion diffusion, particularly the role of constrictions in t-tubules, anastomoses and rete-like structures. We suggest that our models can be applied in computational studies of ion diffusion in the t-system by volume meshing of the t-tubule cavity and numerical solvers for partial differential equations describing diffusion[12].

Our approach can also be applied for modeling cells during development and aging as well as affected by cardiac diseases. Morphological changes of the t-system of myocytes have been described for diseased human ventricles[23] and in addition to changes of protein densities for tachycardia induced heart failure[7]. Effects of these changes are difficult to assess at cellular and tissue level with traditional experimental and analytical approaches. Computational studies based on realistic models of cell anatomy might give insights in these effects and thus complement the traditional approaches.

## References


1. D. M. Bers. *Excitation-Contraction Coupling and Cardiac Contractile Force.* Kluwer Academic Publishers, Dordrecht, Netherlands, 1991.
2. B. A. Block, T. Imagawa, K. P. Campbell, and C. Franzini-Armstrong. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.*, 107(6):2587–2600, 1988.
3. F. Brette and C. Orchard. T-tubule function in mammalian cardiac myocyte. *Circ. Res.*, 92:1182–1192, 2003.
4. J. B. de Monvel, S. Le Calvez, and M. Ulfendahl. Image restoration for confocal microscopy: Improving the limits of deconvolution, with application to the visualization of the mammalian hearing organ. *Biophys J.*, 80:2455–2470, 2001.
5. D. W. Fawcett and N. S. McNutt. The ultrastructure of cat myocardium. I. ventricular papillary muscle. *Cell Biol.*, 42:1–45, 1969.
6. R. C. Gonzalez and R. E. Woods. *Digital Image Processing.* Addison-Wesley, Reading, Massachusetts; Menlo Park, California, 1992.
7. J. He, M. W. Conklin, J. D. Foell, M. R. Wolff, R. A. Haworth, R. Coronado, and T. J. Kamp. Reduction in density of transverse tubules and l-type ca(2+) channels in canine tachycardia-induced heart failure. *Cardiovasc Res*, 49(2):298–307, 2001.
8. W. Heiden, T. Goetze, and J. Brickmann. 'Marching-Cube'-Algorithmen zur schnellen Generierung von Isoflächen auf der Basis dreidimensionaler Daten-

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felder. In M. Frühauf and Martina Göbel, editors, *Visualisierung von Volumendaten*, pages 112–117. Springer, Berlin, Heidelberg, New York, 1991.
9. W. E. Lorensen and H. E. Cline. Marching cubes: A high resolution 3D surface construction algorithm. *Computer Graphics*, 21(4):163–169, 1987.
10. P. J. Mohler, J. Q. Davis, and V. Bennett. Ankyrin-B coordinates the Na/K ATPase, Na/Ca exchanger, and $Insp_3$ receptor in a cardiac t-tubule/SR microdomain. *PLoS Biology*, 3(12):2158–2167, 2005.
11. E. Page and M. Surdyk-Droske. Distribution, surface density, and membrane area of diadic junctional contacts between plasma membrane and terminal cisterns in mammalian ventricle. *Circ. Res.*, 45(2):260–267, 1979.
12. W. H. Press, S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery. *Numerical Recipes in C*. Cambridge University Press, Cambridge, New York, Melbourne, 2 edition, 1992.
13. W. H. Richardson. Bayesian-based iterative method of image restoration. *J Opt Soc Am*, 62:55–59, 1972.
14. V. G. Robu, E. S. Pfeiffer, S. L. Robia, R. C. Balijepalli, Y. Pi, T. J. Kamp, and J. W. Walker. Localization of functional endothelin receptor signaling complexes in cardiac transverse tubules. *J Biol Chem*, 278(48):48154–48161, 2003.
15. F. B. Sachse. *Computational Cardiology: Modeling of Anatomy, Electrophysiology, and Mechanics*, volume 2966 of *Lecture Notes in Computer Science*. Springer, Heidelberg, 2004.
16. E. Savio, J. Frank, M. Inoue, J. I. Goldhaber, M. B. Cannell, J. H. B. Bridge, and F. B. Sachse. High-resolution three-dimensional confocal microscopy reveals novel structures in rabbit ventricular myocyte t-tubules. In *Biophys. J (Annual Meeting Abstracts)*, 2007.
17. E. Savio, J. I. Goldhaber, J. H. B. Bridge, and F. B. Sachse. A framework for analyzing confocal images of transversal tubules in cardiomyocytes. In F. B. Sachse and G. Seemann, editors, *Lecture Notes in Computer Science*, volume 4466, pages 110–119. Springer, 2007.
18. N. Shepherd and H. B. McDonough. Ionic diffusion in transverse tubules of cardiac ventricular myocytes. *Am J Physiol Heart Circ Physiol*, 275:852–860, 1998.
19. C. Soeller and M. B. Cannell. Examination of the transverse tubular system in living cardiac rat myocytes by 2-photon microscopy and digital image-processing techniques. *Circ Res*, 84:266–275, 1999.
20. J. G. Stinstra, B. Hopenfeld, and R. S. MacLeod. On the passive cardiac conductivity. *Ann Biomed Eng*, 33(12):1743–51, 2005.
21. F. Swift, T. A. Stromme, B. Amundsen, O. Sejersted, and I. Sjaastad. Slow diffusion of $K^+$ in the T tubules of rat cardiomyocytes. *J Appl Physiol*, 101:1170–1176, 2006.
22. J. Wernecke. *The Inventor Mentor: Programming Object-Oriented 3D Graphics with Open Inventor*. Addison-Wesley Professional, 1 edition, 1994.
23. C. Wong, C. Soeller, L. Burton, and M. B. Cannell. Changes in transverse-tubular system architecture in myocytes from diseased human ventricles. In *Biophys. J (Annual Meeting Abstracts)*, number 82, page a588, 2002.