Three-dimensional analysis of the tubular networks in mammalian and amphibian skeletal muscle fibres

I.D. Jayasinghe,[1] D. Baddeley,[2] C. Soeller[2] and B.S. Launikonis,[1] [1]School of Biomedical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia and [2]School of Medical Sciences, University of Auckland, Auckland, New Zealand.

The skeletal muscle tubular (t-) system is a series of invaginations of the surface sarcolemma and forms a dense network of tubules throughout the interior of the fibre. Its primary functions are to maintain excitability of the muscle and to carry the action potentials deep into the fibre to effect rapid release of calcium from the intracellular stores, which permits forceful and synchronous contraction by the myofibrils. Furthermore, t-tubules also mediate the movement of ions and biomolecules in and out of the t-system, with different elements of the t-system consisting of different size exclusions to solutes within their lumina (Edwards & Launikonis, 2008). The architecture of the t-system has been extensively studied using electron and fluorescence microscopies, although a complete three-dimensional analysis of its connectivity and a statistical characterization of t-tubule diameters is currently lacking. The re-sealing of the tubular mouths following mechanical skinning provides an ideal assay to trap fluorescent probes within the t-system and high-contrast imaging of the complete connectivity of this network. This technique of dye-trapping was combined with high-resolution 3D confocal fluorescence imaging and a novel image analysis protocol to produce 3D reconstructions of the tubular network within large areas of living fibres from rat *extensor digitorum longus* (EDL) and toad *iliofibularis* . Fluorescence intensity along detected t-tubular connections were calibrated to estimate local tubular diameter by adapting an image analysis previously demonstrated by Soeller & Cannell (1999).

Six-week old male Wistar rats and adult cane toads were euthanized according to the guidelines provided by the Animal Ethics Committee of the University of Queensland. Rats were killed by asphyxiation with CO_2. Toads were stunned by a blow to the back of the head and double-pithed. The EDL muscles and iliofibularis muscles were dissected from the respective species and pinned out within liquid paraffin. Small bundles of fibres from the muscle were isolated and exposed extracellularly to 5 mM Fluo-5N pentopotassium salt or 2 mM fixable dextran-linked Alexa680 in Na-based physiological Ringer solution. Individual fibres were then mechanically skinned, trapping the penetrated dye within the sealed tubular systems. Skinned fibres were transferred into a standard K-based internal solution containing 100 nM free calcium and pinned out in a microscope chamber with a No. 1.5 glass coverslip at the bottom. Confocal z-stacks of samples containing Fluo5N dye were obtained using an inverted Olympus FV1000 confocal microscope with a 488 nm Argon-ion laser excitation and a 60× oil-immersion 1.35NA Olympus objective. Image stacks were deconvolved using an iterative Richardson-Lucy maximum-likelihood deconvolution algorithm implemented in IDL. Image volumes were then subject to 3D skeletonization to trace the connectivity of the t-tubules and the local diameters determined using the calibration protocol used by Soeller & Cannell (1999). Direct stochastic optical reconstruction microscopy (dSTORM) was used for super-resolution imaging of the tubular network within the sub-sarcolemmal region of fibres with fixable dextran-Alexa680 loaded into the t-system. Fibres were fixed with 4% paraformaldehyde and mounted in a medium containing 90% Glycerol and 5 mM β-mercaptoethylamine in phosphate buffered saline. Samples were imaged on a modified Nikon TE2000 inverted TIRF microscope with a 671 nm solid-state laser excitation, a Nikon 60× 1.49NA Nikon TIRF objective and a cooled electron-multiplying CCD camera. Image frames were acquired at an integration time of 50 ms and the single fluorophore events were detected and final images were rendered using custom-written programs implemented in Python.

The local diameters estimated based on confocal data of rat and toad fibres ranged from ˜40 to 150 nm, with ~8% and ~5% of the tubules found extending longitudinally. Regions of densely arranged longitudinal tubules were observed deep within toad fibres. A dense mesh of tubules was observed fully encasing the central nuclei in toad fibres and lining the cytoplasmic face of the peripheral nuclei in rat fibres. This close special association may support their putative role in IP3-receptor-mediated nuclear signalling suggested recently by Escobar *et al.* (2011). We have also observed a dense two dimensional network (the subsarcolemmal tubular network; SSTN) that appeared continuous with the more transversely-ordered t-system. dSTORM images of this network in both skinned and non-skinned fibres have revealed a complex network of tubules with a mean diameter of ~150 nm.

Edwards JN, Launikonis BS. (2008) *Journal of Physiology* **586:** 5077-89.

Escobar M, Cardenas C, Colavita K, Petrenko NB, Franzini-Armstrong C. (2011) *Journal of Molecular and Cellular Cardiology* **50:** 451-459.

Soeller C, Cannell MB. (1999) *Circulation Research* **84: 266-275.**