Journal of Molecular and Cellular Cardiology 76 (2014) 1–11

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Methodological review

Serial block face scanning electron microscopy for the study of cardiac muscle ultrastructure at nanoscale resolutions

Christian Pinali, Ashraf Kitmitto *

Institute of Cardiovascular Sciences, Faculty of Medical and Human Sciences, University of Manchester, M13 9NT, UK

ARTICLE INFO

Article history:
Received 18 June 2014
Received in revised form 31 July 2014
Accepted 12 August 2014
Available online 20 August 2014

Keywords:
Serial block face scanning electron microscopy
Three-dimensional
Transverse-tubules
Sarcoplasmic reticulum
Dyad

ABSTRACT

Electron microscopy techniques have made a significant contribution towards understanding muscle physiology since the 1950s. Subsequent advances in hardware and software have led to major breakthroughs in terms of image resolution as well as the ability to generate three-dimensional (3D) data essential for linking structure to function and dysfunction. In this methodological review we consider the application of a relatively new technique, serial block face scanning electron microscopy (SBF-SEM), for the study of cardiac muscle morphology. Employing SBF-SEM we have generated 3D data for cardiac myocytes within the myocardium with a voxel size of ~15 nm in the X–Y plane and 50 nm in the Z-direction. We describe how SBF-SEM can be used in conjunction with selective staining techniques to reveal the 3D cellular organisation and the relationship between the t-tubule (t-t) and sarcoplasmic reticulum (SR) networks. These methods describe how SBF-SEM can be used to provide qualitative data to investigate the organisation of the dyad, a specialised calcium microdomain formed between the t-ts and the junctional portion of the SR (jSR). We further describe how image analysis methods may be applied to interrogate the 3D volumes to provide quantitative data such as the volume of the cell occupied by the t-t and SR membranes and the volumes and surface area of jSR patches. We consider the strengths and weaknesses of the SBF-SEM technique, pitfalls in sample preparation together with tips and methods for image analysis. By providing a 'big picture' view at high resolutions, in comparison to conventional confocal microscopy, SBF-SEM represents a paradigm shift for imaging cellular networks in their native environment.

Contents

Abbreviations: ECC, excitation–contraction coupling; EM, electron microscopy; FEG, field emission gun; jSR, junctional sarcoplasmic reticulum; LTCC, L-type voltage-gated calcium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SEM, scanning electron microscopy; ssTEM, serial section transmission electron microscopy; SPA, single particle analysis; 3D, three-dimensional; TEM, transmission electron microscopy; t-ts, transverse-tubules; 2D, two-dimensional.

* Corresponding author at: 3rd Floor Core Technology Facility, Cardiovascular Group, Grafton Street, Manchester M13 9NT, UK. Tel.: +44 1613064186; fax: +44 1612751183.
E-mail address: ashraf.kitmitto@manchester.ac.uk (A. Kitmitto).

http://dx.doi.org/10.1016/j.yjmcc.2014.08.010

1. Introduction

Structural studies of the heart have been fundamental to our understanding of cardiac physiology providing mechanistic insights into complex biological processes. For example, the application of high resolution transmission electron microscopy (TEM) to the study of skeletal muscle in the 1950s led to the development of the sliding filament theory describing the process of muscle contraction [1,2]. Since then electron microscopy (EM) has continued to play a major role for unravelling the mechanisms underlying muscle contraction namely excitation–contraction coupling (ECC) [3]. In the heart calcium (Ca^{2+}) regulates ECC through a process termed calcium-induced calcium release (CICR). CICR is a phenomenon whereby depolarisation of the cell membrane leads to an inward current (I_{Ca}) generated by the opening of the L-type voltage-gated Ca^{2+} channel (LTCCs) localised to both the transverse-tubule (t-t) system and the surface sarcolemma. This influx of Ca^{2+} ions into the cytosol triggers the release of a bolus of Ca^{2+} from the sarcoplasmic reticulum (SR), via the ryanodine receptors (RyR) anchored in the SR that activates the myofilaments. Other factors including SR Ca^{2+} content and Ca^{2+} sensing proteins such as calsequestrin also play a key role in maintaining synchronous CICR (for reviews see [4,5]). CICR is proposed to be under a 'local control' mechanism whereby the LTCC Ca^{2+} current (I_{Ca}) directly influences the opening of the RyRs, leading to Ca^{2+} release from the SR with disruption of I_{Ca} resulting in termination of SR Ca^{2+} release [6,7]. The release of Ca^{2+} from the SR into the cytosol gives rise to the calcium transient which is the summation of a series of Ca^{2+} sparks from the RyR [8]. The cellular geometry of the t-ts and SR and spatial distribution of the LTCC and RyR are central to the generation of Ca^{2+} sparks, a process integral for ECC.

Confocal microscopy techniques have been fundamental to unravelling the molecular level processes of CICR, by visualisation of SR Ca^{2+} release in the form of 'sparks' [9,10] and showing that the Ca^{2+} sparks triggered within each dyad contribute to the Ca^{2+} transient of the cell [11–13]. However, it is electron microscopy methods that have revealed how the t-ts and the junctional portion of the SR (jSR) are organised to form a dyad, the site of Ca^{2+} release. This methodological review will focus primarily upon the application of the novel technique of serial block face scanning electron microscopy (SBF-SEM) to the study of cardiac myocyte ultrastructure, and in particular the organisation of the sarcotubular network. Briefly, we shall also consider the other types of EM techniques that have contributed towards furthering our understanding of ECC to provide a context for the potential that SBF-SEM offers.

2. Preparation of the heart myocardium for TEM study; issues of image quality

Key to any EM study is the sample preparation step for preservation of the specimen in as close to the native state as possible, to ensure that the resultant images reflect the morphology and ultrastructure of the tissue. In order for biological specimens to withstand the vacuum and damage from the electron beam tissue samples are routinely chemically fixed and then embedded in plastic blocks.

2.1. Chemical fixation of the left ventricle

A combination of paraformaldehyde and glutaraldehyde (penetration limit of ~2 mm of tissue) is typically used in a cocktail known as the Karnovsky's fixative [14]. A standard recipe for tissue fixation employs 2.5% glutaraldehyde (w/v) in 0.1 M sodium cacodylate buffer (pH 7.2–pH 7.4) and between 2–4% (w/v) paraformaldehyde as a primary fixation step to cross-link the protein components. Many of the protocols for fixation and staining have been optimised using perfusion fixation in small mammal hearts but this is not always practical when working with larger animals or with human samples. For our studies of sheep and rat myocardium we have obtained good sample preservation by immersing small (1–2 mm^3) pieces of left ventricular tissue, taken immediately after sacrifice, in Karnovsky's fixative for 15 min with the paraformaldehyde concentration kept below 2% to avoid cellular shrinkage at room temperature (RT) [15]. The tissue (~1 cm^3) is then chopped into several smaller pieces in a petri dish containing the fixative and the trimmed slices are transferred into fresh fixative for 2–3 h at 4 °C. During the trimming process the tissue must always be immersed in the fixative. We recommend that the sample is not left for more than seven days in the fixative to avoid cell shrinkage. The specimen is then washed (5 × 5 min) in the primary fixation buffer. Note: This is important because at this stage the specimen is still semi-permeable and so washing with water will potentially cause osmotic changes and cellular damage. The next step is postfixing in 2% (v/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide in sodium cacodylate buffer (pH 7.2–pH 7.4; 1 h at room temperature, RT). Osmium tetroxide diffuses into the cell membranes and cross-links the lipid constituents. After treatment with osmium tetroxide the tissue is permeabilised so double-distilled water (ddH_2O) can be used to wash the sample between treatments as described below. To visualise the sarcoplasmic reticulum this secondary fixation step is modified as described in Section 2.2.1.

Biological matter is essentially made up of C, N and O with atomic numbers 6, 7 and 8 and so is barely visible within the microscope, thus there is a need to enhance the contrast and improve the scattering properties. This is achieved by post-staining methods. Uranyl acetate and lead citrate are heavy metal stains commonly used for post-staining but uranyl acetate is generally used for *en bloc* staining. After fixation the tissue is washed (3 × 10 min) in ddH_2O at RT. The sample is then left overnight in 0.5% w/v aqueous uranyl acetate at 4 °C, after which it is washed again with ddH_2O (3 × 10 min). In addition to enhancing the contrast of the biological material by a direct association, staining can be selective for delineation of different cellular features. Phosphotungstic acid for example, attaches to positively charged proteins as well as polysaccharides whereas uranyl acetate binds mostly to negatively charged proteins. After staining, the tissue is washed and then dehydrated in a graded ethanol series at increasing concentrations; 25%, 50%, 75%, 90%, and 100% (twice) v/v in ddH_2O, all steps are carried out at RT. The next step involves transfer into propylene oxide (PO) for 15 min (2×) and then infiltration with increasing concentrations of resin; 2–3 h in resin:PO (v/v) mixes at ratios of 1:1, 1:2 and 2:1. Three further infiltration steps into pure resin (100%) are carried out, two for 2–3 h and one further step in pure resin overnight. The samples are then left to cure at 60 °C for 48 h.

The plastic blocks are then sectioned in an ultramicrotome, standard equipment for any EM laboratory and deposited on EM grids (we routinely use 400-mesh copper grids (Agar Ltd.)). The thickness of the