ESRF	**Experiment title:** Structural changes in the cardiac thick filament induced by phosphorylation of the myosin regulatory light chain	**Experiment number**: LS-2717
Beamline: ID02	**Date of experiment**: from: 19/10/2017 to: 24/10/2017	**Date of report**: 26/02/2018
Shifts: 15	**Local contact(s)**: T. Narayanan	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

*Dr. Elisabetta Brunello, King's College London
*Dr. Luca Fusi, King's College London
*Dr. Jesus Garcia Ovejero, King's College London
*Dr. So-Jin Holohan, King's College London
*Prof. Malcolm Irving, King's College London
Dr. Andrea Ghisleni, King's College London
Dr. Thomas Kampourakis, King's College London
Prof. Jon Kentish, King's College London

Report:

The aim of this project was to directly compare X-ray diffraction patterns from intact and chemically permeabilised cardiac trabeculae dissected from the right ventricle of rat hearts. Thanks to the high spatial resolution FReLoN detector available at the refurbished ID02 beamline we recorded the structural changes in the thin and thick filament structure that follow the permeabilization procedure and used an osmotic agent to recover the physiological interfilament lattice spacing.

Methods. Rats were sacrificed by cervical dislocation after sedation with Isoflurane (in compliance with the Home Office Schedule 1) and the heart was rapidly excised and cannulated via the ascending aorta and retrogradely perfused with Krebs-Henseleit solution saturated with oxicarb (95% O_2, 5% CO_2) to have a constant pH=7.4. The sacrifice of the animals was performed at ID17 and the perfused heart was brought to and dissected in the wet lab close to ID02. Trabeculae were dissected under a stereomicroscope and suitable right ventricular trabeculae were mounted in an experimental trough filled with the same solution between the levers of a force transducer and a motor or chemically permeabilised to allow direct control of the milieu bathing the myofilaments. For the intact preparations, the solution was continuously exchanged in the trough via a laminar flux between two opposite apertures parallel to the transducer levers. Temperature was continuously monitored and kept constant by controlling the temperature and the flux of the incoming solution. The trough was closed with a cover and sealed with silicon grease and was mounted vertically at the beamline to obtain the best spatial resolution on the meridional axis (parallel to the longitudinal muscle axis). Two mica windows placed as closed as possible to the muscle reduced the X-ray path in water. Platinum stimulating electrodes were positioned along the length of the trabecula. The permeabilised samples were stored at -20°C for up to 24h before mounting in relaxing solution on a multi-trough system between the levers of a force transducer and a motor to adjust muscle length. Temperature was continuously monitored and kept constant via a Peltier system. The trough was mounted vertically at the beamline to obtain the best spatial resolution on the meridional axis (parallel to the muscle axis). X-ray exposures were collected in a dedicated air chamber to avoid X-ray absorption in water.

Results. Our first aim was to measure the absolute periodicity of myosin in the thick filaments of cardiac muscle using the high spatial-resolution FReLoN CCD detector (ESRF), active area 4.9 × 4.9 mm^2, 2048 x 2048 pixels, pixel size 24 x 24 μm^2. The sample was moved vertically between X-ray exposures (20 ms per point on the trabecula) to spread the radiation damage (beam dimension on the sample ~300x70 μm^2, HxV; flux ~$7.6*10^{10}$ph/s). First, we recorded X-ray diffraction patterns at 31 m camera length to set the sarcomere length at ~2.0 μm in each sample using the the ultra-low angle reflections arising from the sarcomere periodicities. Secondly, 2D X-ray diffraction patterns were acquired at three different sample-to-detector distances (1.6 m, 2.4 m and 3.2 m), from intact or demembranated trabeculae from rat right ventricle, to record the reflections arising from the myosin-containing thick filament. X-ray patterns were binned by 8 in the horizontal direction before the readout to increase the signal-to-noise ratio on the meridional axis. At each camera length the distribution of diffracted intensity along the meridional axis of the X-ray pattern was calculated by integrating from 0.005 nm^{-1} on either side of the meridian. To get the absolute spacing calibration, the position on the detector of the M3 reflection, reporting the axial repeat of the myosin motors along the thick filament, was calculated as the weighted position of the component peaks measured by fitting multiple Gaussian peaks with the same axial width at each camera length. The scattering angle θ was measured from the linear fitting of the M3 position at the three camera lengths. The energy was calibrated with Au L_3 edge and Pb L_3 edge. The spacing of the M3 reflection was calculated as $d = \lambda/\sin\theta$. The measured values for the spacing of the M3 reflection (S_{M3}) were 14.479 ± 0.007 nm for intact cardiac trabecula in diastole (SL = 1.95 μm, T = 27°C) and 14.479 ± 0.011 nm for demembranted trabeculae in relaxing solution in the presence of 3% dextran T500 to reverse the swelling induced by the permeabilization procedure (SL = 2.14 ± 0.04 μm, T = 27°C). Therefore, under these experimental conditions, the axial repeat of the myosin motors along the myosin filament is not affected by the permeabilization procedure. Our second aim was to investigate the effect of the demembranation protocol on the structure of the thick filament by comparing low angle X-ray diffraction patterns from intact and demembranated trabeculae under similar experimental conditions. Therefore, at 1.6m camera length, we recorded the first myosin layer line (ML1), associated with the helical order of the myosin motors on the thick filament, the meridional myosin-based reflections (M1, …, M6), orders of a fundamental periodicity of ca. 43 nm, and the equatorial reflections, reporting the distribution of mass in the filament lattice, in intact and demembranated trabeculae. In the demembranted trabeculae the interfilament lattice spacing d_{10} and the equatorial intensity ratio I_{11}/I_{10} typical of intact trabeculae were recovered by the addition of 3% dextran T500 to the physiological solution. Moreover addition of dextran increased the intesities of the first myosin layer line I_{ML1} and of all the meridional reflections arising from myosin, close to the values observed in the patterns from intact trabeculae in diastole.

Conclusion. 1) We determined the absolute periodicity of myosin in the thick filaments of cardiac muscle. 2) The comparison of 2D X-ray diffraction patterns from intact and demembranated cardiac trabecuale from the right ventricle of the rat hearts showed that the physiological relaxed structure of the thick filament in demembranated trabeculae is recovered by addition of 3% dextran T-500. These data provide the basis for the design of a novel experimental approach using demembranated trabeculae as a physiological experimental model to investigate regulatory mechanisms of cardiac contractility based on calcium- and phoshorylation-dependent structural changes in the thick filament.