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Report:

The aim of this project was to optimise cardiac muscle preparations and protocols for high-resolution X-ray diffraction studies aimed at answering key questions about the regulation of the heart in health and disease. In particular we aimed at recording the fine structure of the axial myosin-based reflections during the physiological cardiac cycle, because the resolution of these reflections is a fundamental requirement for future studies of thick-filament based regulation of cardiac muscle contractility. Firstly we set up the optimal physiological conditions to collect X-ray patterns from electrically-paced intact rat ventricular trabeculae, which are a good compromise in terms of sample length and diffracting mass and have been used in previous X-ray diffraction experiments. Secondly we were able to collect X-ray diffraction patterns from intact preparations isolated from the mouse, which are much smaller than those from the rat, to test the feasibility of X-ray studies of mouse hearts for future work on transgenic disease models. Finally we evaluated the quality of the patterns from relaxed rat and mouse trabeculae in which the cell membrane was permeabilised to enable future studies using demembranated cardiac preparations.

Methods. Rats (mice) were sacrificed by cervical dislocation (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 (rats/mice) 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 (or papillary muscles) were mounted in an experimental trough filled with the same solution between the levers of a force transducer and a motor. The solution was continuously exchanged through 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.

Results. Intact rat trabeculae were stimulated at 27.5ºC at 1Hz; under these conditions the sample could be costantly paced for hours. The sample was moved vertically between X-ray exposures to spread the radiation damage and we estimated that the maximum full-beam time-exposure per frame was 10ms to avoid local radiation damage (beam dimension on the sample ~350x60 μm^2, HxV; flux ~10^{12}ph/s). The total exposure for each sample was ~400 ms. Under these conditions we exploited the flexibility of the ID02 beamline, both

in terms of detector pool and range of camera lengths (0.6m to 31m from the sample position). Most of the experiments used the FReLoN detector because of the higher spatial resolution and dynamic range and the possibility to use differential binning (8x1) to further increase the S:N on the meridional axis of the X-ray pattern.

In each experiment we collected X-ray patterns using a sample-to-detector distance of 31m, in order to measure on-line the sarcomere length in the trabecula both during diastole (Fig 1A) and during systole (Fig 1B) and to estimate the extent of shortening during force development. Data were recorded at 1.6m camera length (Fig. 1C-D) to record the fine structure of the myosin-based reflections arising from the axial periodicities of the myosin motors in the thick filament. The X-ray pattern during diastole showed a myosin layer line reflection (ML1) arising from the helical arrangement of the myosin motors, and axial reflections (M1-6) indexing at a periodicity of 43nm (Fig 1C), similar to the X-ray pattern from resting skeletal muscle. During systole all the reflections became weaker and both M3 and M6, associated with the myosin motors and the thick filament backbone respectively, changed their spacing. This is the first record, at our knowledge, of changes in intensity, spacing and fine structure of the myosin-based reflections from diastole to systole. We also started to investigate the effects on the X-ray patterns during diastole and systole induced by increasing the temperature to 32ºC, closer to the physiological value, and the sarcomere length from 1.9 µm to 2.2 µm in order to study the phenomenon of length-dependent activation, a fundamental auto-regulatory mechanism in the heart. In one experiment we recorded time-resolved changes in the X-ray pattern during a single cardiac twitch using the Pilatus detector to collect 35 17ms-frames with 3ms readout between each frame (beam attenuated to have 10ms total exposure at each point along the trabecula). Despite the lower spatial resolution of this detector this experiment showed that it is possible to follow the structural changes in the meridional reflections occurring throughout the whole cardiac contraction cycle.

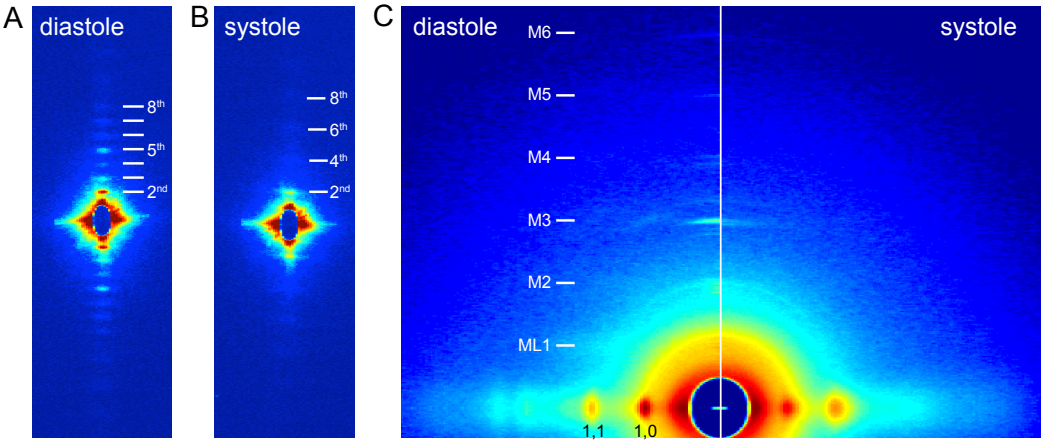

Fig. 1. *X-ray diffraction patterns from intact rat ventricular trabeculae at 30m (A and B) and 1.6m (C and D) camera length during diastole (A and C) and systole (B and D). Data collected with FReLoN detector at ID02 with exposure time 2ms (A and B) or 20 ms (C and D). The vertical axis is parallel to the fibre axis: reflections in A and B are orders of the sarcomere length periodicity (2.06 µm, A; 1.80 µm, B); reflections in C and D are orders of the myosin-based reflections with periodicity ca. 43 nm. Temperature, 27.5ºC; stimulation frequency, 1Hz.*

The high quality of the data collected from intact rat trabeculae prompted us to test other cardiac preparations. Therefore we collected data from rat ventricular papillary muscles which have bigger diffracting mass and can be used in future experiments to record the weaker meridional or layer line reflections. To test the feasibility of potential future studies on cardiac muscle disease using transgenic mouse models, we also used trabeculae and papillary muscles from the mouse. Although these samples have a smaller diffracting mass, we were able to record X-ray patterns with clear meridional reflections by averaging data from consecutive contraction cycles. Finally X-ray patterns with adequate signal-to-noise were also collected from demembranated trabeculae in relaxing solution.

Conclusion. We efficiently exploited the allocated beamtime with a new team and a new setup built explicitly for these experiments: we collected data from 21 samples in 15 shifts and we are currently analysing them. X-ray diffraction patterns in which the fine structure of the myosin-based reflections is resolved can be recorded from electrically paced mouse and rat intact cardiac preparations and from demembranated ventricular trabeculae. The high spatial resolution of the X-ray patterns from the heart will be exploited by a new generation of X-ray studies aimed at elucidating the structural dynamics of the thick filament during the cardiac cycle. Moreover it has become now possible to test the mechanosensing properties of the cardiac thick filament by measuring with subnanometer precision changes in the conformation of the myosin motors associated with stress-induced changes in the thick-filament backbone.