	Experiment title: Molecular structural basis of relaxation in skeletal muscle	**Experiment number**: LS3206
Beamline: ID02	**Date of experiment**: from: 13 June 2023 to: 16 June 2023	**Date of report**: 13 Sep 2023
Shifts: 9	**Local contact(s)**: Theyencheri Narayanan	*Received at ESRF:*

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Report: Muscle contraction is driven by sliding between thick (myosin-containing) and thin (actin-containing) filaments, and triggered by a transient increase in calcium ion concentration in the muscle cells. The calcium ions bind to the thin filaments, causing a structural change that permits binding of the myosin motor domains to actin, providing a start signal for contraction. The strength, speed and metabolic cost of contraction are controlled by a second set of regulatory structural changes in the thick filaments in which the myosin motors are released from the helical folded conformation on the surface of the thick filaments that made them unavailable for binding actin in resting muscle[1]. Release of the motors from the helical folded state is triggered mechanically, by the strain in the thick filament[2]. This intrinsic mechano-sensing mechanism couples the number of active motors to the external load on the muscle, maximizing the efficiency of contraction. The positive feedback loop implicit in this mechanism, together with the fact that actin-bound motors can themselves activate the *thin* filaments in a second positive feedback loop, are likely to have evolved to maximise the speed of muscle activation. Conversely, they are strongly detrimental to rapid muscle relaxation following the falling phase of the calcium transient. The mechanisms by which muscle escapes these positive feedback loops in order to relax rapidly have remained mysterious, although they are known to involve a phase transition from slow 'isometric relaxation', in which the filaments are locked in an activated state, to 'chaotic relaxation', involving transient yielding of local regions of the muscle cell[3]. The yield point is also mechanically determined, but the molecular structural basis of the yield process is unknown and it has received little attention, despite the fact that rapid relaxation is essential to the normal performance of both skeletal and cardiac muscle and is impaired in muscle disease. Better understanding of the normal mechanisms of relaxation is needed to underpin the development of improved therapies for muscle disease.

Time-resolved X-ray diffraction uniquely provides nm-scale resolution of changes in filament structure and myosin motor conformation on the millisecond timescale in living muscles in physiological conditions[4-6].

The upgraded ID02 beamline with Eiger 2-4M detector is currently the best facility worldwide for such studies. In particular, the combination of large area and small pixel size of this detector allows the intensity of the actin-based second layer line, reporting the activation state of the thin filament, to be recorded at the same time as the low-angle myosin-based reflections that report the activation state of the thick filament and the motor conformations. The ability to move the detector between 2.0 and 31 m in the same experiment allows sarcomere length to be measured in the same preparations and protocols, a capability unique to this beamline.

In LS 3206 isolated intact extensor digitorum longus (EDL) muscles of the mouse were dissected at the animal facility of the beamline ID17 and then transferred to the beamline ID02, where they were mounted vertically at sarcomere length 2.4μm at ID02 in a temperature-controlled trough in oxygenated Krebs solution at 27°C. Electrical stimulation, force measurement, and control of muscle length used the standard methods of our lab[6]. X-ray diffraction patterns were recorded with the Eiger 2-4M detector. X-ray exposure was controlled using fast shutters and monitored with a pin diode. The muscle was moved vertically between exposures to reduce radiation damage. Data were analysed with SAXS utilities (M. Sztucki, ESRF), SAXS Package (P. Boesecke, ESRF) and Igor Pro (WaveMetrix Inc).

Muscles were stimulated repetitively at 130 Hz for 100 ms at 27°C, with a recovery period of 3 min between successive contractions. Before the stimulus muscles were stretched by 5% in 8 ms in order to investigate the hypothesis that the resting state is stabilized by binding of the N-termini of myosin binding protein-C to the thin filaments whilst its C terminus is strongly bound to the thick filaments. The 5% stretch would be expected to detach these links. Muscle length was held fixed during the stimulation and for the first 15 ms after stimulation, during which intracellular calcium concentration decreases substantially[7]. Ramp shortening of 5% was then imposed in 8 ms to reduce force to zero, and thereby determine the role of mechano-sensing in relaxation. We recorded an average of 40 runs per muscle in 10 muscles including fixed-end controls, using a combination of 2.0, 3.2m and 31m sample-to-detector distances and 2-ms time frames (1.8ms live time). We measured the second actin-based layer line that reports the azimuthal position of tropomyosin from the 2.0-m data. We measured changes in equatorial reflections, myosin-based layer lines, myosin-based meridional reflections (in particular the interference fine-structure of the M3 reflection from the axial repeat of the myosin motors that also reveals the region of the thick filament in which motors are activated[6]), the spacing of the M6 reflection that mediates mechano-sensing in the thick filament backbone, troponin-based meridional reflections, all from the 3.2m data. We measured sarcomere length from the 31m data. Data analysis is in progress.

References [1]Irving M 2017 *Biophys J* 113: 2579. [2]Linari M *et al*. 2015. *Nature* 528:276. [3]Poggesi et al. 2005. *Eur J Physiol*. 449:505. [4]Piazzesi G *et al.* 2002 *Nature* 415:659, 2007. [5]Piazzesi G *et al.* 2007 *Cell* 131:784. [6]Hill C *et al*. 2021 *eLife* 10:e68211. [7]Caputo *et al* 1994. J. *Physiol* 478:137.