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X-ray Diffraction of Intact Murine Skeletal Muscle as a Tool for Studying the Structural Basis of Muscle Disease

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Corresponding Author:	Thomas Irving, Ph.D. Illinois Institute of Technology Chicago, IL UNITED STATES
Corresponding Author's Institution:	Illinois Institute of Technology
Corresponding Author E-Mail:	irving@iit.edu
Order of Authors:	Weikang Ma
	Thomas Irving, Ph.D.
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TITLE:

X-ray Diffraction of Intact Murine Skeletal Muscle as a Tool for Studying the Structural Basis of Muscle Disease

AUTHORS AND AFFILIATIONS:

Weikang Ma[1], Thomas C. Irving[1]

[1]BioCAT, Dept. of Biological Sciences, Illinois Institute of Technology, Chicago, IL, USA

Email addresses of co-authors:

Weikang Ma (wma6@iit.edu)

Thomas C. Irving (irving@iit.edu)

KEYWORDS:

skeletal muscle; X-ray diffraction; acto-myosin interaction; sarcomere structure; skeletal muscle myopathy; skeletal muscle physiology

SUMMARY:

We present detailed protocols for performing small-angle X-ray diffraction experiments using intact mouse skeletal muscles. With the wide availability of transgenic mouse models for human diseases, this experimental platform can form a useful test bed for elucidating the structural basis of genetic muscle diseases

ABSTRACT:

Transgenic mouse models have been important tools for studying the relationship of genotype to phenotype for human diseases including those of skeletal muscle. Mouse skeletal muscle has been shown to produce high quality X-ray diffraction patterns on third generation synchrotron beamlines providing an opportunity to link changes at the level of the genotype to functional phenotypes in health and disease by determining the structural consequences of genetic changes. We present detailed protocols for preparation of specimens, collecting the X-ray patterns and extracting relevant structural parameters from the X-ray patterns that may help guide experimenters wishing to perform such experiments for themselves.

INTRODUCTION:

Synchrotron small-angle X-ray diffraction is the method of choice for studying the nm-scale structure of actively contracting muscle preparations under physiological conditions. Importantly, structural information from living or skinned muscle preparations can be obtained in synchrony with physiological data, such as muscle force and length changes. There has been increasing interest in applying this technique to study the structural basis of inherited muscle diseases that have their basis in point mutations in sarcomeric proteins. The muscle biophysics community has been very active in generating transgenic mouse models for these human disease conditions that could provide ideal test beds for structural studies. Recent publications from our group[1-3] and others[4,5] have indicated that the X-ray patterns from the mouse extensor digitorum longus (EDL) and soleus muscles can provide all the diffraction information available from more

traditional model organisms such as frog and rabbit psoas skeletal muscle. An advantage of the mouse skeletal muscle preparation is the ease of dissection and performing basic membrane-intact, whole muscle physiological experiments. The dimensions of the dissected muscle have sufficient mass to yield highly detailed muscle patterns in very short X-ray exposure times (~millisecond per frame) on third generation X-ray beamlines.

Muscle X-ray diffraction patterns consist of the equatorial reflections, the meridional reflections as well as the layer line reflections. The equatorial intensity ratio (ratio of the intensity of the 1,1 and 1,0 equatorial reflections, I_{11}/I_{10}), is closely correlated to the number of attached cross-bridges, which is proportional to the force generated in mouse skeletal muscle[2]. The meridional reflections that report periodicities within the thick and thin filaments can be used to estimate filament extensibility[1,3,6,7]. Diffraction features not on the meridian and the equator are called layer lines, which arise from the approximately helically ordered myosin heads on the surface of thick filament backbone as well as the approximately helically ordered thin filaments. The intensity of myosin layer lines is closely related to the degree of ordering of myosin heads under various conditions[2,8]. All of this information can be used study the behaviors of sarcomeric proteins in situ in health and disease.

Synchrotron X-ray diffraction of muscle has been historically done by teams of highly specialized experts but advances in technology and the availability of new data reduction tools indicate that this need not always be the case. The BioCAT Beamline 18ID at the Advanced Photon Source, Argonne National Laboratory has dedicated staff and support facilities for performing muscle X-ray diffraction experiments that can help newcomers to the field get started in using these techniques. Many users choose to formally collaborate with BioCAT staff, but an increasing number of users find they can do the experiments and analysis themselves reducing the burden on beamline staff. The primary goal of this paper is to provide training that provides potential experimenters with the information they need to plan and execute experiments on the mouse skeletal muscle system either at the BioCAT beamline or at other high flux beamlines around the world where these experiments would be possible.

PROTOCOL:

All animal experiments protocols were approved by the Illinois Institute of Technology Institutional Animal Care and Use Committee (Protocol 2015-001, Approval date: 3 November 2015) and followed the NIH "Guide for the Care and Use of Laboratory Animals"[9].

1. Pre-experiment preparation

1.1. Prepare 500 mL of Ringer's solution (contains: 145 mM NaCl, 2.5 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10.0 mM HEPES, 11 mM glucose, pH 7.4) freshly for each day of the experiment.

1.2. Fill 200 mL of Ringer's solution in a spray bottle and store at 4 °C fridge. Fill a Petri dish (10 cm in diameter) with Ringer's solution and perfuse with 100% oxygen by connecting the tube from an oxygen cylinder to an aquarium air stone. The Petri dishes ("dissecting dishes") were

previously coated with an elastomer compound to allow inserting pins during the dissection.

1.3. Prepare metal mounting hooks. Cut two pieces of stainless-steel wire, 0.5 mm in diameter, to the appropriate length and bend the wire at both ends to form hooks. Arrange all the dissecting tools, scissors, suture tying forceps, micro-scissors handy for use.

NOTE: The hook part should be about 3 mm long. The longer wire (ending in a hook) should be about 5 cm long, and the shorter wire (also ending in a hook) should be about 1 cm long in order to fit the custom chambers used at BioCAT and allow for a sufficient range of motion for the transducer arm.

1.4. Connect and turn on all the equipment. This includes a combined motor/force transducer, motor/force transducer controller a high-power bi-phasic current stimulator, and a computer controlled data acquisition/control system.

1.4.1. Turn on the data acquisition system and calibrate it before beginning the experiment[10]. Briefly, calibrating the force by adding a set of known weights, covering up to 50% of the maximum force measured by the force transducer in a linear progression, on the force transducer and recording the output voltage changes. Calibrate the length by applying a set of known output voltage to the lever arm and measure the length change of the arm.

1.4.2. Connect the hoses from the thermal block on the sample holder to a refrigerated circulating bath and set the temperature to maintain the desired temperature in the chamber to between 10 °C and 40 °C. Determine this empirically ahead of time by setting the circulating bath to a range of temperatures and measuring the temperature in the chamber with a thermocouple.

2. Muscle preparation

2.1. Euthanizing the mouse

2.1.1. Euthanize the mouse by carbon dioxide inhalation followed by cervical dislocation.

2.1.2. Spray the skin on the hind limb with cold Ringer's solution to prevent hair from blowing into the preparation. Remove the skin by cutting it away around the thigh using fine dissection scissors and quickly pull the skin down using #5 forceps to expose the muscles.

2.1.3. Amputate the hind limb and transfer it to a dissecting dish that has been filled with oxygenized Ringer's solution, and then place under a binocular dissecting microscope.

2.2. Preparing a soleus muscle

2.2.1. Pin the hind limb down in the dissecting dish with the gastrocnemius muscle facing upwards. Cut the distal tendon of the gastrocnemius/soleus muscle group and lift the muscles gently and slowly by cutting away the fascia on either side of the gastrocnemius muscle using

fine scissors. Isolate the gastrocnemius/soleus muscle group from the limb after freeing the proximal tendon of the soleus muscle.

2.2.2. Pin the muscle group containing the gastrocnemius muscle and the distal tendon down in the dissecting dish. Lift the soleus muscle gently via the proximal tendon and separate it from the gastrocnemius muscle leaving as much of the soleus distal tendon intact as possible.

2.3. Preparing an extensor digitorium longus (EDL) muscle

2.3.1. Pin the hind limb down in the dissecting dish with the tibialis anterior muscle facing upwards. Cut the fascia along the tibialis anterior (TA) muscle and pull it clear using forceps. Identify and cut the distal tendon of the TA muscle. Lift the TA muscle and cut it out carefully without pulling on the EDL muscle.

2.3.2. Cut open the lateral side of the knee and expose the two tendons. Cut the proximal tendon, leaving as much of the tendon as possible still attached to the muscle, and lift the EDL muscle (medial muscle) by gently pulling the tendon. Cut the distal tendon once it is exposed.

2.4. Mounting the muscle

2.4.1. Pin down the muscle via the tendons, and trim all the extra fat, fascia and tendon away as much as possible. Insert one tendon into a pre-tied knot and tie the suture tightly with suture tying forceps. Tie the second knot on around the metal hook.

2.4.2. Repeat the same procedure with the long hook on the other end of the tendon. Make sure that none of the body of the muscle is contacted by the sutures. This will damage the preparation.

2.4.3. Attach the short hook to the bottom of the experimental chamber and the long hook to the dual mode force transducer/motor. Bubble the solution in the experimental chamber with 100% oxygen.

2.5. Optimizing stimulation protocols and muscle length

2.5.1. Stretch the muscle by adjusting the micromanipulators attached to the transducer/motor to generate a baseline tension between 15 to 20 mN before finding the best stimulus parameters. Set the stimulation voltage to 40 V. The stimulation current is systematically increased until there is no additional increase in twitch force. The highest current found is increased by about 50% to ensure supra-maximal activation.

2.5.2. Find the optimal length, L_0, defined as the muscle length that give maximum twitch force, by increasing the muscle length and activating the muscle with a single twitch until the active force (peak force minus baseline force) stops increasing.

2.5.3. Perform a short tetanic contraction (1 s activation) to test the mounting and stretch the muscle back to optimal baseline force if necessary. Record the muscle length in mm with a digital caliper.

3. X-ray diffraction

NOTE: The following description is for X-ray diffraction experiments done using the small angle X-ray diffraction instrument on the BioCAT beamline 18ID at the Advanced Photon Source, Argonne National Laboratory but similar methods could be employed on other beamlines such as ID 02 at the ESRF (France) and BL40XU at SPring8 (Japan). Beamline 18ID is operated at a fixed X-ray beam energy of 12 keV (0.1033 nm wavelength) with an incident flux of ~10^{13} photons per second in the full beam.

3.1. Choose a specimen to detector distance (camera length). Use a 1.8 m camera length for experiments examining the 2.7 nm actin and high order myosin reflections such as 2.8 nm meridional reflections. Use a 4-6 m camera for other experiments, where one is primarily interested in fine detail on the meridian and layer lines

3.2. Optimizing the position of the sample in beam

3.2.1. Determine the beam position by using a piece of X-ray sensitive paper that produces a dark spot in response to X-rays ("a burn"). Then use a video cross-hair generator to create a cross-hair aligned with the burn mark on the paper or simply make a mark on the video screen with a marker pen.

3.2.2. Use the BioCAT supplied graphical user interface to the sample positioner to move the muscle to be centered on the beam position. Oscillate the sample chamber at ~ 10-20 mm/s by moving the sample stage in order to spread the X-ray dose over the muscle during the exposure. Observe the sample as it moves to avoid large regions of fascia (contains collagen which will pollute the diffraction patterns) and to ensure that it stays illuminated during the entire path of its travel.

NOTE: The exact steps required in sections 3.3 and 3.4 to make the required settings and actions using the beamline-supplied graphical user interface will be beamline and detector specific. Ask beamline staff as to how to perform these operations.

3.3. Setting up the CCD (charge coupled device) detector for high resolution patterns from muscle in defined static states (resting, or during isometric contraction)

3.3.1. Set up the exposure time and exposure period in the graphical user interface to the control software. Take a dark background image before taking the exposure and repeat this procedure every 2 hours or after changing of exposure time to correct any drift in the detector readout electronics.

3.3.2. Attenuate the X-ray beam to desired value for the exposure. Then take an image. It is not possible to take sequences of images with this detector. The CCD detector also needs several seconds to read out an individual image.

3.4. Setting up the pixel array detector for a time resolved experiment

3.4.1. Set up the number of images, exposure time, exposure period in the graphical user interface. The pixel array detector used here needs at least 1 ms to readout. The maximum frame frequency for photon counting detector is 500 Hz. Use the photon counting detector output signal to control the X-ray shutter.

3.4.2. Attenuate the beam to the desired intensity. Arm the detector and wait for the trigger from the data acquisition system. Synchronize the mechanical and X-ray data by triggering them at the same time. The X-ray patterns are collected continuously throughout the protocol a with a 1 ms exposure time and a 2 ms exposure period.

NOTE: The exact exposure time and exposure period should be determined on a case by case basis for the information desired and the observed lifetime of the sample in the beam. Attenuate the beam in order to use no more X-ray beam than is needed to provide analyzable data in the chosen exposure period.

4. Post-experiment muscle treatment

4.1. Recover and weigh the muscle after each mechanical and X-ray experiment. Calculate the cross-sectional area of the muscle using the measured muscle length and the muscle mass[11] assuming a muscle density of 1.06 g/mL[12].

4.2. Stretch the muscle to the experimental length and fix the muscle in 10% formalin for 10 min. Separate the fixed muscle into a series of fiber bundles selected from locations throughout the entire muscle cross section[3].

4.3. Measure the sarcomere length using a video sarcomere length measuring system.

REPRESENTATIVE RESULTS:

Isometric tetanic contraction. Any kind of classic muscle mechanical experiment, such as isometric or isotonic contractions, can be performed with simultaneous acquisition of X-ray patterns. **Figure 1A** shows the experimental setup for mechanical and X-ray experiments. An example force trace for an isometric tetanic contraction is shown in **Figure 1B**. The muscle was held at resting for 0.5 s before activated for 1 s. The mechanical recording stops 1 s after the stimulus. The X-ray patterns were collected continuously throughout the protocol at 1 ms exposure time at 500 Hz.

X-ray diffraction patterns. The muscle X-ray diffraction pattern can give nanometer resolution structural information from structures inside the sarcomere. Muscle X-ray diffraction patterns

are composed of four equivalent quadrants divided by the equator and the meridian. The equatorial pattern arises from the myofilament packing within the sarcomere perpendicular to the fiber axis, while the meridional patterns report structural information from the myofilaments along the muscle axis. The remaining reflections not on the equator or the meridian are called layer lines. Layer lines (e.g., features labeled MLL4 and ALL6 in **Figure 2A**) arise from the approximately-helical arrangement of molecular subunits within the myosin containing thick filaments and the actin containing thin filaments. The myosin-based layer lines are strong and sharp in patterns from resting muscle (**Figure 2A**), while actin-based layer lines are more prominent in patterns from contracting muscle (**Figure 2B**). Difference patterns obtained by subtracting the resting pattern from the contracting pattern (**Figure 2C**) can shed light on structural changes during force development in healthy and diseased muscle. By following these structural changes at the millisecond time scale of the molecular events during muscle contraction, the X-ray diffraction patterns can reveal substantial structural information (**Figure 2D**).

Data Analysis using MuscleX. Here is an example of equatorial reflections analysis using the "equator" routine in the MuscleX package (**Figure 3**). MuscleX is an open-source analysis software package developed at BioCAT[13]. The equatorial intensity ratio ($I_{1,1}/I_{1,0}$) is an indicator of the proximity of myosin to actin in resting muscle (**Figure 3A**), while it is closely correlated to the number of attached cross-bridges in contracting (**Figure 3B**) murine skeletal muscle[2]. The intensity ratio, $I_{1,1}/I_{1,0,}$ is about 0.47 in resting muscle and about 1.2 in contracting muscle. The distance between the two 1,0 reflection ($2*S_{1,0}$) is inversely related to the inter-filament spacing. Detailed documentations and manuals for MuscleX are available online[13].

FIGURE AND TABLE LEGENDS:

Figure 1. Mechanical and X-ray experiment setup and protocol. (**A**) The muscle is mounted on one end to a hook inside the experimental chamber and the other end to a dual mode motor/force transducer. It is held between two Kapton film windows to allow the X-rays to pass through. The chamber is filled with Ringer's solution perfused with 100% oxygen throughout the experiment. (**B**) The mechanical protocol for X-ray experiments on a muscle during tetanic contraction.

Figure 2. EDL X-ray diffraction patterns. EDL muscle X-ray diffraction pattern from resting (**A**) and contracting (**B**) muscle. (**C**) The difference pattern between resting and contracting pattern. The blue region indicates high intensity in resting pattern, while the yellow region represents high intensity in contracting pattern. (**D**) X-ray diffraction pattern from a 1 ms exposure with EDL muscle. MLL1: First order myosin layer line; MLL4: Fourth order myosin layer line; ALL1: First order actin layer line ALL6: Sixth order actin layer line; ALL7: Seventh order actin layer line; Tm: tropomyosin reflection (indicated by a white box); M3: third order meridional reflection; M6: sixth order meridional reflection.

Figure 3. Data analysis of equatorial patterns using MuscleX. The background subtracted equatorial intensity ratio profile (while area) and first five orders (green lines) were fit to calculate

the intensity of each peak.

DISCUSSION:

Recent publications from our group showed that X-ray patterns from the mouse skeletal muscle can be used to shed light on sarcomeric structural information from muscle in health and disease[1-3] especially with the increased availability of genetic modified mouse models for various myopathies. High resolution mechanical studies on single fibers or small bundles combined with X-ray diffraction is best done by experts. If, however, more modest mechanical information will suffice for your purposes, the whole muscle preparation allows collection of detailed X-ray patterns from a simple preparation.

A clean dissection is key to a successful combined mechanical and X-ray experiment. It is very important not to pull on the target muscle as well as other muscles associated with the soleus or EDL muscles during dissection since this could tear parts of the muscle and lead to reduced force. It can also lead to damaged internal structure that will degrade the X-ray patterns. Since everything will scatter in the X-ray beam, it is important to cleaning away any extra fat, the collagen in fascia as well as any hairs or loose bits of tissue while doing the following protocol. To reduce additional compliance in the muscle preparation, it is also important to securely tie the tendons to the hooks, as close as possible to the muscle body without damaging it.

Different X-ray exposure times can provide different kinds of information from the same muscle. Using the full beam on 18ID, an analyzable equatorial pattern can be obtained in a 1 ms exposure (See **Figure2D**). For an analyzable first myosin layer line reflection, a 10 ms total exposure time is typically required. To collect higher order meridional reflections such as the M15 (2.8 nm myosin meridional reflection) and the 2.7 nm actin meridional reflection, typically at least 1 s total exposure is required but more than 2 s total exposure is recommended for high accuracy measurements.

The choice of the optimal X-ray detector for the experiment is important. For the most detailed X-ray patterns a customized CCD detector, such as the one at BioCAT with ca. 40 µm pixels and ~65 µm point spread functions in the phosphor, can provide patterns with high dynamic range and good spatial resolution but can only take one frame at a time. For time resolved experiments, the photon counting pixel array detector at BioCAT can collect X-ray patterns at 500 Hz. The 172 µm pixel size with this detector, however, does not provide sufficient spatial resolution for detailed studies of the inner part of the meridian but is adequate for most other purposes. BioCAT acquired a high-resolution photon counting detector providing 75 µm real resolution at maximum frame rate of 9,000 Hz. Similar detectors of this type are expected to supplant current detectors for muscle studies over the next few years.

With the very high fluxes of X-rays at third generation synchrotrons, radiation damage is a serious concern. It is always a good choice to attenuate the beam to deliver no more beam than is needed to observe the desired diffraction features. The same total X-ray exposure can be achieved by prolonging the exposure time from an attenuated beam. An advantage of photon counting pixel array detectors is that individual frames can be summed together with no noise penalty. Even

then, radiation damage is possible. Signs of radiation damage includes drop of maximum force of contraction, smearing of layer line reflections, even change of muscle color.

One of the limitations of the intact mouse skeletal muscle preparation is the difficulty in obtaining sarcomere length from the intact muscle during the experiments. The muscles are too thick for video microscopy and laser diffraction. While with future developments it may be possible to estimate sarcomere length directly from the diffraction patterns[14], in the near term the only option is to measure it after the experiment as described here.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

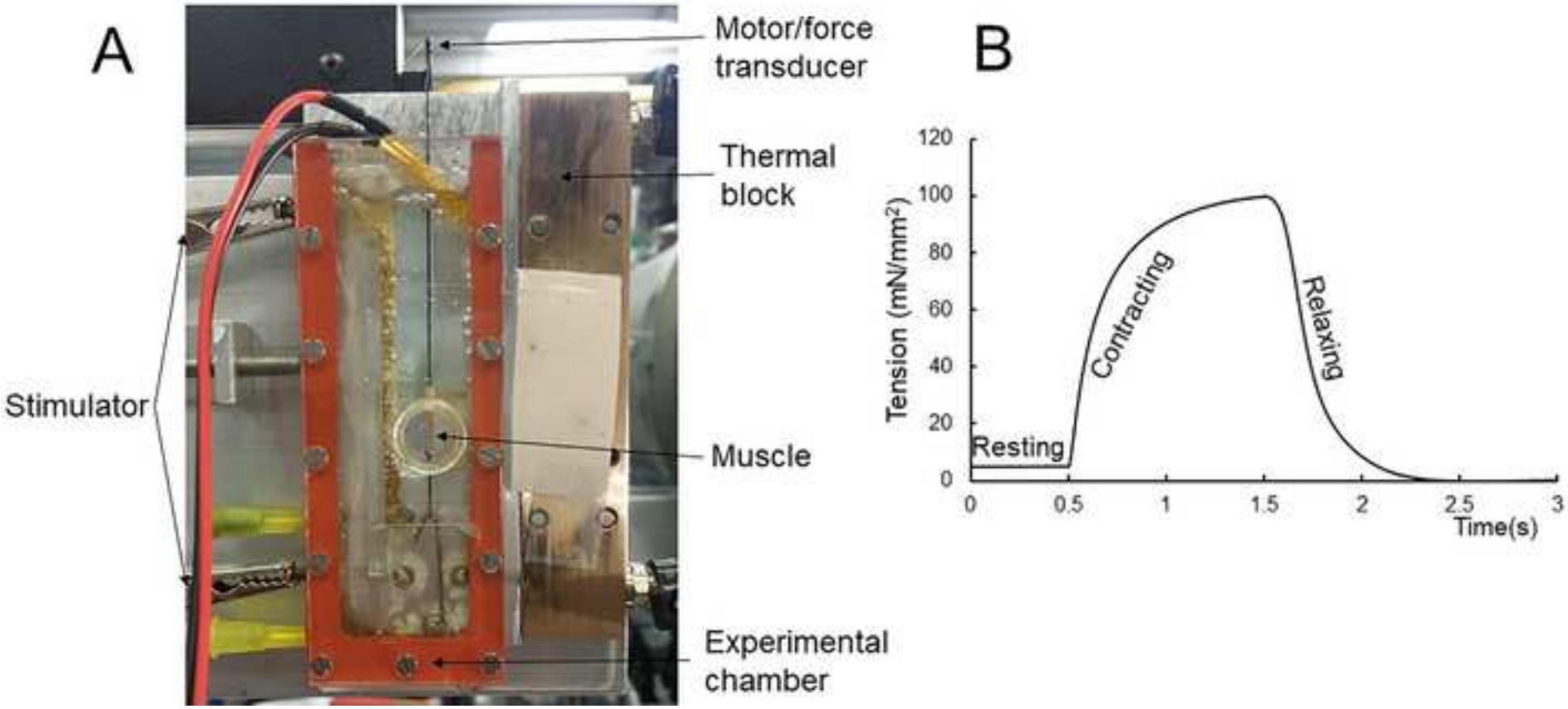

Figure 2

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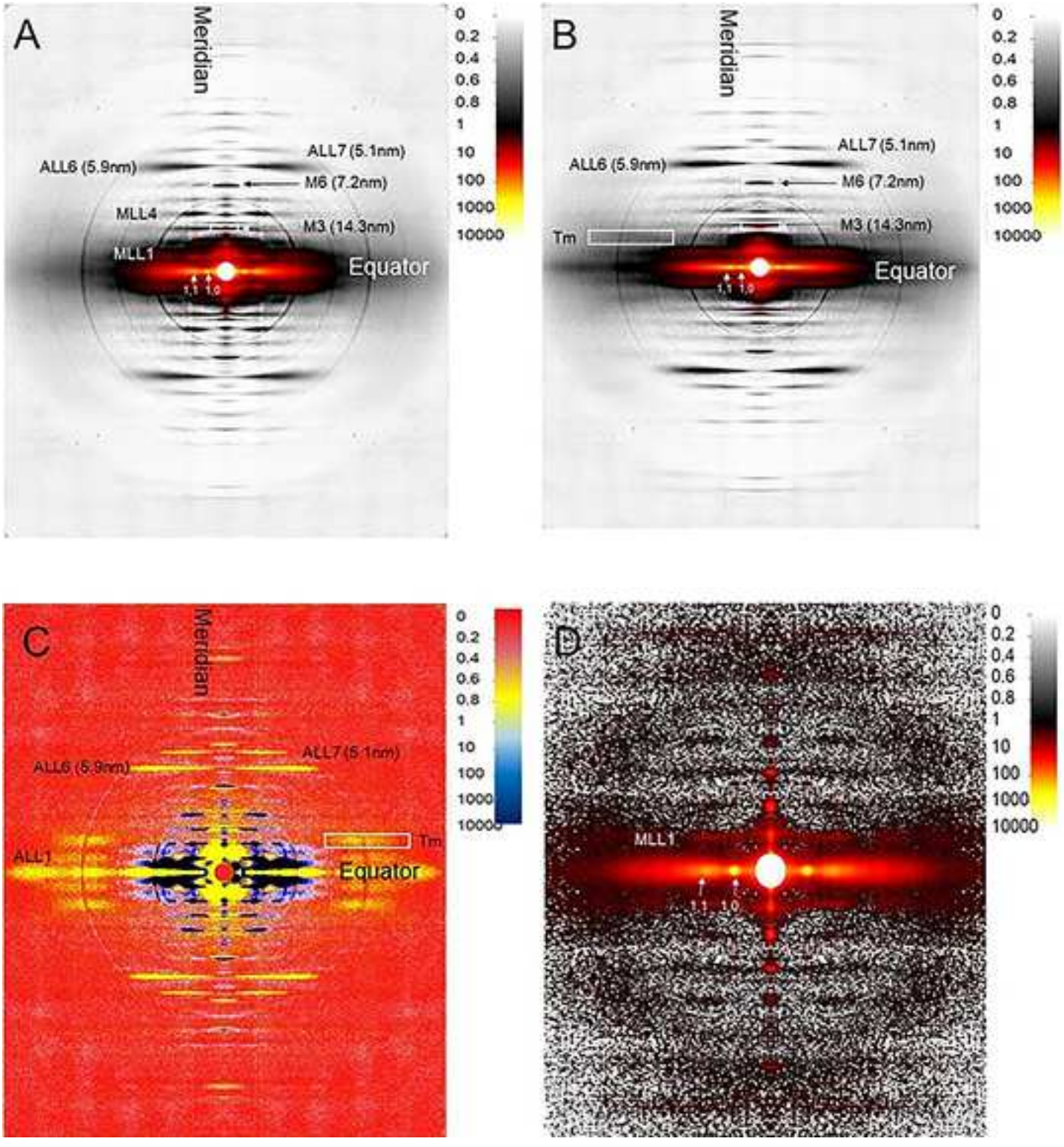
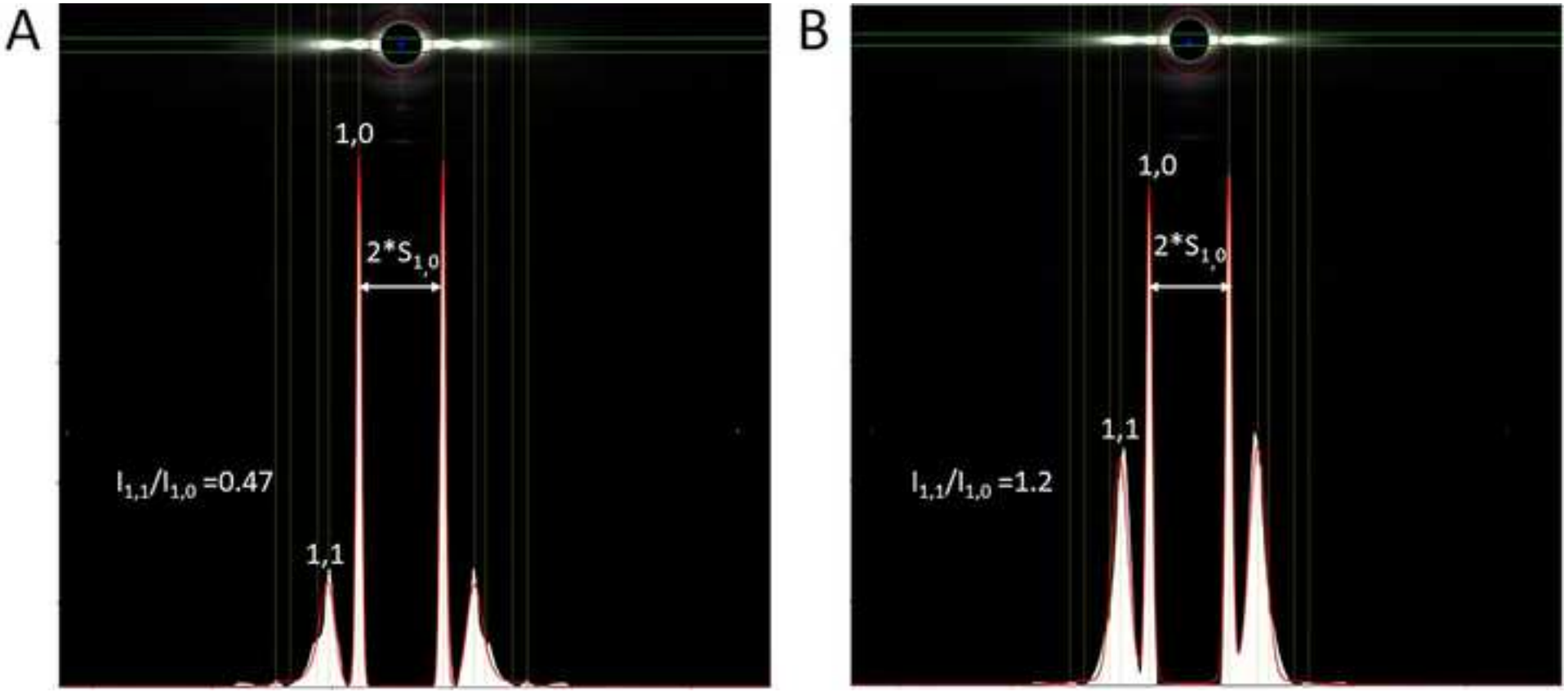


Figure 3



Name of Material/ Equipment	Company	Catalog Number
#5 forceps	WPI	500342
4/0 surgical suture	Braintree Sci	SUT-S 108
aquarium air stone	uxcell	
$CaCl_2$	Sigma-Aldrich	C5670
CCD detector	Rayonix Inc	MAR 165 CCD
data accquisition system	Aurora Scientific Inc	610A
elastomer compound	Dow Corning	Sylgard 184
Glucose	Sigma-Aldrich	G8270
HEPES	Sigma-Aldrich	H3375
High resolution photon counting detector	Dectris Inc	EIGER X 500K
high-power bi-phasic current stimulat	Aurora Scientific Inc	701
Iris Scissors	WPI	501263-G
KCl	Sigma-Aldrich	P9541
$MgSO_4$	Sigma-Aldrich	M7506
micro scissor	WPI	503365
motor/force transducer	Aurora Scientific Inc	300C-LR
NaCl	Sigma-Aldrich	S9888
petri-dish	Sigma-Aldrich	CLS430167
photon counting detector	Dectris Inc	Pilatus 3 1M
Stainless Steel wire	McMaster-carr	8908K21
Suture Tying Forceps	WPI	504498
Video sarcomere length measuring sys	Aurora Scientific Inc	900B

Comments/Description

a regular air stone from a pet store would be fine

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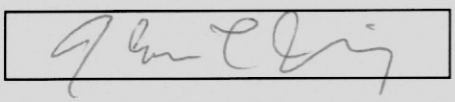
CORRESPONDING AUTHOR

Name:	Thomas C. Irving
Department:	Biological Sciences
Institution:	Illinois Institute of Technology
Title:	Professor
Signature:	
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Dear Dr. Steindel,

Re: JoVE59559 "X-ray Diffraction of Intact Murine Skeletal Muscle as a Tool for Studying the Structural Basis of Muscle Disease,"

We would like to thank you and the reviewers for their kind words and constructive comments. We have addressed each point raised by the editor and the reviewers individually as outlined below. Changes made in the text in response to each point are identified by a blue font in both the text and the response below. Our response to reviewer's specific points, given below, are shown in a red font.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please ensure manuscript is formatted per JoVE guidelines–letter (8.5" x 11") paper size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Done

3. Please define all abbreviations before use.

We have defined all abbreviations at the time of first usage in the text

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For example: Aurora Scientific Inc.

Fixed

Keywords:

1. Please provide at least 6 keywords or phrases.

We have added more keywords:

skeletal muscle; X-ray diffraction; acto-myosin interaction; sarcomere structure; skeletal muscle myopathy; skeletal muscle physiology

Protocol:

1. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

All animal experiments protocols were approved by the Illinois Institute of Technology Institutional Animal Care and Use Committee (Protocol 2015-001, Approval date: 3 November 2015) and followed the NIH publication "Guide for the Care and Use of Laboratory Animals"[9].

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:
1. 1.1: How large is the Petri dish? It is not in the Table of Materials. How is it perfused with oxygen?

We have modified this section to read:

Fill a petri-dish (10cm in diameter) with Ringer's solution and perfuse with 100% oxygen by connecting the tube from an oxygen cylinder to an aquarium air stone. The petri-dishes ("dissecting dishes") were previously coated with an elastomer compound to allow inserting pins during the dissection.

2. 1.2: Are the metal hooks custom-built? If so, how, exactly?

Section 1.2 has been modified as follows:

Prepare metal mounting hooks. Cut two pieces of stainless steel wire, 0.5mm in diameter, to the appropriate length and bend the wire at both ends to form a hook. The hook part should be about 3mm long. The longer hook should be about 5cm long, and the shorter hook about 1cm long in order to fit the custom chambers used at BioCAT and allow for a sufficient range of motion for the transducer.

3. 2: Please be more specific about instruments used in this section; e.g., what is used to remove skin in 2.1.2?

We have modified this as follows:

Remove the skin by cutting it away around the thigh using fine dissection scissors and quickly pull the skin down using #5 forceps to expose the muscles.

4. 2.5: Is the transducer/motor being used in this section? It's not clear. Please provide more

details on how exactly the steps here are carried out; e.g., how is the muscle stretched to between 12 and 20 mN in 2.5.1?

We have modified this section as follows:

Stretch the muscle by adjusting the micromanipulators attached to the transducer/motor to generate a baseline tension between 15 to 20mN before finding the best stimulus parameters.

5. 3.2.1: How exactly is alignment done?

This sentence was poorly written. This section was modified as:

Determine the beam position by using a piece of X-ray sensitive paper that produces a dark spot in response to X-rays ("a burn"). You can then use a video cross-hair generator to create a cross-hair aligned with the burn mark on the paper or you can simply make a mark on the video screen with a marker pen.

6. 3.2.2: 'Move the muscle to the middle of the muscle'-this is unclear.

This has been modified to read:

Use the BioCAT supplied graphical user interface to the sample positioner to move the muscle to be centered on the beam position.

7. 3.3/3.4: Please describe exact software steps used in these sections; e.g., 'click', 'select', etc.

The operations needed to set the parameters and perform the operations described in section 3.3 and 3.4 will be specific to the exact make and models of detectors that are being used at a particular beamline, and this is subject to change. Furthermore, the graphical user interfaces used to communicate with the detectors are generally developed in-house by beamline staff and, at least in the case of BioCAT, are under constant development. So anything we would write here is likely to be inappropriate or obsolete by the time this paper is published.

To address this situation, we have added the following note on line 198:

Note: the exact steps required in sections 3.3 and 3.4 to make the required settings and actions using the beamline-supplied graphical user interface will be beamline and detector specific. You will need to be trained by beamline staff as to how to perform these operations.

8. 4.2: Please provide more detail or a reference here.

Reference 3 is appropriate so we have modified the section to read:

Stretch the muscle to the experimental length and fix the muscle in 10% formalin for 10 min. Separate the fixed muscle into a series of fiber bundles selected from locations throughout the entire muscle cross section[3]. Measure the sarcomere length using a video sarcomere length measuring system

Results:
1. "Isometric tetanic contraction": Figure 1B does not show a diffraction pattern.

This was a mistake. We have replaced

An example diffraction pattern taken during an isometric tetanic contraction is shown in Fig. 1B**.**

With:

An example force trace for an isometric tetanic contraction is shown in Fig. 1B.

Figures:
1. Figure 2: Please define the abbreviations here (MLL1, ALL6, Tm, etc.) in the legend. What are the boxed regions in panels B and D?

We have modified the caption as follows:

Figure 2. EDL X-ray diffraction patterns. EDL muscle X-ray diffraction pattern from resting (**A**) and contracting (**B**) muscle. (**C**) The difference pattern between resting and contracting pattern. The blue region indicates high intensity in resting pattern, while the yellow region represents high intensity in contracting pattern. (**D**) X-ray diffraction pattern from a 1ms exposure with EDL muscle. MLL1: First order myosin layer line; MLL4: Fourth order myosin layer line; ALL1: First order actin layer line ALL6: Sixth order actin layer line; ALL7: Seventh order actin layer line; Tm: tropomyosin reflection (indicated by a white box); M3: third order meridional reflection; M6: sixth order meridional reflection.

References:
1. Please ensure references have a consistent format. Please do not abbreviate journal names.

We used the JoVE format template for Endnote downloaded from the website. It appears then that this template is incorrect and needs to be fixed. In order to proceed, we have manually spelt out all journal names in response to your request.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Done

2. Please order the Table of Materials alphabetically by name of material.
Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This clearly written paper describes the techniques to record X-ray diffraction patterns from intact skeletal muscles from transgenic mice in synchrotron radiation facilities. The techniques described here will serve as powerful tools for investigating the mechanisms of inherited myopathies, and will benefit the communities of clinical science as well as basic science. My impression is that many of the descriptions are applicable to a specific synchrotron radiation facility, and the paper looks more like an instruction manual than a methods paper. Nevertheless, the underlying ideas are applicable to other facilities as well. The paper may be published almost as is, but can be even better if the following points are considered.

Major Concerns:
There is no major concern.

Minor Concerns:
It would be better to describe the necessity and the procedures of obtaining institutional approval of animal experiments and experiments using living genetically modified organisms.

Done

line 51: "The equatorial intensity ratio (ratio of the intensity of the 1,1 and 1,0 equatorial reflections, I11/I10), is proportional to the number of attached cross-bridges which closely correlated to the force generated in mouse skeletal muscle" This description may not be very accurate, although I agree that the two quantities are positively correlated with each other.

We agree. We have modified the text (starting on line 53 to read"

The equatorial intensity ratio (ratio of the intensity of the 1,1 and 1,0 equatorial reflections, I11/I10), is closely correlated to the number of attached cross-bridges which is proportional to the force generated in mouse skeletal muscle

line 56: "quasi-helically ordered" What is meant by quasi-helical?
Although "quasi-helical" seems to be preferred by some investigators, perhaps "approximately helical" might be more widely understood. We have modified the text to read:

Diffraction features not on the meridian and the equator are called layer lines which arises from

the approximately helically ordered myosin heads on the surface of thick filament backbone as well as the approximately helically ordered thin filaments

line 164: It is truly regrettable that the BL45XU beamline of SPring-8 is no longer available for small-angle scattering experiments.

It is very regrettable indeed that BL45XU is no longer available. We have changed the reference to BL 40XU which is still available, as far as we know.

Figures: It would be more helpful if an anatomical diagram of the muscle is provided.

Our expectation is that the high quality videos produced by JOVE will be more effective than an anatomical diagram.

Reviewer #2:
This manuscript highlights the relevance of using X-ray diffraction in order to investigate sarcomeric structural changes caused by genetic muscle diseases. The authors provided detailed information about how to elucidate these structural changes by the proposed methods. The manuscript is clear and well written.

In general, the authors:
provided a clear and explicit idea of the objective showed the applicability of a well-established technique reported strong evidences that this is a valid method (through their own results and results from the literature) gathered all the collected data and discussed important topics mentioned future plans/improvements/updates mentioned the limitations of the method, very informative
I liked how the authors explained the difference between equatorial/meridional patterns. They provided some background knowledge and that helps the reader to understand and interpret the data.

Minor Issues:
- Data Visualization/Interpretation: The written part of the results section is well-written but I had some problems to link the written part to the figures. Especially when the authors mentioned layer lines (first time reading about it). So it was not clear to me where they are. Maybe it is something simple that I could not identify which could be the same problem for the readers.

We have changed the following sentence to make it clearer:

Layer lines (e.g. features labeled MLL4 and ALL6 in Figure 2A) arise from the approximately-helical arrangement of molecular subunits within the myosin containing thick filaments and the actin containing thin filaments.

- Line 123: how did the authors pin the hind limb down in the petri dish (Sylgard plate)?

As described above under 1.2 we have added. The petri-dishes ("dissecting dishes") were previously coated with an elastomer compound to allow inserting pins during the dissection

Having defined "dissecting dish" we have replaced all subsequent instances of "petri dish" with "dissecting dish"

e.g.
2.2.1) Pin the hind limb down in the dissecting dish with the gastrocnemius muscle facing upwards.

- Representative Results: lines 230-231. The sentence was a little bit confusing and not clear

The X-ray patterns are collected continuously throughout the protocol a with a 1ms exposure time and a 2ms exposure period

- The authors did not provide much details about data acquisition system calibration

We added a brief description on how to calibrate the system as well as a reference for more details.

Briefly, the force is calibrated by sequentially attaching a set of known weights to the force transducer, covering up to 50% of the maximum force measured by the force transducer in a linear progression, and recording the output voltage changes. Calibrating the length is calibrated by applying a set of known output voltage to the lever arm and measure the length change of the arm.

In the section 2.3.2 the authors mentioned cutting the tendon (how specifically?) and right after in the section 2.4.1 they mentioned pinning down the muscle via the tendons. I believe there is a specific way to cut these tendons that was not clearly described.

We have rewritten section 2.3.2 to read:

Cut open the lateral side of the knee and expose the two tendons. Cut the proximal tendon, leaving as much of the tendon as possible still attached to the muscle, and lift the EDL muscle (medial muscle) by gently pulling the tendon. Cut the distal tendon once it is exposed.

- The "post-experimental muscle treatment" section 4.2 was a little bit confusing. The authors mention that it is hard to get the sarcomere length information because of the thickness of the preparation. However, in the 4.2 section, they measure it. If I understood well, they fixed the entire tissue with formalin 10% for 10 min (after stretching it) in order to fix the sarcomere length (closer to experimental length). Then, they were able to dissect the tissue into small bundles to measure the sarcomere length. Am I correct?

We have rewritten this section as (same as point 8 for reviewer 1):

Stretch the muscle to the experimental length and fix the muscle in 10% formalin for 10 min. Separate the fixed muscle into a series of fiber bundles selected from locations throughout the entire muscle cross section[3]. Measure the sarcomere length using a video sarcomere length measuring system

Recommendations:
- The authors did not mention risks, training or any ethical standard. I am not sure if those are important topics to be discussed in this case

On line 75 we inserted:

All animal experiments protocols were approved by the Illinois Institute of Technology Institutional Animal Care and Use Committee (Protocol 2015-001, Approval date: 3 November 2015) and followed the NIH "Guide for the Care and Use of Laboratory Animals".

- Line 56, they wrote arises and it should be arise

Fixed

- Lines 180/181 "to move the muscle to the middle of the muscle". It does not sound right
See above how this was fixed

Line 230: "the x-ray patterns was" ... should be "were"-
Fixed

The authors should also mention that X-ray diffraction can be done in resting muscle since Figures 2A and 3A are patterns obtained from extensor digitorium longus muscle under resting conditions

This is addressed on line 264:

The myosin-based layer lines are strong and sharp in patterns from resting muscle (Fig 2A), while actin-based layer lines are more prominent in patterns from contracting muscle (Fig 2B).

- It would have been nice having pictures of "muscle preparation section" since it is a crucial step

Our expectation is that this will be addressed in the high quality videos that will be produced by the JOVE production team