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Application of Consistent Massage-Like Perturbations on Mouse Calves and Monitoring the Resulting Intramuscular Pressure Changes

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Corresponding Author:	Yasuhiro Sawada National Rehabilitation Center for Persons with Disabilities Tokorozawa, JAPAN
Corresponding Author's Institution:	National Rehabilitation Center for Persons with Disabilities
Corresponding Author E-Mail:	ys454ind@gmail.com
Order of Authors:	Naoyoshi Sakitani
	Takahiro Maekawa
	Kumiko Saitou
	Shuhei Murase
	Masakuni Tokunaga
	Keisuke Sawada
	Atsushi Takashima
	Motoshi Nagao
	Toru Ogata
	Yasuhiro Sawada
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April 19, 2019

Vineeta Bajaj, Ph.D.
Science Editor, *JoVE*

Dear Dr. Bajaj:

Following the editor's comments, we have extensively revised our manuscript (both text and figures). Some of the reviewer's comments were unclear (Comments 18 and 24). We think we have provided adequate information related to those comments. As you realized in our Response Letter, we have addressed all the other points raised by the editor.

Thank you for handling our submission.

Sincerely,

Yasuhiro Sawada, M.D., Ph.D.
Department of Clinical Research,
National Rehabilitation Center for Persons with Disabilities

TITLE

Application of Consistent Massage-Like Perturbations on Mouse Calves and Monitoring the Resulting Intramuscular Pressure Changes

AUTHORS AND AFFILIATIONS

Naoyoshi Sakitani[1*], Takahiro Maekawa[1*], Kumiko Saitou[1,2], Shuhei Murase[1,3], Masakuni Tokunaga[1], Keisuke Sawada[4], Atsushi Takashima[5], Motoshi Nagao[1], Toru Ogata[1], Yasuhiro Sawada[6]

[1]Department of Rehabilitation for Motor Functions, National Rehabilitation Center for Persons with Disabilities, 4-1 Namiki, Tokorozawa, Saitama, Japan
[2]Graduate School of Sport Sciences, Waseda University, Mikajima, Tokorozawa, Saitama, Japan
[3]Department of Orthopaedic Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo, Japan
[4]Department of Pathology, Children's Hospital of Philadelphia, Philadelphia, PA, USA
[5]Department of Assistive Technology, National Rehabilitation Center for Persons with Disabilities, 4-1 Namiki, Tokorozawa, Saitama, Japan
[6]Department of Clinical Research, National Rehabilitation Center for Persons with Disabilities, 4-1 Namiki, Tokorozawa, Saitama, Japan

*These authors contributed equally.

Email Address of Corresponding Author:

Yasuhiro Sawada (ys454-ind@umin.ac.jp)

Email Addresses of Co-Authors:

Naoyoshi Sakitani (7asdfghjklzxcvbnm@gmail.com)
Takahiro Maekawa (silenthawk712@gmail.com)
Kumiko Saitou (saito935@gmail.com)
Shuhei Murase (m990095@yahoo.co.jp)
Masakuni Tokunaga (masa201501nyusps@gmail.com)
Keisuke Sawada (sawadak@email.chop.edu)
Atsushi Takashima (takashima-atsushi@rehab.go.jp)
Motoshi Nagao (nagao-motoshi@rehab.go.jp)
Toru Ogata (togata16@gmail.com)

KEYWORDS

immobilization, disuse muscle atrophy; massage; inflammation; macrophage; local cyclical compression; MCP-1

SUMMARY

Here we describe the protocols for applying defined mechanical loads to mouse calves and for monitoring the concomitant intramuscular pressure changes. The experimental systems that we have developed can be useful for investigating the mechanism behind the beneficial effects of physical exercise and massage.

ABSTRACT

Massage is generally recognized to be beneficial for relieving pain and inflammation. Although previous studies have reported anti-inflammatory effects of massage on skeletal muscles, the molecular mechanisms behind are poorly understood. We have recently developed a simple device to apply local cyclical compression (LCC), which can generate intramuscular pressure waves with varying amplitudes. Using this device, we have demonstrated that LCC modulates inflammatory responses of macrophages in situ and alleviates immobilization-induced muscle atrophy. Here, we describe protocols for the optimization and application of LCC as a massage-like intervention against immobilization-induced inflammation and atrophy of skeletal muscles of mouse hindlimbs. The protocol that we have developed can be useful for investigating the mechanism underlying beneficial effects of physical exercise and massage. Our experimental system provides a prototype of the analytical approach to elucidate the mechanical regulation of muscle homeostasis, although further development needs to be made for more comprehensive studies.

INTRODUCTION

Massage is generally recognized to be beneficial for both pain relief and improvement of the physical performance among competitive athletes and non-athletes alike[1,2]. In fact, previous studies have shown that massage suppresses local inflammation[3] and prompts recovery from the post-exercise muscle damage[4,5]. Molecular mechanisms underlying the beneficial effects of massage remain largely unknown.

One of the difficulties with the mechanistic investigation on massage relates to the reproducibility of experimental techniques by which massage-like interventions are tested. In previous studies, experimental procedures that mimic massage mostly involve the application of physical interventions using practitioners' body parts, such as palms and fingers[6-8]. This makes it is difficult to precisely reproduce their magnitude, frequency, duration, and mode.

Many devices have been developed to apply defined mechanical loads to the target tissues. For example, Zeng et al. have developed a pneumatic system for the length-wise mechanical loading to rats' hindlimbs[9] and Wang et al. have developed a mechatronic device that can apply massage-like mechanical loads to hindlimbs of rats and rabbits with real-time feedback control[10]. Compared to them, our local cyclical compression (LCC) system is much simpler, demanding far less cost for construction. Nonetheless, we can reproduce the intramuscular pressure changes that are generated during the mild muscle contraction. Using this device, we have successfully demonstrated that the massage-like mechanical interventions modulate local interstitial fluid dynamics and alleviate immobilization-induced muscle atrophy[11].

Here, we describe the details of our device and the protocol, which may help explore the molecular mechanisms behind the positive effects of exercises and massages. The schematics of the protocol is presented as **Supplementary Figure 1**.

PROTOCOL

All animal experiments were conducted under the approval by the Institutional Animal Care and Use Committee of the National Rehabilitation Center for Persons with Disabilities.

1. Immobilization of the mouse bilateral hindlimbs

NOTE: Male C57BL/6 mice were used for experiments at the age of 11 - 12 weeks after acclimation for at least 7 days.

1.1. Adequately anesthetize a mouse using sodium pentobarbital (50 mg/kg i.p.). Make sure that mice do not respond to a hindlimb toe pinch.

NOTE: Conduct the procedure of immobilization between 10 a.m. and 7 p.m. to minimize the possible effects on the feeding activity of mice.

1.2. Apply surgical tapes to the bilateral hindlimbs of the mouse laid in a supine position with the knee joints extended and ankle joints plantar-flexed.

1.3. Place an aluminum wire (see **Table of Materials**) on the trunk at L4-5 spine level and coil the wire in a spiral configuration around the hindlimbs with 5 mm gaps between each turn of the spiral layer (**Figure 1A**). Make sure not to coil the wire too tightly and avoid disturbing the local blood flow.

1.4. To minimize the possibility of escape from wiring, immobilize the hip joints at the position of 90° abduction by manually adjusting the configuration of aluminum wire.

1.5 Return the mice to their original cages. 3 h later, make sure that they recover from anesthesia and access to food and water as usual.

1.6. House 3 - 6 immobilized mice per cage as before immobilization.

2. Measurement of intramuscular pressure of mouse gastrocnemius muscles

NOTE: Several different weights of cylindrical units (36 g, 66 g, and 200 g) were tested in the pressure-monitoring experiments combined with LCC. This measurement was conducted separately from the experiments to analyze muscle inflammation and atrophy (see step 3 - 5 for more details) i.e., the mice subjected to pressure measurement were not used for histological analyses.

2.1. Because pressure measurement involves more invasive procedures (e.g., skin incision and needle insertion) as compared to hindlimb wiring and LCC, use a mixture of three anesthetic agents (medetomidine 0.75 mg/kg, midazolam 4.0 mg/kg, and butorphanol 5.0 mg/kg). Make sure that mice do not respond to the hindlimb toe pinch.

2.2. Lay the mouse in a prone position, make a 2-mm incision with a scalpel on the posterior calf after depilating with an electric shaver and semi-sterilizing the skin surface with 70% ethanol-soaked absorbent cotton.

2.3. Insert a 20 G indwelling needle into the gastrocnemius muscle at an obtuse angle (150° – 170°) to the skin surface.

2.4. Using the plastic sheath of the needle as a guide, place a sensor of the blood pressure telemeter (see **Table of Materials**) in the mid-belly of gastrocnemius muscle.

2.5. After suturing the skin with 4-0 nylon suture, apply LCC with several different weights of cylindrical units to the calf in the mice (see step 3 for more details), and monitor the intramuscular pressure using software for biological signal analysis (see **Table of Materials**).

2.6 Return the mice to their original cages. 3 h later, make sure that they recover from anesthesia and have access to food and water as usual.

3. Local cyclical compression (LCC) on mouse calves

3.1. Except for the intramuscular pressure measurement and euthanizing (i.e., cervical dislocation), use sodium pentobarbital (50 mg/kg i.p.) for anesthesia.

3.2. Disengage the mouse from hindlimb wiring and lay it in a prone position with the knee joints extended and the ankle joints plantar-flexed so that the calves faced upward. Do not fix the mouse hindlimbs on the stage.

3.3. Apply LCC to the calf by vertically moving a cylindrical weight unit (**Figure 1B**) covered with a cushion pad (**Figure 1C**) at 1 Hz for 30 min per day, 7 days.

3.4. After each bout of daily LCC, re-wire the mouse hindlimbs.

4. Immunohistochemical analysis of gastrocnemius

4.1. Euthanize the mouse by cervical dislocation under adequate anesthesia using a mixture of three anesthetic agents (medetomidine 0.75 mg/kg, midazolam 4.0 mg/kg, and butorphanol 5.0 mg/kg).

4.2. After depilating the posterior calf surface, make a skin incision, and dissect gastrocnemius muscles by separating from tibio-fibular bone using a surgical scissor and quickly freeze them in an optimal cutting temperature compound solution.

4.3. Using a cryostat, prepare cryo-section samples of gastrocnemius muscles on glass slides. Store the samples in a -80 °C freezer until analysis.

4.4. Take out the gastrocnemius cryo-section samples to be analyzed from the freezer and dehydrate them by air drying at room temperature.

4.5. Use a liquid blocker pen to draw an area that includes all the cryo-sections on the slide. The circle will prevent the solutions from flowing off the slide.

4.6. Avoid drying of the samples by placing the slides in a tray in which a moist environment is created with water-soaked paper cloth.

4.7. Apply 100 µL of blocking buffer (phosphate-buffered saline (PBS) containing 0.25% casein, carrier protein, and 0.015 M sodium azide) for 30 min at room temperature.

4.8. Rinse the slides twice by incubating with PBS-T (PBS containing 0.1% polyoxyethylene sorbitan monolaurate (see **Table of Materials**) for 5 min.

4.9. Apply 100 µL of primary antibody diluted with PBS on each sample, cover the tray with a lid, and incubate overnight at room temperature.

4.10. Wash 3 times with PBS-T (5 min for each wash).

4.11. Apply 100 µL of secondary antibody diluted with PBS on each sample and incubate for 1 h at room temperature.

NOTE: For anti-laminin staining, use Alexa Fluor 568-conjugated secondary antibody. For anti-F4/80, anti-MCP-1, and anti-TNF-α, use Alexa Fluor 568- or 488-conjugated secondary antibody.

4.12. Wash 3 times with PBS-T (5 min for each wash).

4.13. Apply 100 µL of DAPI solution diluted with PBS-T on each sample and incubate for 3 min at room temperature.

4.14. Wash 3 times with PBS-T (5 min for each).

4.15. Mount the samples with mounting medium and cover them with coverslips.

5. Histo-morphometric analysis of gastrocnemius

5.1. Place the sample slides on a fluorescence microscope (see **Table of Materials**) and view the samples using a 20× objective with appropriate filters (DAPI-B, 360/40 nm for excitation and 460/50 nm for emission; GFP-B, 470/40 nm for excitation and 535/50 nm for emission; FRITC, 540/25 nm for excitation and 605/55 nm for emission.

5.2. Using the software for image analysis (see **Table of Materials**), measure the cross-sectional area (CSA) of each myofiber, and count the number of F4/80-, MCP-1-, and TNF-α-positive cells.

NOTE: Determine CSA of each myofiber by tracing the internal margin of the basement membrane visualized with anti-laminin-2 immunostaining.

REPRESENTATIVE RESULTS

Consistent with our previous observations[12], the CSA of gastrocnemius myofibers were significantly decreased by hindlimb immobilization (**Figures 2A**,**B**). Furthermore, our immunofluorescence staining analysis revealed that cells expressing MCP-1 and TNF-α, both of which play key roles in regulating inflammatory processes[13,14], significantly increased in gastrocnemius muscle tissues of immobilized hindlimbs (MCP-1: **Figures 2C,F,H**; TNF-α: **Figures 2D,G,I**). Together with the increase in cells positively stained with F4/80, a marker for macrophages (**Figures 2C-E,H,I**), hindlimb immobilization appeared to instigate calf muscle atrophy involving local inflammatory responses including macrophage accumulation. We then sought to examine whether LCC, a massage-like mechanical intervention, modulated this immobilization-induced muscle inflammation and atrophy.

Among several different LCC magnitudes that we tested by changing the weight of the cylindrical unit, the one corresponding to 50 mmHg intramuscular pressure waves (LCC with 66 g, **Figure 3A**) appeared to most efficiently alleviate the immobilization-induced decrease in myofiber CSA and increase in macrophage accumulation in gastrocnemius muscles (**Figure 3B**). Based on the results of myofiber CSA and macrophage accumulation, we employed 66 g LCC for further studies. Notably, the LCC-induced intramuscular pressure waves, whose peak magnitudes were dependent on the cylindrical unit weight, were highly uniform (**Figure 3A**), indicating the consistency and reproducibility of LCC as a mechanical intervention on skeletal muscles.

LCC (1 Hz, 30 min per day, 7 days) significantly alleviated the immobilization-induced decreases in myofiber CSA of gastrocnemius muscles (**Figures 4A,B**). Furthermore, LCC partially tempered the immobilization-induced decrease in contracting force of triceps surae muscles (**Figure 4C**). In addition, LCC tempered the increases in F4/80-positive, TNF-α-positive, F4/80-, MCP-1-, and TNF-α-positive cells in gastrocnemius muscle tissues of immobilized hindlimbs (F4/80, **Figures 4D,F**; MCP-1, **Figures 4D,G**; TNF-α, **Figures 4E,H**). Collectively, LCC, which generates intramuscular pressure waves with an amplitude of 50 mmHg, alleviated immobilization-induced muscle atrophy and local inflammatory responses including macrophage accumulation.

FIGURE AND TABLE LEGENDS

Figure 1: Mouse bilateral hindlimb immobilization and local cyclical compression (LCC) application. (**A**) Bilateral mouse hindlimbs were immobilized by spiral wiring with the hip joints abducted, the knee joints extended, and the ankle joints plantar-flexed. (**B**) LCC device. (**C**) Experimental set-up for LCC on the mouse calf.

Figure 2: Mouse hindlimb immobilization, which atrophies calf muscles, induces a local inflammatory response. (**A**) Cross-sectional micrographic images of anti-laminin-2 immunofluorescence staining of gastrocnemius muscles. High magnification images (right) refer to the areas indicated by rectangles in low magnification images (left).

Scale bars, 100 µm. (**B**) Immobilization induced muscle atrophy. CSA of gastrocnemius myofibers decreased with the period of hindlimb immobilization. To quantify CSA, 100 myofibers were randomly chosen. Data are presented as means ± S.D. *, $P < 0.05$, one-way ANOVA with post hoc Bonferroni test (n = 3 mice for each group). (**C and D**) Micrographic images of anti-MCP-1 (green in C) and anti-TNF-α (green in D) and anti-F4/80 (red) immunostaining. For merged presentation (green and red), low and high magnification images are laid as in (A). Arrows point to double positive cells for F4/80 and MCP-1 (C) or TNF-α (D) Scale bars, 100 µm. (**E-I**) Quantification of anti-MCP-1, anti-TNF-α, and anti-F4/80 immunostaining. Effects of immobilization were analyzed with reference to the period of bilateral hindlimb immobilization. Statistical analysis was conducted with reference to the 'Day 0' samples (gastrocnemius muscles from mice that were not subjected to immobilization). Data are presented as means ± S.D. *, $P < 0.05$, one-way ANOVA with post hoc Bonferroni test (n = 3 mice for each group). This figure has been modified with permission[11].

Figure 3: Effects of LCC with different magnitudes on immobilization-induced muscle atrophy and inflammation response. (**A**) Application of different magnitudes of LCC by changing the weight of the cylindrical unit. Scale bar, 1 s. 36-g, 66-g and 200-g cylindrical units produced 45 mmHg, 50 mmHg and 140 mmHg intramuscular pressure waves, respectively. (**B**) Comparison of the effects of LCC application to immobilized hindlimbs with 36-g, 66-g and 200-g cylindrical units. CSA of gastrocnemius myofibers (left) and F4/80-positive cells (right) of LCC-applied calf were quantified as relative values to those of the control hindlimb, which was not exposed to LCC, in each mouse. Data are presented as means ± S.D. *, $P < 0.05$, one-way ANOVA with post hoc Bonferroni test (n = 4 mice for each group). This figure has been modified with permission[11].

Figure 4: LCC attenuates immobilization-induced muscle atrophy and inflammatory response. (**A,B**) Alleviation of immobilization-induced muscle atrophy by LCC application. CSA of gastrocnemius myofibers (B) was analyzed as in **Figure 2B**. Data are presented as means ± S.D. *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA with post hoc Bonferroni test (n = 6 mice for each group). (**C**) The decrease in contracting force of triceps surae muscles after immobilization and its partial restoration by LCC. Data are presented as means ± S.D. *, $P < 0.05$, paired Student's t test (n = 4 mice for control, n = 5 mice for immobilization group). (**D,E**) Micrographic images of anti-MCP-1 (green in D), anti-TNF-α (green in E) and anti-F4/80 (red) immunofluorescence staining of gastrocnemius muscles of mobilized (top) and immobilized hindlimbs without (middle) and with (bottom) LCC application are presented as in **Figures 2C**,**D.** Scale bars, 100 µm. (**F-H**) Quantification of anti-MCP-1, anti-TNF-α, and anti-F4/80 immunostaining. We compared calf muscles of immobilized hindlimbs with and without LCC application. Data are presented as means ± S.D. *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA with post hoc Bonferroni test (n = 6 mice for each group). This figure has been modified with permission[11].

Supplementary Figure 1: Schematic representation of experimental protocols.

DISCUSSION

We have described a method for applying a massage-like mechanical stimulus, which has anti-inflammatory effects. Our system has following advantages even when compared with those reported previously. First, previous studies did not quantitatively define the mechanical forces applied[2] or defined their magnitudes based on the measurement at the body surface, but not inside the tissues[10]. In contrast, we measured intramuscular pressure using a blood pressure telemeter. Second, the simple structure of our device (**Figure 1B**) allowed us to construct the system with high consistency and reproducibility (**Figure 3A**) at a relatively low cost. Third, our intervention (LCC) relates to physical activity (mild muscle contraction) with regard to intramuscular pressure changes (50 mmHg[15]). Our approach will provide a scientific basis for massage-like intervention as a possible therapeutic/preventative procedure that lessens the demerit of physical inactivity[16].

The most critical step in our protocol is the positioning of mouse hindlimbs (Protocol step 3.3). We need to apply LCC in the direction perpendicular to calf muscles; otherwise, muscle tissues will be partly squeezed and damaged even when the 66-g cylindrical unit is used.

The limitation of the LCC method includes the requirement of anesthesia, which may have some effects on muscle metabolism. Also, we cannot entirely preclude the influences of tiny muscle contraction that may be caused as a reflex to sharp impacts during LCC application.

In conclusion, we have demonstrated that interstitial fluid movement mediates the LCC effects[11]. We may be able to induce interstitial flow more efficiently by modifying the mode of cyclical compression. For example, compression of sinusoidal mode may be better as compared to sharp strokes used in our current study.

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DISCLOSURES:

The authors declare that there are no competing interests associated with the manuscript.

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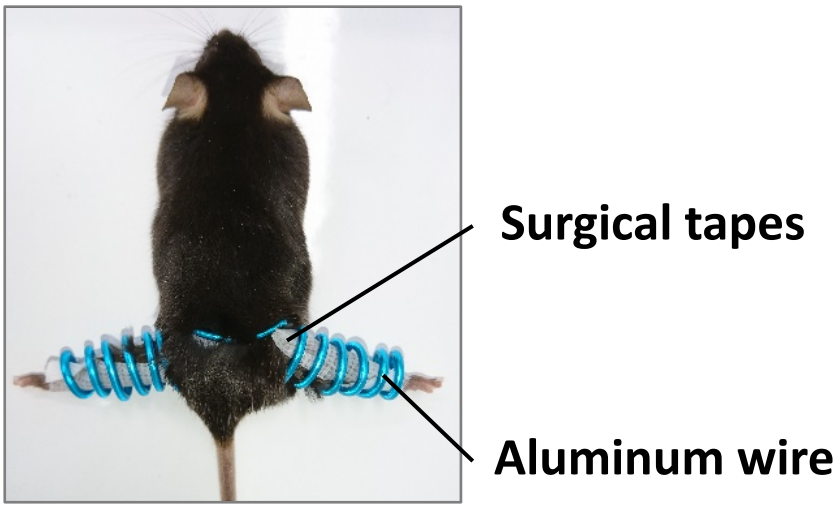
Figure 1

A



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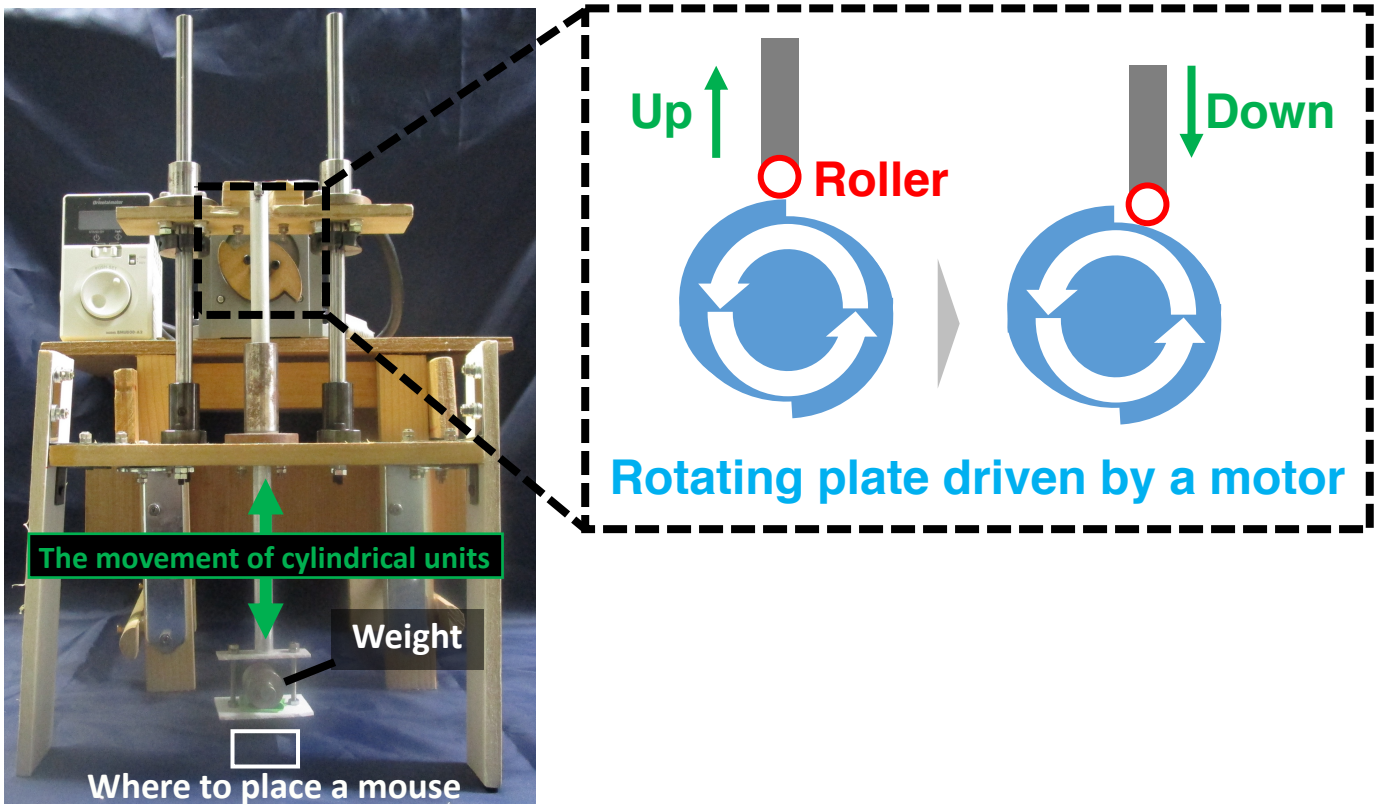

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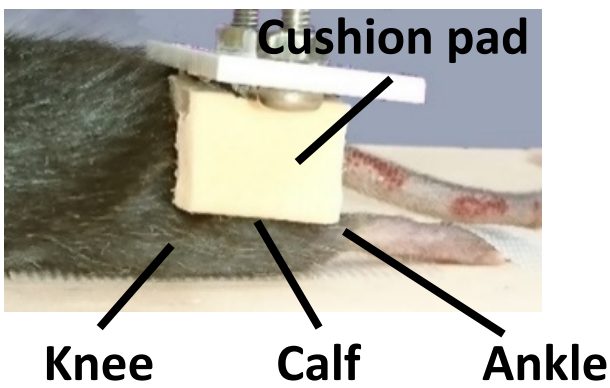


Figure 2

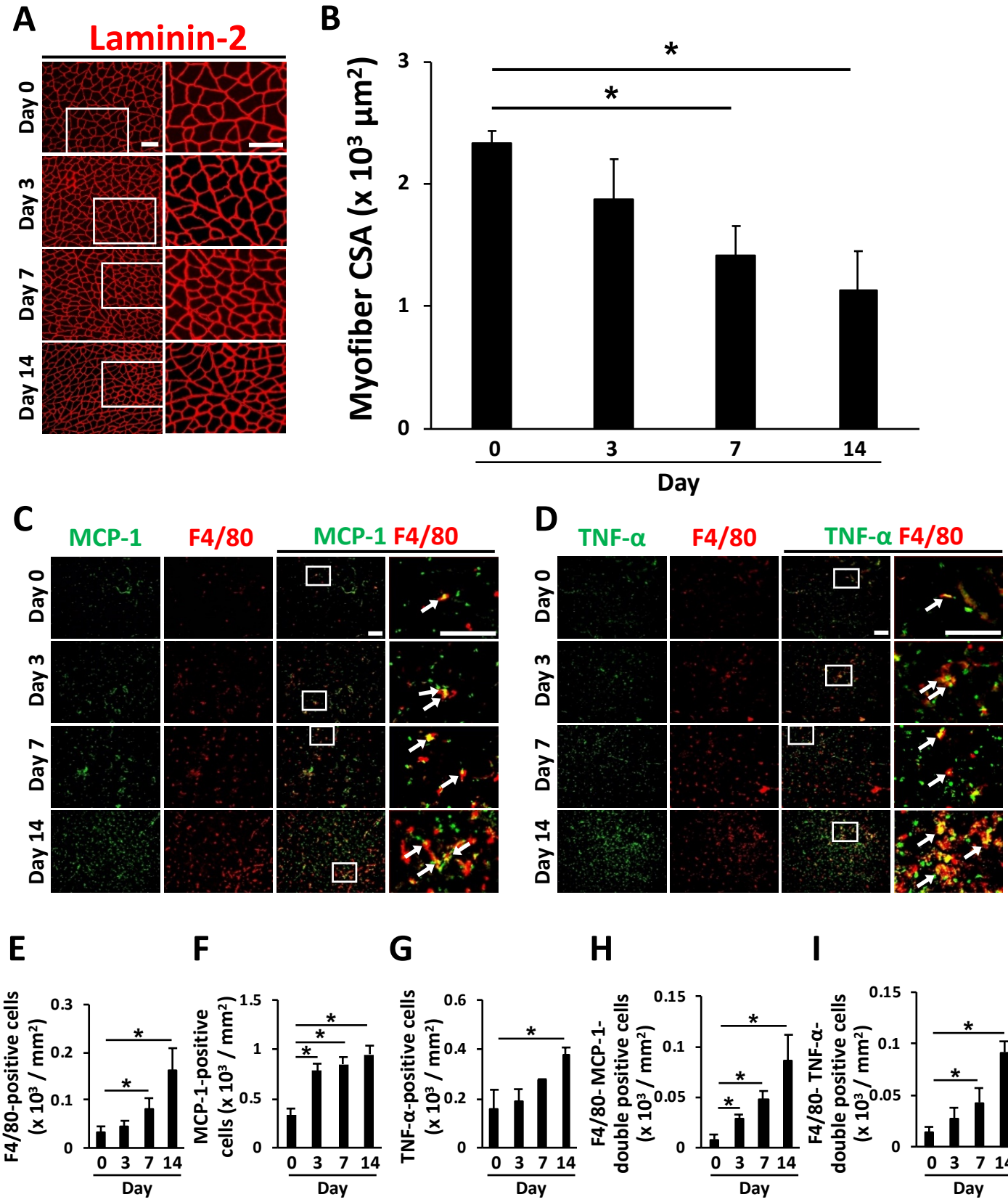


Figure 3

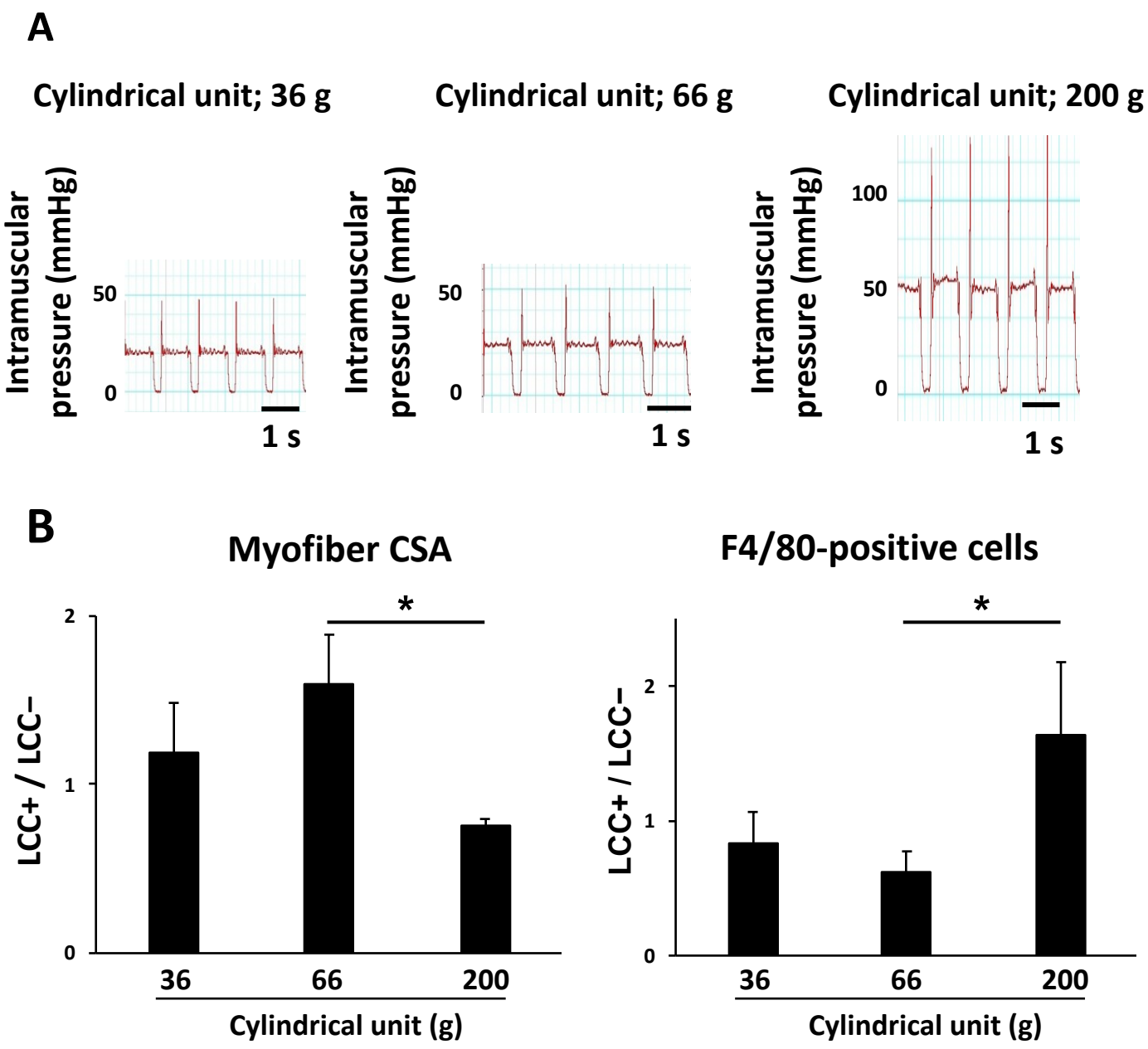
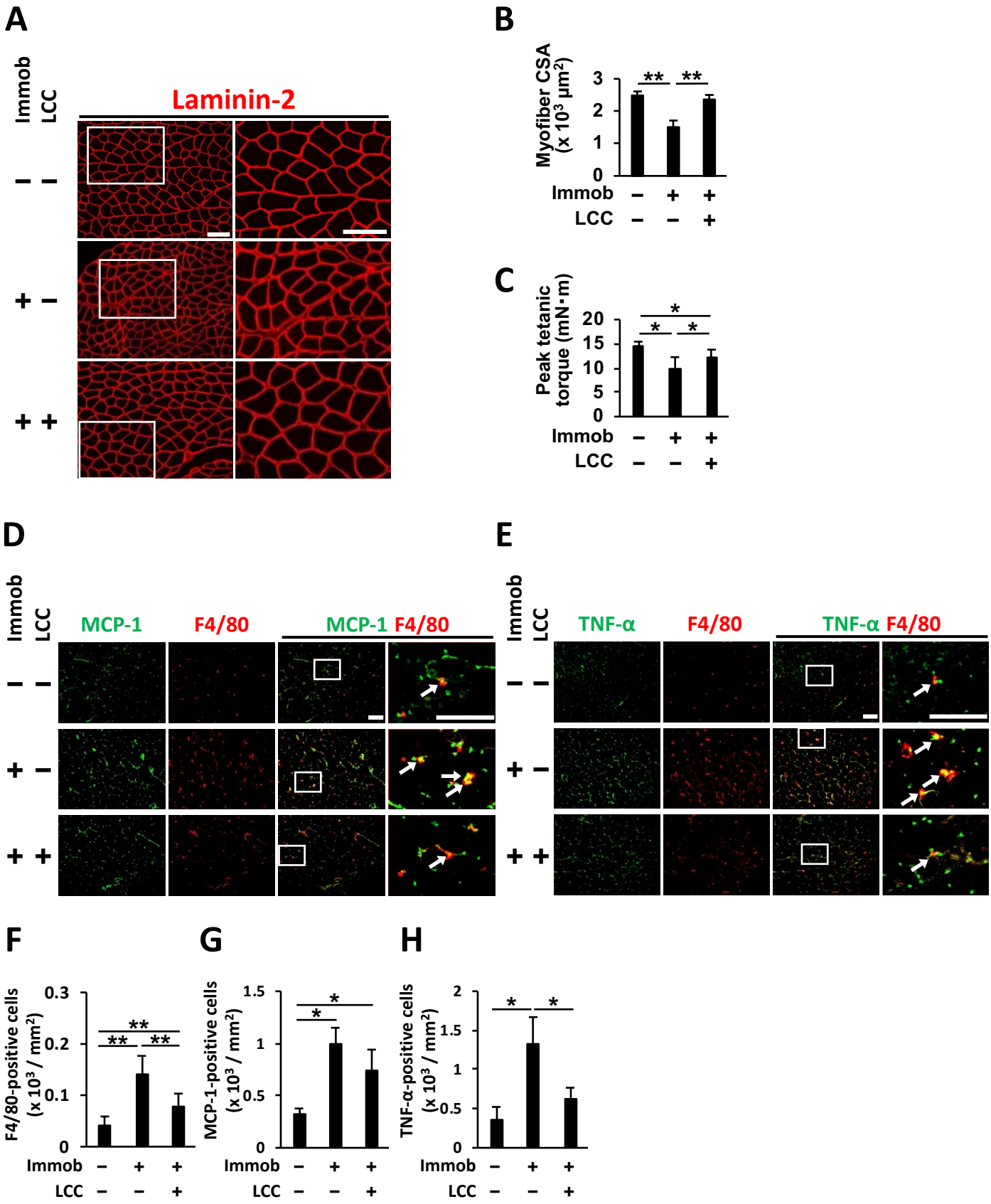


Figure 4

Figure 4

Click here to access/download;Figure;190419-2 re-revised figures (Figure 4).pdf



Name of Material/ Equipment	Company	Catalog Number
Aluminum wire	DAISO JAPAN	B028
Blood pressure telemeter	Millar	SPR-671
DAPI	Thermo Fisher Scientific	D1306
Goat anti-rabbit Alexa Fluor 488 (Dilution ratio, 1:500)	Invitrogen	A11034
Goat anti-rat Alexa Fluor 568 (Dilution ratio, 1:500))	Invitrogen	A11077
ImageJ	NIH	N/A
LabChart8	ADInstrumens	
Prolong gold	Thermo Fisher Scientific	P36930
Protein Block Serum-Free	Dako	X090930-2
Rat monoclonal anti-laminin-2 antibody (Dilution ratio, 1:1000)	Sigma Aldrich	L0663
Rat monoclonal anti-F4/80 antibody (Dilution ratio, 1:500)	Abcam	ab6640

Rabbit polyclonal anti-MCP-1 antibody (Dilution ratio, 1:1000)	Abcam	ab25124
Rabbit polyclonal anti-TNF-α antibody (Dilution ratio, 1:1000)	Abcam	ab66579
Surgical tape	3M Japan	1530EP-0

Comments/Description

An aluminum wire is used to avoid escaping restriction by the wire
A blood pressure telemeter is used to mesure intramuscular pressure.

DAPI is a fluorescent probe which is commonly used to stain DNA for fluorescent microscopy.
Antibody for immunohistochemical staining.
Antibody for immunohistochemical staining.
Analysis software for image
Analysis software for acquiring biological signals.

Prolong gold is for mounting stained samples.

For blocking non-specific background staining in immunohistochemical procedures.
Antibody for immunohistochemical staining.
Antibody for immunohistochemical staining.

Antibody for
immunohistochemical
staining.
Antibody for
immunohistochemical
staining.
Surgical tape is used to restrict joint movement.

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Author(s): Naoyoshi Sakitani, Takahiro Maekawa, Kumiko Saitou, Shuhei Murase, Masakuni Tokunaga, Keisuke Sawada, Atsushi Takashima, Motoshi Nagao, Toru Ogata, Yasuhiro Sawada

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CORRESPONDING AUTHOR

Name:	Yasuhiro Sawada
Department:	Department of Clinical Research
Institution:	National Rehabilitation Center for Persons with Disabilities
Title:	M. D., Ph. D.
Signature:	Yasuhiro Sawada
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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

Comment 1

Please expand the Introduction to include all of the following with more citations.

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

Response 1

We have revised the Introduction accordingly (page 3, lines 114 - 116 in our revised manuscript).

Comment 2 and 3

Citations? Please number the citations in order. So 5 should be followed by 6, 7, 8. Presently. 6-8 are missing.

Response 2 and 3

By a careless mistake, we have deleted citation numbers (6 - 8) from our revised manuscript. We have added the citation numbers in the latest version of our manuscript.

Comment 4

Presently the protocol steps are lacking connection between the individual sections (from 1-2 and 2-3 and 3-4). Please bring out this clarity. Maybe including a timeline to show at what time which step is performed may help?

Response 4

We have added Supplementary Figure 1 to clarify the connection between the individual sections.

Comment 5

How is the mouse placed in this case?

Response 5

The mouse was laid in a supine position (page 4, lines 141 - 142 in our revised manuscript).

Comment 6

Any specifics of the wire to be used?

Response 6

The relevant information is given in Table of Materials. We have clarified this in the Protocol section (page 4, line 145).

Comment 7

Do you perform surgery to do the same? Also in the figure there seem to be something else present along with the wire, please comment on the same.

Response 7

No surgical or other invasive procedures were involved in the wiring process. The white material seen beneath the wire (Figure 1A) is the surgical tape used to ease the handling of the hindlimbs. We have added this information in Figure 1A of our revised manuscript.

Comment 8

How is this done?

Response 8

We manually adjusted the configuration of aluminum wire (page 4, lines 150 - 152).

Comment 9

Please comment on post anesthesia steps as well. Do you leave the mouse in the single housed cage? Is the mouse able to maintain normal movement?

Also, what is the control in this case?

Response 9

We just returned the mice to their original cages. Yet, we made sure that 3 hours later, they recovered from anesthesia and accessed to food and water as usual (page 4, lines 154 - 157). With regard to the control, we have modified the sentence to clarify the samples used as a reference for statistical analysis (pages 7 - 8, lines 325 - 327).

Comment 10

Please quantify the weights.

Response 10

We tested 36-g, 66-g, and 200-g. We have added this information in our revised manuscript (page 4, line 163).

Comment 11

Where are the steps for analysis of muscle inflammation and atrophy? Please include the step numbers here to bring out clarity.

Response 11

We have added relevant information (page 4, line 166).

Comment 12

Is this the same mouse from step 1?

Response 12

We have clarified that the mice subjected to pressure measurement were not used for histological analyses (page 4, lines 166 - 167 and Supplementary Figure 1).

Comment 13

Is there a specific reason to use different anesthesia in this case?

Response 13

Because pressure measurement involved more invasive procedures (e.g., skin incision and needle insertion) as compared to hindlimb wiring and LCC (page 4, lines 169 - 170).

Comment 14

We cannot have phrases like could be, should be, would be, etc. Please reword in imperative tense.

Response 14

We have revised the text (page 4, line 172).

Comment 15

Before performing the incision, do you shave the fur, do you sterilize the area? Please include all specific details.

Response 15

We depilated with an electric shaver and semi-sterilizing the skin surface with 70% ethanol-soaked absorbent cotton (page 4, lines 175 - 176).

Comment 16

With respect to?

Response 16

We inserted a needle at an obtuse angle (150° - 170°) to the skin surface (page 4, line 179).

Comment 17

Also please include post anesthesia recovery steps.

Response 17

Please refer to Response 9.

Comment 18

Please provide more details on how to monitor the intramuscular pressure. Button clicks in the software etc?

Again how were the controls treated?

Response 18

We compared 33-g, 66-g, and 200-g. There was no control for pressure measurement. We think we have provided sufficient information on pressure monitoring.

Comment 19

Is this mouse the same as in step 2 or 1? Please bring out clarity with respect to the step number. Also, if same, please mention after how many days is this step performed?

Response 19

As mentioned in Response 12, the mice subjected to pressure measurement were not used for histological analyses. With regard to the protocol for 7-day LCC experiments, we describe the details in 3.3 (page 5, lines 201 - 202) and Supplementary Figure 1.

Comment 20

This needs more clarity. Please mention what part of the LCC instrument contains the cylindrical weight, how is the movement performed, where is the mouse placed etc. Maybe mark the position of weights in the instrument shown. Do you perform any button clicks, knob turns etc to bring the weights onto the mouse?

What is the range of weights tested on the mouse?

What is the control in this case?

Response 20

We have added more detailed information in Figure1B.

Comment 21
Dosage?

Response 21
We have described the information (page 5, lines 209 - 210).

Comment 22
How is this done.

Response 22
After depilating the posterior calf surface, we made a skin incision, and dissected gastrocnemius muscles by separating from tibio-fibular bone using a surgical scissor (page 5, lined 212 - 213).

Comment 23
What are appropriate filters in your case.

Response 23
We have provided relevant information (page 6, lines 259 - 261).

Comment 24
How is this done, please provide all the button clicks in the software, graphical user interface, etc.

Response 24
We think we have provided sufficient information.

Comment 25
Please include a one liner title for each figure with all the panels combined.

Please upload each figure individually to your editorial manager account. Please keep the panels together.

Response 25

We have added figure titles (page 7, lines 306 - 307 and 312 - 313; page 8, lines 331 - 332, 343 - 344, and 360).

Comment 26

Please make the borders thicker in between the panels.

Response 26

We have revised Figure 2A accordingly

Comment 27

Data for this ?

Response 27

Statistical analysis was conducted with reference to the ‘Day 0’ samples (gastrocnemius muscles from mice that were not subjected to immobilization). We have clarified this in the figure legend (page 7 - 8, lines 325 - 327)

Comment 28

Please do not write the discussion in pointwise manner. Please use paragraph style instead.

Please expand the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

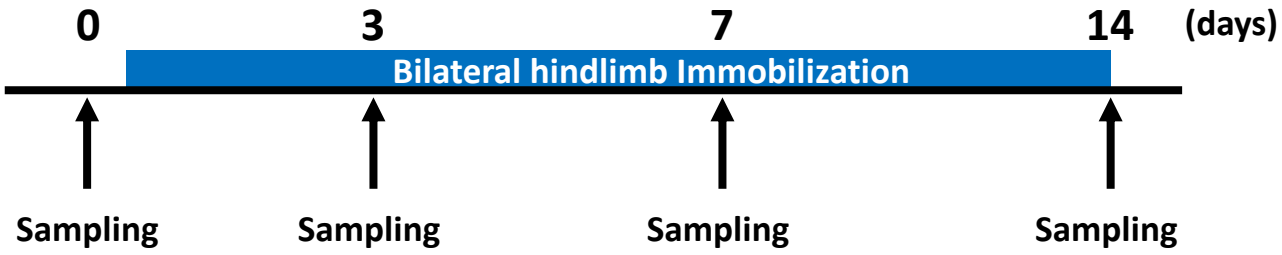
e) Any future applications of the technique

Response 28

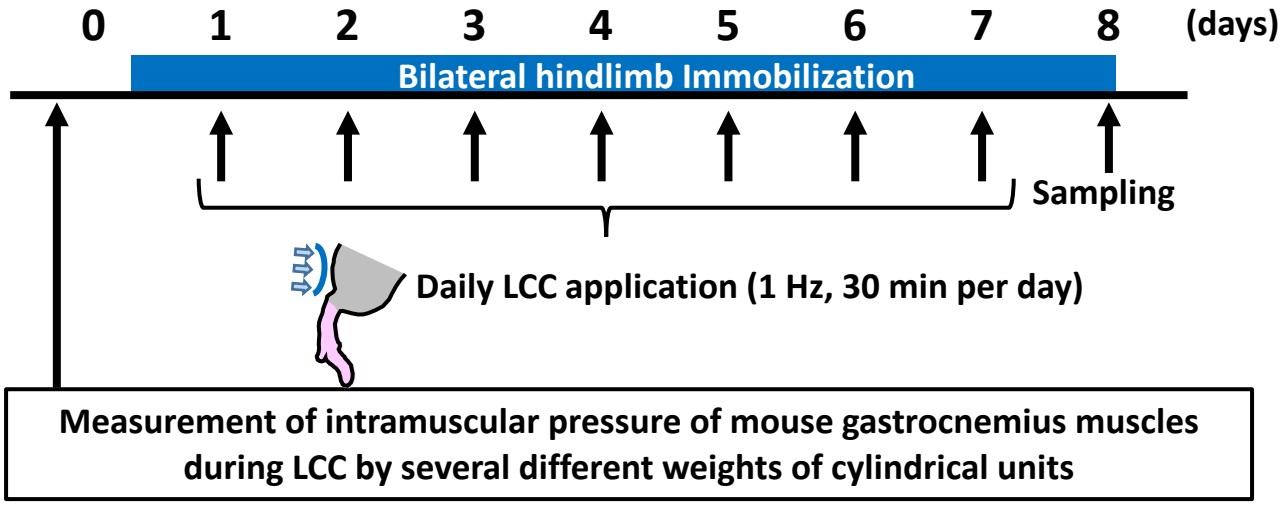
We have revised the manuscript accordingly.

Supplementary Figure 1

Experiment for Figure 2



Experiment for Figures 3 and 4



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