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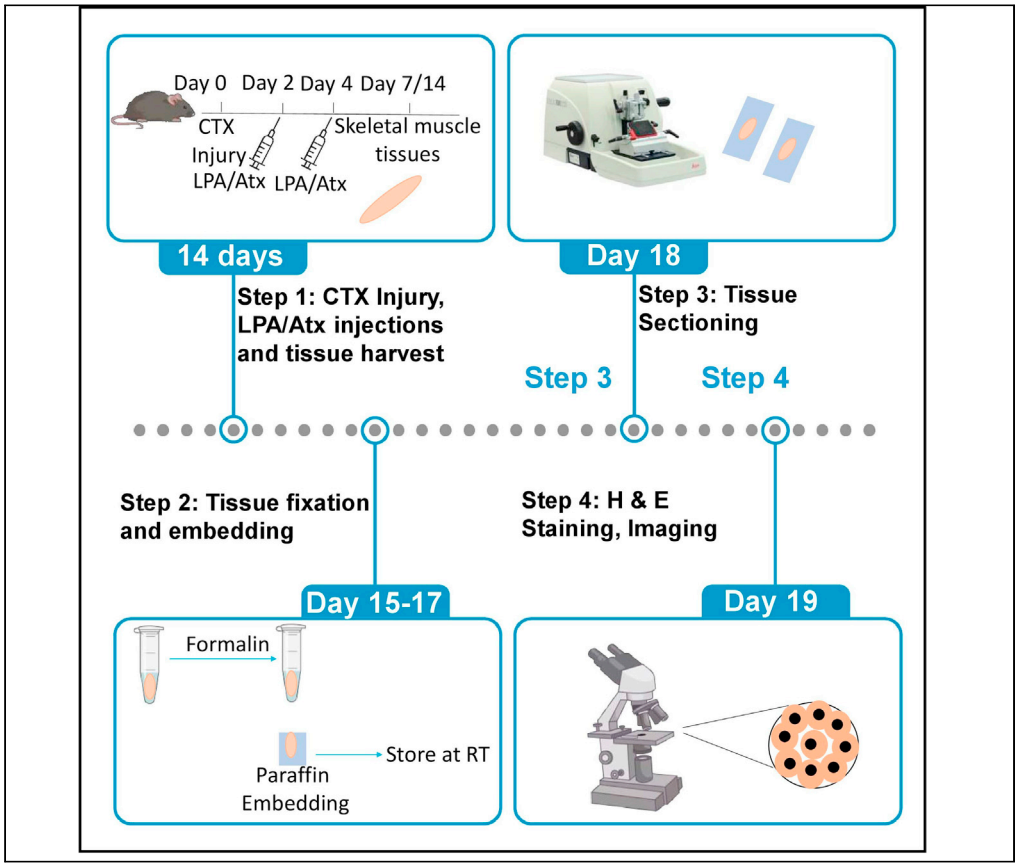
Protocol for accelerated skeletal muscle regeneration and hypertrophic muscle formation in mice



Skeletal muscle system is the major organ associated with movement of the body. Myogenesis and regeneration induced post-injury contribute to muscle formation and maintenance. Here, we provide detailed protocol for the accelerated repair of injured skeletal muscles and generation of hypertrophic muscle fibers. This protocol includes cardiotoxin induced muscle injury and also describes isolation of satellite cells from skeletal muscle tissues of mice. This protocol can be used to study the mechanisms associated with accelerated muscle repair and hypertrophy.

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Highlights

Satellite cell isolation from mouse skeletal muscle tissue and induction of hypertrophy

An optimized protocol for accelerated muscle regeneration in mice

Protocol for generation of hypertrophic muscles in mice

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Protocol for accelerated skeletal muscle regeneration and hypertrophic muscle formation in mice

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SUMMARY

The skeletal muscle system is the major organ associated with movement of the body. Myogenesis and regeneration induced post-injury contribute to muscle formation and maintenance. Here, we provide detailed protocol for the accelerated repair of injured skeletal muscles and generation of hypertrophic muscle fibers. This protocol includes cardiotoxin induced muscle injury and also describes isolation of satellite cells from skeletal muscle tissues of mice. This protocol can be used to study the mechanisms associated with accelerated muscle repair and hypertrophy.

For complete details on the use and execution of this protocol, please refer to Ray et al. (2021).

BEFORE YOU BEGIN

Mouse satellite cells are isolated from tibialis anterior, gastrocnemius and EDL muscles of C57BL/6 mice according to ethical guidelines. All experiments are conducted in Class II biosafety cabinets using standard sterile techniques. All cells are cultured and propagated in a humidified 37°C incubator and contain 5% CO_2. Before initiation of the protocol, prepare the media, solutions, and collagen-coated plates. Similarly, the media should be pre-warmed to 37°C for 30 min. Please refer to the key resources table for a comprehensive list of reagents and resources. Please refer to materials and equipment for complete recipe tables.

1. A complete list of reagents and labware can be found in the key resources table.
2. All procedures, except for the dissociation of skeletal muscle tissue, are performed under sterile conditions in a biological safety cabinet.
3. Cool centrifuges to 4°C.
4. Prepare the following media and sterile-filter using a 0.22 μm filter (see "materials and equipment" for detailed recipes):
   a. Satellite cells culture medium (typically 50 mL)
   b. Differentiation medium (20 mL)
   c. Hypertrophy induction medium (20 mL)
5. Satellite Cells Culture Medium: Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco, catalog number: 11995065), supplemented with 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin, filter sterilized. 10 ng/mL basic fibroblast growth factor (bFGF).
6. Differentiation medium: DMEM, 1% penicillin/streptomycin and 2% Horse serum.
7. Hypertrophy Inducing medium: DMEM, 1% penicillin/streptomycin 2% horse serum and 10 μM Lysophosphatidic Acid (LPA, Sigma, catalog number: L7260).

Initial preparations before satellite cell culture

Timing: 15–20 min (needs to be prepared in advance, before satellite cell isolation)

8. Collagen solution 5 mg/mL

Add 10 mL PBS into 50 mg of collagen vial to make a stock solution of 5 mg/mL. Make 100 μL aliquots and store in −20°C for up to 3 months.

9. Collagenase type I solution (0.2% solution in DMEM or DPBS)

Prepare a fresh 0.2% collagenase type I in DPBS or DMEM medium. 2 mL of this 0.2% collagenase solution is used for tissue dissociation from one mouse.

10. Collagen-coated culture plates
    a. Aliquot the collagen type I (100 μL each) in 1.5 mL Eppendorf tubes and store at −20°C freezer for future use. Thaw one collagen aliquot on ice and resuspend it in DPBS at 0.1 mg/mL.
    b. Coat 35 mm culture dishes and/or 6 well plates with collagen type I for a minimum of 60 min in a 37°C cell culture incubator and 5% CO_2. Alternatively, plates can also be prepared by incubating for 8–12 h at 4°C and stored at 4°C for up to 1 month after sealing it with parafilm to avoid drying of the plates.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pax7 (1:100 dilution used)	Developmental Studies Hybridoma Bank	Pax7
MyHC (1:100 dilution used)	Developmental Studies Hybridoma Bank	MF20
Dystrophin (1:100 dilution used)	Developmental Studies Hybridoma Bank	MANDRA1 (7A10)
myod1 (1:100 dilution used)	Santa Cruz Biotechnology	sc760
myogenin (1:100 dilution used)	Santa Cruz Biotechnology	sc-398002
Alexa Fluor 647 Goat Anti -Mouse IgG (1:500 dilution used)	Thermo Fisher	A21236
Alexa Flour 488 Goat Anti -Rabbit IgG (1:500 dilution used)	Thermo Fisher Scientific	A11034
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Institute of Life Sciences, Bhubaneswar	N/A
Chemicals and recombinant proteins		
Collagenase	Sigma-Aldrich	C5894
Cardiotoxin	Sigma-Aldrich	C9759
basic fibroblast growth factor (bFGF)	Thermo Fisher Scientific	RFGFB50
Insulin	Sigma-Aldrich	12643
Collagen	Sigma-Aldrich	C9791
Horse Serum	Thermo Fisher Scientific	26050070
FBS	Thermo Fisher Scientific	10082147
DMEM, high glucose	Thermo Fisher Scientific	11995065
Penicillin and Streptomycin	Thermo Fisher Scientific	15070-063
LPA	Sigma-Aldrich	L7260
Active Atx	Echelon Biosciences	E-4000
DPBS	PAN-Biotech	P04-36500
Cell isolation kit		
Satellite cell isolation kit	Miltenyi Biotec	130-104-268
Other		
96-well flat bottom plates	BD Falcon	353072
1.5 mL Eppendorf tube	BD Falcon	N/A
15 mL Centrifuge tube	BD Falcon	352096

(*Continued on next page*)

Continued		
REAGENT OR RESOURCE	SOURCE	IDENTIFIER
50 mL Centrifuge tube	BD Falcon	352070
5 mL serological pipette	BD Falcon	356551
10 mL serological pipette	BD Falcon	356551
Scalpel (surgical blade)	N/A	N/A
35 mm cell culture dish	BD Falcon	353001
Block heater	Eppendorf	N/A
70 μm Cell strainer	BD Falcon	352350
Millex-GV Syringe Filter Unit, 0.22 μm	Merck Millipore	SLGV033R
37°C Water bath	N/A	N/A
37°C and 5% CO2 tissue culture incubator	Thermo Fisher Scientific	N/A
Inverted microscope (such as Zeiss)	Zeiss	N/A
Biological safety cabinets	N/A	N/A

MATERIALS AND EQUIPMENT

Preparation of reagents

This step prepares the media and reagents, which are made on the morning of the experiment.

> ***Alternatives:*** Left-over media can be re-used for a later experiment, provided they are stored as stated below.

All media and solutions are prepared using sterile techniques.

FGF-basic 100 μg/mL

Dissolve 50 μg of FGF-basic in 0.5 mL sterile water to make a 100 μg/mL stock solution. A working concentration of 10 ng/mL FGF-basic would be needed per well. Make 100 μL aliquots and store in −20°C for up to 3 months.

LPA 2 mM

Dissolve 1 mg fresh LPA in 1 mL of DPBS to make a ~2.20 mM stock solution. A working concentration of 10 μM LPA would be needed for mouse injections or satellite cell treatment.

Collagen coating

To coat the tissue culture dishes or chambered slides with collagen, incubate the dishes 8–12 h at 4°C or 37°C for 60 min with 0.1 mg/mL solution of collagen in sterile DPBS or 5 $\mu g/cm^2$. Aspirate the solution and dry the plates before seeding the cells.

Satellite cells culture medium

Prepare culture medium as follows:

- Thaw frozen aliquots of bFGF on ice.
- First bring DMEM into a conical tube, then add the growth factor and 20% FBS.
- Filter through a 0.22 μm filter.
- Add penicillin and streptomycin
- Store the medium at 4°C (up to 2 weeks).

Culture medium		
Reagent	Final concentration	Amount
DMEM	–	16 mL
FBS	20%	4 mL
Pen/Strep (100×)	1%	200 μL
bFGF	10 ng/mL	

⚠ CRITICAL: b-FGF should be added to the media immediately before use.

Differentiation medium

Prepare differentiation medium as follows:

- First bring DMEM into a conical tube, then add 2% horse serum.
- Filter through a 0.22 μm filter.
- Add penicillin and streptomycin
- Store the medium at 4°C (up to 2 weeks).
- Add LPA to the differentiation medium immediately before adding to the cells

Reagent	Final concentration	Amount
DMEM	–	19.4 mL
FBS	2%	400 μL
Pen/Strep (100×)	1%	200 μL
LPA	10 μM	

⚠ CRITICAL: LPA should be added to the media immediately before use.

Tissue dissociation medium

Prepare dissociation medium as follows:

- Bring Collagenase to 25°C–37°C.
- Prepare a 0.2% collagenase solution by dissolving in DPBS or DMEM. Filter through a 0.22 μm filter.

Reagent	Final concentration	Amount
DPBS	–	2 mL
Collagenase I	0.2%	4 mg

⚠ CRITICAL: Collagenase solution should be freshly prepared and used immediately.

STEP-BY-STEP METHOD DETAILS

In this step, muscle stem or satellite cells are isolated from skeletal muscle tissues of mice, cultured to form myoblasts and differentiated further to generate hypertrophic myotubes. LPA is added to differentiating satellite cells to induce hypertrophy as reported in Ray et al. (2021).

Isolation of satellite cells from skeletal muscle tissues

Timing: 1–2 h

1. Prepare a collagen coated 6-well plate and 4 or 8 well chambered slides (refer to collagen plate coating) and keep the culture medium warmed to 37°C ready.
2. Tibialis anterior, gastrocnemius and extensor digitorum longus (EDL) muscles of C57BL/6 mice are subjected to enzymatic dissociation 0.2% collagenase solution for 45 min after which non-muscle tissues are gently removed and the muscle is minced under a dissection microscope, followed by 45-min incubation in the same collagenase solution.
3. The cell suspension is filtered through a 70-μm nylon filter to get a single cell suspension, followed by red blood lysis (RBC) lysis and washing.

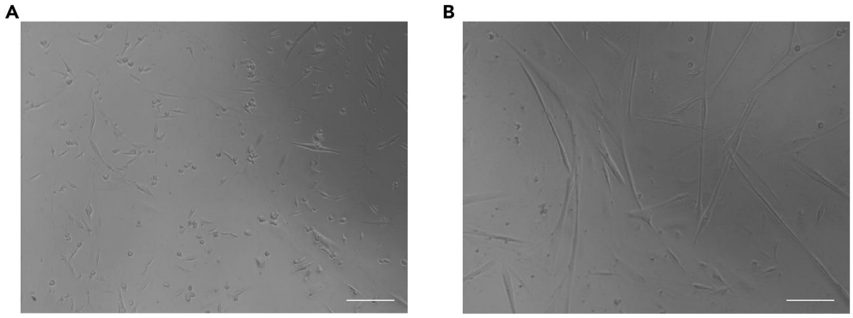

Figure 1. Light microscopy showing satellite cells and differentiated myotubes

(A and B) Light microscopic images of (A) mouse satellite cells and (B) differentiated myotubes from the satellite cells. Scale bar, 100 um.

4. Following washing, cells are isolated using a negative selection kit (130-104-268, Miltenyi Biotec) according to the manufacturer's protocol.
5. Isolated satellite cells are cultured in growth medium consisting of DMEM, 20% FBS, 10 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen), and penicillin and streptomycin. We typically use 10,000–15,000 cells/well for culture of satellite cells in 4 or 8 chambered slides.

Note: FBS can markedly enhance the proliferation of satellite cells. Tissues can be incubated in collagenase medium for a maximum of 2 h with frequent observation for tissue dissociation. The isolated cells are >92% pure and we usually get approximately 50,000–70,000 satellite cells from the skeletal muscle tissues of the hindlimbs of an adult mouse.

⚠ CRITICAL: Do not incubate the tissues in an enzymatic solution for more than 2 h.

Feeding satellite cells

Timing: 30 min

6. The next day warm culture medium and DPBS to 37°C. Remove the spent medium and wash the cells once with 1 mL prewarmed DPBS to remove debris, then add 2 mL (35 mm dish) or 400 μL (for 4/8 well chambered slides) fresh and prewarmed culture medium to continue feeding. Refresh culture medium every alternate day until cells become 50%–60% confluent.

⚠ CRITICAL: Cell morphology is monitored under an inverted microscope. Proliferating satellite cells display round colony or polygonal morphology, while the differentiated cells exhibit changed morphology (e.g., enlarged cells with cylindrical morphology). Scrape off large, differentiated colonies with a P200 pipette tip by visual recognition. Small fibroblasts cells will spontaneously disappear upon replenishment of satellite cell specific culture medium (Figure 1).

7. Proceed to differentiation of satellite cells and induction of hypertrophic myotubes when they reach 50%–60% confluency (approx. 4–5 days).

Note: For optimal results, cells should be approximately 50%–60% confluent after 4–5 days in culture. If cells do not reach this confluency, adjust the timing to start the differentiation later.

8. Prewarm satellite cells differentiation medium and DPBS to 37°C.
9. Aspirate the spent medium from the satellite cells to be induced for differentiation. Rinse the cells with 2 mL DPBS, and then add 2 mL (35 mm dish) or 400 μL (for 4/8 well chambered slides) of differentiation medium supplemented with 10 μM LPA per well for inducing hypertrophic myotubes.

10. Incubate the cells in hypertrophy inducing medium for 3–4 days with replenishment of LPA containing differentiation medium every day to the differentiating cells.

Accelerated regeneration of cardiotoxin injured skeletal muscle tissues

Timing: 2–4 weeks

Skeletal muscle regeneration is induced by initiating skeletal muscle injury by the intramuscular injection of cardiotoxin (CTX), a snake venom toxin that induces myofiber damage and a strong regenerative response (Chargé and Rudnicki, 2004; Goddeeris et al., 2013; Price et al., 2014). Here we describe the steps in induction of muscle injury and initiation of accelerated repair by injecting LPA to regenerating muscles as reported in Ray et al. (2021).

11. C57BL/6 mice between the ages of 6–8 weeks are injected with Cardiotoxin (CTX) injections for skeletal muscle injury.
12. Wipe the hindlimbs of C57BL/6 mice with ethanol wipes.
13. Inject 10 μM (50 μL) cardiotoxin (CTX) diluted in PBS intramuscularly (i.m.) into tibialis anterior muscles with approximately 25 μL volume injected at two different sites in the TA muscles. CTX is injected in both legs. The needle for injection is inserted into the center of the TA avoiding it from going too deep or beyond muscle. (The needle can be inserted 2/3 mm deep and at an approximately 20° angle).
14. Mice are left for 48 h for the induction of muscle injury or myofiber damage and initiation of regenerative process.
15. 20 μM LPA is injected (i.m.) every alternate day in regenerating muscles until tissues are harvested for histological analysis.
16. Tissues are harvested at different time intervals (day 7, 14 or as required in experiments) for histological analysis.

Induction of hypertrophic skeletal muscle formation

Timing: 2–4 weeks

17. Direct intramuscular (i.m.) injection of 3 μg Atx (in 50 mL PBS-BSA) or 10 μM final concentration of LPA in 50 μL PBS-BSA (50 μL PBS-BSA used as a vehicle control) is conducted into skeletal muscles of C57BL/6 mice.
18. Atx or LPA is injected for five consecutive days or until tissues are harvested for analysis.
19. Skeletal muscle tissues are harvested 7 and 14 days after CTX injury for histological analysis.

Pause point: Harvested tissues can be stored in formalin for 24–48 h. Paraffin embedded tissues can be stored for extended periods.

EXPECTED OUTCOMES

By using this model protocol, induction of accelerated skeletal muscle regeneration can be carried out in mice and generate hypertrophic muscles for muscle regeneration or muscle hypertrophy studies. *In vitro* generated thicker myotubes can be used in identifying signaling events/mechanisms implicated in hypertrophy. Together, this protocol helps in studying the skeletal muscle system. This protocol can be modified/tuned with varying LPA doses to determine the molecular mechanisms associated with skeletal muscle hypertrophy or muscle regeneration.

LIMITATIONS

In vitro myotube formation efficiency may depend on the muscle harvesting protocol, age and strain of mice, protocol for satellite cell isolation and initial cell density used for induction of myotube

differentiation. Importantly, myotube formation is strongly dependent on the proximity of myoblasts. It is critical that a fresh differentiation medium with LPA is refreshed every day to differentiating cells, which also helps in removal of debris. Progress must regularly be monitored under the light microscope. Of note, the glucose content of the culture medium used for proliferating satellite cells should be high, as these cells have high nutrient demands and might proliferation efficiency.

Accelerated skeletal muscle regeneration depends on the injected LPA. It is critical to use fresh LPA resuspended immediately in DPBS for injection into regenerating muscles.

TROUBLESHOOTING

Problem 1

Poor satellite cells recovery after skeletal muscle dissociation (steps 2–5 of "Isolation of satellite cells from skeletal muscle tissues").

Potential solution

Recovery of satellite cells could be low due to the age of mice used. Make sure the mice used is young 6–8 weeks of age. The tissue pieces are constantly dissociated in collagenase at 37°C. The tissue should be cut into small pieces with maximum removal of adipose tissues for appropriate penetration of the collagenase. Further, addition of dispase can also aid in the tissue dissociation for better recovery of satellite cells.

Prolonged dissociation time decreases cell viability. We recommend enzymatic dissociation for not longer than 2 h. Once single cells and (few) cell clusters are visible under the microscope, immediately stop the enzymatic reaction.

Adding bFGF and extra serum is essential for satellite cells growth. Failing to add extra serum in the medium will result in fewer satellite cells. Therefore, always add extra serum in the culture medium and refresh on alternate days. The correct composition of the culture medium is critical.

Problem 2

Satellite cells do not form myotubes (steps 7–10 of "feeding satellite cells").

Potential solution

If not, enough satellite cells are present in a well for myotube induction, cells from several wells can be pooled together. While mechanically removing cells from different wells to pool satellite cells together, pre-wet the tips with FBS which helps to avoid adherence of the cells inside the tip and loss of cells. It is important that cells have a high density to facilitate their fusion and myotube formation. Further extended culture of satellite cells can also impact their differentiation characteristics.

Problem 3

Skeletal muscle injury is not reproducible (steps 13 and 14 of "accelerated regeneration of cardiotoxin injured skeletal muscle tissues").

Potential solution

For skeletal muscle injury try to inject the CTX into 2–3 different sites in the skeletal tissue of mice. CTX injections at multiple sites ensure proper dispersion of the cardiotoxin for injury to take place.

Problem 4

Hypertrophic muscle formation is not reproducible (steps 17 and 18 of "induction of hypertrophic skeletal muscle formation").

Potential solution

For proper hypertrophic muscle formation or accelerated muscle regeneration, always inject freshly prepared LPA into the skeletal muscle tissues of the mice. Frequency of LPA or Atx injection is also critical and should be injected daily or consecutively for many days because of the very low half-life of LPA.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vivek Rai (vivekrai.a@gmail.com).

Materials availability

All the materials used in this protocol are commercially available.

Data and code availability

Data are available upon request from the lead contact

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AUTHOR CONTRIBUTIONS

R.R. developed the methods and performed all experiments. V.R. contributed to method development and analysis of data. R.R. wrote the first draft of the manuscript. V.R. acquired funding and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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