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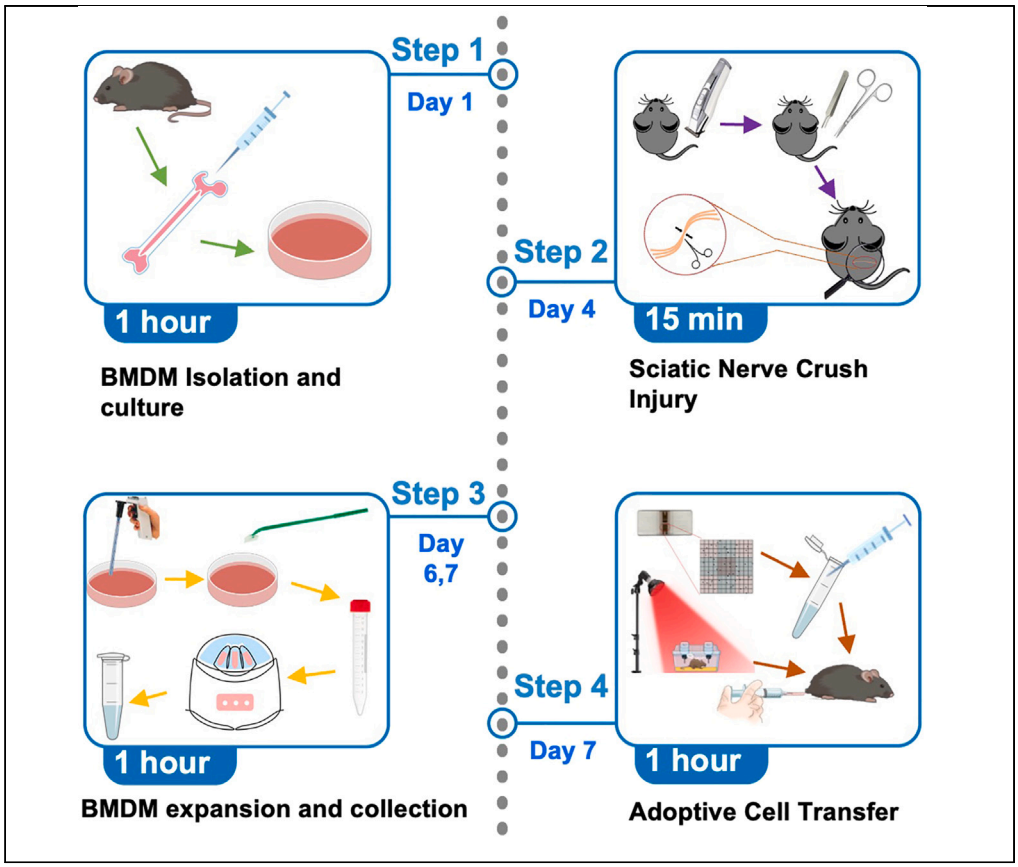
Protocol

Adoptive cell transfer of macrophages following peripheral nerve injury in mice



Macrophages are key innate immune cells involved in multiple biological processes, including peripheral nerve regeneration. Here, we describe a protocol for the adoptive cell transfer of bone-marrow-derived macrophages (BMDMs) following sciatic nerve crush injury (SNCI). This procedure involves isolating BMDMs from a donor mouse, potentially manipulating them *ex vivo*, and reintroducing them into an animal following SNCI. Preclinical studies show that BMDMs can infiltrate injured nerves and impact functional recovery, potentially providing a novel therapy for nerve injuries.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Isolating bone-marrow-derived macrophages from mice

Adoptive cell transfer of macrophages by tail-vein injection

Macrophages injected into tail vein localize to injured nerves

Assessment of peripheral nerve regeneration by sciatic nerve crush

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Adoptive cell transfer of macrophages following peripheral nerve injury in mice

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SUMMARY

Macrophages are key innate immune cells involved in multiple biological processes, including peripheral nerve regeneration. Here, we describe a protocol for the adoptive cell transfer of bone-marrow-derived macrophages (BMDMs) following sciatic nerve crush injury (SNCI). This procedure involves isolating BMDMs from a donor mouse, potentially manipulating them *ex vivo*, and reintroducing them into an animal following SNCI. Preclinical studies show that BMDMs can infiltrate injured nerves and impact functional recovery, potentially providing a novel therapy for nerve injuries.

For complete details on the use and execution of this protocol, please refer to Jha et al.[1]

BEFORE YOU BEGIN

Institutional permissions

Mice are required for this protocol both for a) generation of bone-marrow-derived macrophages (BMDM) and b) sciatic nerve crush injury (SNCI) model, and thus regulatory approval is necessary before beginning this procedure. The procedure must comply with animal surgery guidelines, including aseptic surgical techniques to prevent possible infection and appropriate anesthesia and analgesia to minimize pain. The Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University approved animal experiments in this procedure.

Preparation of L929 conditioned medium

Timing: 10 days

L929 (ATCC) is a mouse fibroblast line that secretes macrophage colony-stimulating factor (M-CSF). M-CSF is a growth factor that regulates the activation, proliferation, and differentiation of BMDMs from hematopoietic stem cells.

1. Culture L929 cells in complete DMEM/F12 medium in T-25 flask cell culture dishes at 37°C in 5% CO_2 atmosphere.
2. At about 80% confluency, discard the media and wash with 5 mL of PBS pre-warmed to 37°C. Add 1 mL of trypsin, swirl the trypsin solution to cover the cell surface, and return the flask to the CO_2 incubator for 5 min.
3. Confirm cell lifting by light microscopy and add 10 mL of PBS to stop the trypsinization. Collect the cells and centrifuge at 500 g for 5 min.

4. Resuspend the cell pellet in 10 mL of fresh complete DMEM/F12 media and seed the cells in T175 flask.
5. Grow L929 cells until confluent and supplement with fresh media. Let the media stay for 4–5 days. Some cell death may occur at this time, but dead cells do not affect the quality of the conditioned medium.
6. Centrifuge the media at 500 g for 5 min and filter with 0.22 μm filter to remove cells and cellular debris.
7. Aliquot and store the media at −80°C. It can be stored for up to 1 year when stored properly.
8. Before use, we recommend a pilot experiment differentiating BMDMs to ensure appropriate expression of M-CSF.

Reagent substitution

Similar products of equal quality from a different supplier may substitute for the reagents listed. M-CSF (10 ng/mL) or macrophage migration inhibitory factor (MIF) (10 ng/mL) may substitute for 20% L929 conditioned media.[2–5] DMEM/F12 can be substituted with RPMI1640.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM/F12	Thermo Fisher Scientific	Cat #11330032
RPMI1640	Thermo Fisher Scientific	Cat #11875119
Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher Scientific	Cat #15070063
GlutaMAX Supplement	Thermo Fisher Scientific	Cat #35050061
Trypsin-EDTA solution	Thermo Fisher Scientific	Cat #25-053-CI
Fetal bovine serum (FBS)	Sigma	Cat #12306C
Sterile phosphate-buffered saline (PBS) (pH 7.4)	Thermo Fisher Scientific	Cat #AM9624
Red blood cell lysis buffer (NH_4Cl)	Thermo Fisher Scientific	Cat #00-4333-57
99.9% isoflurane	N/A	N/A
Experimental models: Cell lines		
L929	ATCC	CCL-1
Experimental models: Organisms/strains		
C57BL/6J mice, male and female, 6–8 weeks of age	The Jackson Laboratory	Stock no: 000664
Other		
Microtubes, 1.7 and 2 mL	Fisher Scientific or of user choice	N/A
Pipette tips, 10, 200, and 1000 μL	Fisher Scientific or of user choice	N/A
Syringes, 1 and 3 mL	Fisher Scientific or of user choice	N/A
Needle, 27G	Fisher Scientific or of user choice	
Falcon centrifuge tubes, 15 and 50 mL, sterile	Fisher Scientific or of user choice	N/A
Serological pipettes, 5, 10, and 25 mL	Fisher Scientific or of user choice	N/A
Cell culture plates, 6-well	Fisher Scientific or of user choice	N/A
Membrane filter system, 0.22 μm	Fisher Scientific or of user choice	Cat #09-761-107
Hemocytometer	Fisher Scientific or of user choice	Cat #02-671-54
100 mm × 20 mm tissue culture-treated dish, sterile	CELLTREAT or of user choice	Cat #229620
150 mm × 20 mm tissue culture-treated dish, sterile	CELLTREAT or of user choice	Cat #229651
70 μm Cell strainer, individually wrapped, sterile	CELLTREAT or of user choice	Cat #229483
Cell lifters	CELLTREAT or of user choice	Cat #229306
Surgical scissors, forceps, and blades	N/A	N/A
Tabletop centrifuge	N/A	N/A
Ethanol	Of user choice	N/A
Infrared lamp	Amazon	N/A
Mice restrainer	Fisher Scientific	Cat #NC0772753

MATERIALS AND EQUIPMENT

Complete DMEM/F12 media		
Reagent	Final concentration	Amount for 500 mL
FBS	10%	50 mL
Penicillin-Streptomycin	1%	5 mL
GlutaMAX	1%	5 mL
DMEM/F12	N/A	440 mL

Macrophage differentiation media		
Reagent	Final concentration	Amount for 500 mL
FBS	10%	50 mL
Penicillin-Streptomycin	1%	5 mL
GlutaMAX	1%	5 mL
LCM	10%	50 mL
DMEM/F12	N/A	390 mL

STEP-BY-STEP METHOD DETAILS

Bone-marrow-derived macrophages (BMDM) are primary macrophage cells, derived from bone marrow cells *in vitro* in the presence of growth factors. Macrophage colony-stimulating factor (M-CSF) is a lineage-specific growth factor responsible for the proliferation and differentiation of committed myeloid progenitors into macrophage/monocyte lineage cells.[5]

Major step 1: Bone marrow isolation

Timing: 1 h (n = 2 animals)

Day 1

Extraction of bone marrow cells from mice.

1. Euthanize and dissect the mouse.
   a. Sacrifice the mouse using a CO_2 asphyxiation followed by cervical dislocation.
   b. Immediately spray the mouse with 70% EtOH before dissection.
   c. Use surgical scissors and forceps to dissect the whole leg, from hip to ankle, and immediately soak the leg in 70% EtOH for 5 min in a 10-cm Petri dish.
   d. Use forceps and scissors to remove the skin and skeletal muscle tissue from the bone and wash the bone with cold PBS in a 10 cm Petri dish. Be careful not to cut bone as this will compromise the sterility of the bone marrow.
   e. Place the bone in a new 10 cm Petri dish containing PBS and separate the femur and tibia by cutting at the knee joint.
   f. Clean away any remaining tissue and wash again with PBS in a new dish.
   g. Discard mouse carcass and all excess tissues according to institutional policy.

   Note: Bones must be thoroughly cleaned of skeletal muscle to avoid contamination from the tissue cells.

2. Extract bone marrow from the femur and tibia.
   a. Fill a 1-mL syringe with cold bone marrow isolation buffer (BMIB).
      i. BMIB: Add 1.5 mL FBS to 48.5 mL PBS (3% final concentration FBS)
      ii. Make fresh prior to use and keep on ice.

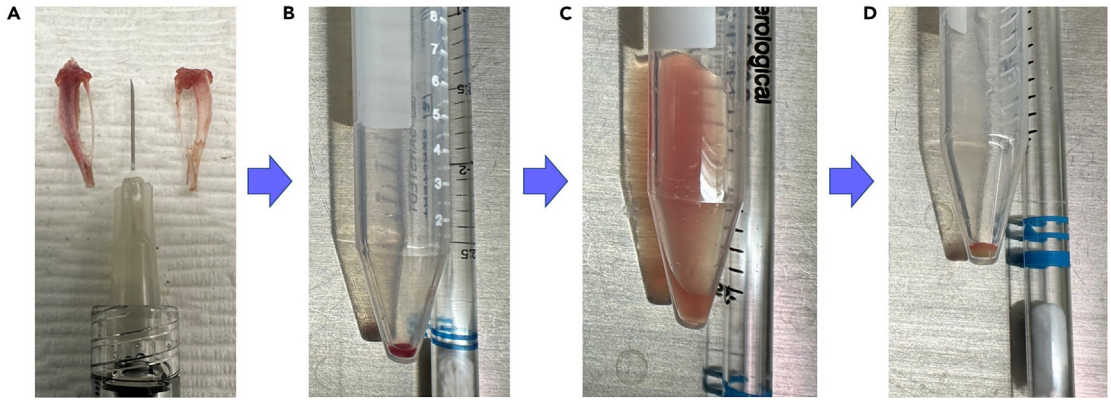

Figure 1. Preparation and harvesting of bone-marrow-derived macrophages (BMDMs)
(A) Mice tibia and femur before (left) and after (right) flushing the bone marrow.
(B–D) (B) Bone marrow pellet (C) Cell suspension in RBC lysis buffer (D) Cell pellet after RBC lysis.

b. Cut off the ends of the femur bone and gently insert a 27-gauge needle attached to the syringe into the marrow cavity.
c. Flush the bone marrow into a 6-well plate or 10-cm Petri dish by expelling the BMIB through the cavity.
d. Flush the bone from both ends using the 1-mL syringe with the cold BMIB.
e. Refill the syringe with BMIB and repeat this step until you have flushed out all the bone marrow material into the 6 well plate or dish.
f. Repeat the process for tibia bones or bones from additional animals.

Note: The color of the bone changes from dark red to white when the bone marrow is completely flushed out (see Figure 1). The amount of bone marrow cells obtained depends on the mouse's age and genetic background. Usually, 6–8-week-old mice are ideal BM donors.

⚠ CRITICAL: It is essential to maintain sterility throughout this procedure. Perform the entire procedure a laminar flow hood, and use generous amounts of 70% EtOH to sterilize surgical equipment periodically.

3. Prepare bone marrow cells for culture.
   a. Gently pipette the mixture up and down several times using a pipette to homogenize the bone marrow.
   b. Transfer the solution into a 15 mL conical tube and centrifuge at 500 g for 5 min at 4°C to pellet cells.
   c. Aspirate and discard the supernatant.
   d. Resuspend the cells in 1 mL Red Blood Cell Lysis Buffer and incubate for 3–5 min at room temperature to lyse red blood cells.
   e. Add 9 mL of BMIB to neutralize the Red Blood Cell Lysis Buffer.
   f. Centrifuge at 500 g for 5 min at 4°C to pellet cells.
   g. Discard supernatant and resuspend cells in 5 mL BMIB.
   h. Pass the resuspended cell suspension through a 70-μm cell filter to get rid of cellular debris and clumps.

Note: Pre-wet the cell strainer.

   i. Centrifuge at 500 g for 5 min at 4°C to pellet cells.

Note: Pool together cells from all the animals to get a homogeneous cell population for the recipient animals.

Major step 2: Bone marrow culture and macrophage differentiation

Timing: 7 days

Day 1

Following isolation of murine bone marrow, culture and differentiate macrophage progenitors into BMDMs.

4. Resuspend the cells from bone marrow in 5 mL of complete DMEM/F12 containing 10% LCM medium pre-warmed to 37°C, and gently mix the cell pellet.
5. Prepare each 150 mm Petri dish with 25 mL DMEM/F12 containing 10% LCM medium.
6. Add 1 mL of cell suspension per dish and swirl to distribute the cells evenly in medium. Therefore, you will need five plates for each mouse.
7. Incubate plates at 37°C in 5% CO_2 atmosphere.
8. Supplement with fresh media on day 3 by adding 15 mL DMEM/F12 containing 10% LCM medium pre-warmed to 37°C.
9. Replace all media on day 6 with 25 mL fresh DMEM/F12 + 10% LCM. Check under a microscope to ensure that cells have a spindle-like appearance, which signifies that these cells are macrophages.
10. On Day 7, BMDMs are ready to harvest. At this stage, you may also treat BMDMs further to induce specific macrophage phenotypes.
11. Aspirate media from each plate.
12. Use a cell lifter to scrape cells from the Petri dishes or add 5 mL of Cellstripper (Mediatech) per plate and incubate at 37°C for 10 min to allow the cells to lift off the plate.
13. Collect the cell suspension and add 10 mL of DMEM/F12 medium to neutralize the Cellstripper.
14. Centrifuge at 500 g for 5 min at 4°C to pellet cells. Resuspend the cell pellet in PBS or DMEM/F12 media and count the cell number using a hemocytometer.

Note: The cell culture process will take a week, allowing the macrophage progenitors to attach to the adherent surface and divide until there is a confluent monolayer of macrophages.

Major step 3: Sciatic nerve crush

Timing: 1 h

Day 4

Sciatic nerve crush is a commonly used research technique to evaluate peripheral nerve regeneration. The axons are severed in this technique, while the epineurium and connective tissue are not disturbed.[6–8]

15. Perform the surgery in a sterile location to avoid any potential infection in mice.
16. Start by cleaning the work area with 70% ethanol.
17. Prepare the surgery area by placing a clean drape on a heating pad.
18. Anesthetize adult mice (~100 days old) with 2% isoflurane by nose cone.
19. Inject mice with analgesic, Meloxicam, at 2 mg/kg body weight subcutaneously.
20. Apply ophthalmic ointment to the eyes using sterile cotton-tipped applicators.
21. Carefully shave both hindquarters using shavers. You may complete this step the day before to save time on the day of surgery.
22. Clean the skin with 70% ethanol Prep Pads, and betadine surgical scrub.
23. Cover the surgical field with a sterile drape. Sterilize all instruments by autoclave or hot bead sterilization and the surgeon wears a mask, gown, and sterile gloves.

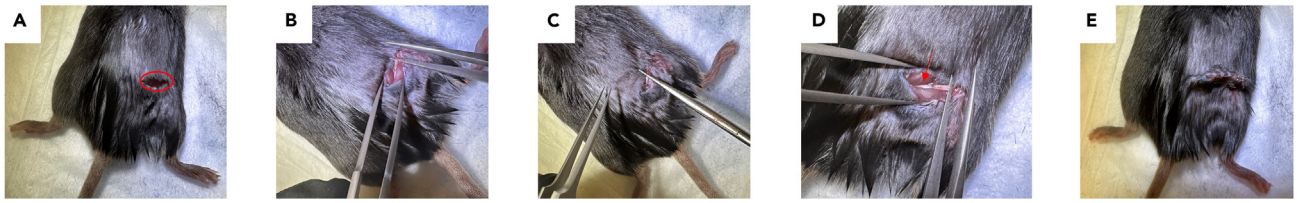

Figure 2. Sciatic nerve crush

(A) Subcutaneous incision is made above the femur.

(B) Muscle fascia is opened to expose the sciatic nerve.

(C) Sciatic nerve is crushed for 20 s using a smooth hemostat.

(D) The nerve post-crush demonstrates interruption of the white myelin sheath (see red arrow).

(E) The crushed nerve is repositioned under muscle fascia and the skin incision is closed with 9 mm reflex clips.

24. Incise the skin just below the lateral aspect of the femur bone (See Figure 2 and Methods video S1).
25. Open the fascial plane between the gluteus maximus and the anterior head of the biceps femoris with blunt scissors to reveal the sciatic nerve.
26. Gently free the sciatic nerve from the surrounding connective tissue without damaging or stretching the nerve.
27. Crush the sciatic nerve with smooth forceps for 20 s at 3 clicks of the forceps—being careful not to stretch the nerve.

Note: Following nerve crush, a pink line across the nerve will identify the crush site.

28. Place the nerve carefully back in its original position and close the muscle fascia.
29. Finally, close the skin incision using 9 mm reflex clips. If 9 mm reflex clips restrict movement, one can use smaller reflex clips or 6-0 or absorbable sutures instead.

Post-operative care

30. Following the procedure, place animals on a heating pad at 37°C until they show signs of movement.

Note: If a heating pad is unavailable, an infrared lamp can be used.

31. After recovery, return animals to their home cage, where water and food are readily accessible on the floor in the form of Hydrogel and wet food.

Major step 4: Counting and preparing BMDMs for adoptive transfer

Timing: 1 h

Day 7

32. Divide the bone marrow cells from one animal (see Step #3) into five 150 mm dishes for bone marrow differentiation. Keep the cells on ice.
33. On day 7, wash the cells with PBS, scrape in 5 mL of PBS using a cell lifter, and collect in 15 mL tubes.
34. Spin the cells twice with PBS at 500 g for 5 min to eliminate any carry-over media and FBS, which can be immunogenic when injected into animals.
35. Pool the cells from all the tubes in one 15 mL tube and gently resuspend in 5 mL of PBS to make a homogeneous cell suspension.

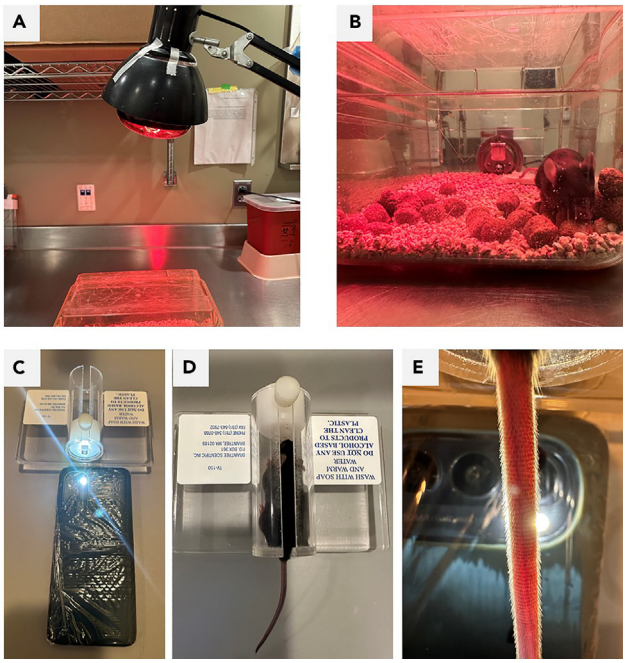

Figure 3. Setup and heat treatment using an infrared lamp for mouse tail-vein injection

(A and B) Infrared lamp setup.

(C) Setup for the tail vein injection with a phone flashlight and mouse restrainer.

(D) A mouse is shown in restrainer.

(E) Mouse tail vein visualized by trans-illumination.

36. Count the cells using a hemocytometer.

Note: Each animal yields around 15–20 million BMDMs. Ideally, cells isolated from each donor mouse will be sufficient to adoptive cell transfer into 3 recipient mice, though this depends on cell death during cell isolation or treatment, if any.

37. Centrifuge the cell suspension again at 500 g for 5 min.
38. Resuspend the cells in PBS and gently mix the cells to produce a cell suspension such that each 100 μL contains 5 × 10^6 cells.

Note: Cells should be injected within 15 minutes once the cells are ready to avoid cell death.

Major step 5: Adoptive cell transfer of BMDMs

Timing: 1 h

Day 7

Sciatic nerve crush occurred on Day 4 of this protocol; thus, the nerves are now three days post-crush. At this time post-crush, there is maximal macrophage infiltration following injury to peripheral nerves.[9,10]

39. Heat tail regions of mice under the infrared lamp for 10–15 min to prepare them for injections, as heat from the infrared lamp dilates the lateral veins of the tail, facilitating the injection process.
40. Prepare syringes with cells for injection and keep them on ice.
41. Move a single mouse to the mouse retainer (See Figure 3).

42. Pull the tail towards the investigator, hold the syringe in your dominant hand, and position the syringe parallel to the tail. Insert the needle with the bevel up in the lateral vein, and slowly inject the cells (See Methods video S2).

Note: Inject cells smoothly with minimal resistance. If there is resistance to the injection or white blisters appear above the injection site, withdraw the needle, and try a different injection site proximal to the first one. As the fluid will escape via the first site, do not inject distal to the initial injection site.

43. Inject each animal 100 μL of BMDM suspension (See step 31).
44. Remove the needle and press firmly with the thumb to prevent backflow of the injected solution and blood. Apply gentle compression with a clean gauze/wipe or tissue until the bleeding has stopped.

⚠ CRITICAL: The mice should not be kept under the infrared lamp for more than 15 minutes, as mice are sensitive to overheating.

Note: Keep two mice under the infrared lamp at a time.

EXPECTED OUTCOMES

This technique yields robust infiltration of injected BMDMs into the injured regions of nerves. As detailed in our prior publication, the cells are functional and contribute to nerve regeneration.

LIMITATIONS

Unless injected into immunodeficient mice, the BMDMs must be from the same background and gender as the recipient mice.

TROUBLESHOOTING

Problem 1

L929 cells are difficult to trypsinize and thus do not easily lift from plates (related to "before you begin" Step 2).

Potential solution

- Flush the cells carefully with trypsin after incubation for 20–25 min.
- Use pre-warmed trypsin.

Problem 2

Ineffective tail-vein injections of BMDMs, either due to missing the vein or leak of cells following injections.

Potential solution

- Tail vein injection will require practice and some expertise.
- Make sure to hold the site of injection to prevent leakage of BMDMs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Brett M Morrison, bmorris7@jhmi.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102624.

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AUTHOR CONTRIBUTIONS

M.K.J. and B.M.M. participated in the initial conceptualization and design of the study. A.R. optimized the BMDM generation techniques. M.K.J. optimized the adoptive transfer techniques. A.R., M.K.J., and B.M.M. optimized the sciatic nerve injury model. B.M.M. secured funding for the project. A.R. wrote the initial draft of the manuscript. All authors read and contributed comments to the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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