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Rapid isolation of dorsal root ganglion macrophages

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April 16, 2019

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TITLE:

Rapid Isolation of Dorsal Root Ganglion Macrophages

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KEYWORDS:

Macrophage, DRG, FACS, nerve injury, sensory neuron, MAFIA, CSF1R, CX3CR1

SUMMARY

Here we present a mechanical dissociation protocol to rapidly isolate macrophages from the dorsal root ganglion for phenotyping and functional analysis.

ABSTRACT:

There are growing interests to study the molecular and cellular interactions among immune cells and sensory neurons in the dorsal root ganglia after peripheral nerve injury. Peripheral monocytic cells, including macrophages, are known to respond to a tissue injury through phagocytosis, antigen presentation, and cytokine release. Emerging evidence has implicated the contribution of dorsal root ganglia macrophages to neuropathic pain development and axonal repair in the context of nerve injury. Rapidly phenotyping (or "rapid isolation of") the response of dorsal root ganglia macrophages in the context of nerve injury is desired to identify the unknown neuroimmune factors. Here we demonstrate how our lab rapidly and effectively isolates macrophages from the dorsal root ganglia using an enzyme-free mechanical dissociation protocol. The samples are kept on ice throughout to limit cellular stress. This protocol is far less time consuming compared to the standard enzymatic protocol and has been routinely used for our Fluorescence-activated Cell Sorting analysis.

INTRODUCTION:

There is now considerable evidence that immune cells contribute to the neuropathic pain following peripheral nerve injury[1,2]. Peripheral monocytic cells, including mature macrophages, are known to respond to tissue injury and systemic infection through phagocytosis, antigen presentation, and cytokine release. Paralleling the nerve injury-induced microglia activation in the spinal dorsal horn, macrophages in the dorsal root ganglia (DRG) also expand significantly after nerve injury[3,4]. Notably, there are growing interests to determine if macrophages

contribute to neuropathic pain development after peripheral nerve injury by interacting with sensory neurons in the DRG[5-11]. Moreover, recent studies also implicate the contribution of DRG macrophages in the axonal repair after nerve injury[12,13]. Another study further suggests that macrophage subpopulations (i.e., $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6C^{low/-}$ cells) may play a different role in the mechanical hypersensitivity[14]. Therefore, rapidly phenotyping the response of DRG macrophages in the context of nerve injury may help us identify neuroimmune factors contributing to neuropathic pain.

Conventionally, the protocol to isolate macrophages in the DRG involves multiple steps including enzymatic digestion[15,16]. The technique is often time consuming and can be costly for large-scale experiments. Although mild digestion with collagenase type II (4 mg/mL) and dispase type II (4.7 mg/mL) for 20 min was recommended previously[15], it is conceivable that cells after the exposure to this enzyme are prone to cell damage or cell death, which may lead to low yield. In addition, the difference in the quality of enzymes from batch to batch may further impact the efficiency of this process. More importantly, macrophages exposed to the enzyme digestion might be undesirably stimulated and thus can be very different from the in-vivo status. The changes may potentially complicate the outcome of the functional study.

Here we describe an enzyme-free protocol to rapidly isolate DRG macrophages at 4 °C using mechanical dissociation. The samples are kept on ice to limit cellular stress. As a result, our approach provides an advantage to maintain consistency of the isolation, and the isolated cells are presumably healthier and less stimulated. We further present the evidence to validate the quality of the isolated cells with Fluorescence-activated Cell Sorting (FACS) analysis.

PROTOCOL:

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

1. Collect lumbar DRG from experimental mice

1.1. Before starting the experiment, prepare the working solution of the density gradient medium (e.g., Percoll) by mixing 9 volumes of the medium with 1 volume of Ca^{++}/Mg^{++}-free 10x HBSS. Keep it on ice.

1.2. Anesthetize the mouse with 2.5% Avertin. Confirm that the animal is fully anesthetized by the lack of response to the hind paw pinch.

NOTE: Both male and female mice aged 6-8 weeks were used.

1.3. Perfuse the mouse transcardially with 10 mL of pre-chilled 1x PBS.

1.3.1. Place the mouse in the supine position with four paws secured with the tape inside a chemical fume hood. Lift the skin below the rib cage by the forceps, and make a small incision with surgical scissors to expose the liver and diaphragm.

1.3.2. Continue to use scissors to cut the diaphragm and the rib cage, open the pleural cavity to expose the beating heart.

1.3.3. Quickly cut the right atrial appendage with iris scissors. Once the bleeding is noted, insert a 30 G needle into the posterior end of the left ventricle, and slowly inject 10 mL of pre-chilled 1x PBS to perfuse the animal.

1.4. Perform dorsal laminectomy[15] on the mouse placed at the prone position.

NOTE: If cell culture is planned, spray the mouse with 70% ethanol before incision and use pre-sterilized surgical instruments for dissection.

1.4.1. Use a size 11 scalpel to make two longitudinal lateral deep incisions starting from the thoracic region down to the sacral region. Remove the skin by the scissors to expose the dorsal muscle layer.

1.4.2. Use Friedman-Pearson Rongeur to peel off the connective tissues and muscles until the lumbosacral spine processes and bilateral transverse processes are exposed.

1.4.3. Use a Noyes Spring Scissor to carefully open the dorsal spinal column, then switch to a Friedman-Pearson Rongeur to remove the vertebral bones to expose the spinal cord with intact spinal nerves attached.

1.5. Carefully dissect ipsilateral and contralateral lumbar DRG (L4/L5 DRG in our study) and place it into 1 mL of ice-cold Ca^{++}/Mg^{++}-free 1x HBSS in a Dounce tissue homogenizer. Now the tissues are ready for step 2.

NOTE: Trim the spinal nerve attached to the DRG if possible.

2. Isolate single cells from mouse lumbar DRG

2.1. Homogenize the DRG tissue with a loose pestle in the Dounce homogenizer for 20-25 times.

2.2. Place a sterile 70 μm nylon cell strainer in a sterile 50 mL conical tube. Wet the cell strainer with 800 μL of ice-cold 1x HBSS, and the flow-through is collected in the conical tube.

2.3. Collect the homogenized tissue suspension from the homogenizer using a pipette and pass through the wet 70 μm nylon cell strainer into the 50 mL conical tube.

2.4. Rinse the homogenizer twice with 800 μL of ice-cold 1x HBSS and then decant into the same 50 mL conical tube with cell strainer to increase the yield.

2.5. Add 1.5 mL of equilibrated ice-cold isotonic density gradient medium (prepared in step 1.1) into a sterile 5-mL polystyrene FACS tube.

2.6. Transfer the cell homogenate from the 50 mL conical tube (in step 2.4) into the FACS tube, mix well with the density gradient medium by pipetting up and down. Add an additional 500 μL of 1x HBSS to seal the top.

2.7. Pellet the cells by centrifugation at 800 *x g* for 20 min at 4 °C.

2.8. Carefully aspirate the supernatant containing myelin in the medium without disturbing the cell pellet at the bottom of the FACS tube.

2.9. Resuspend the cells in PBS or FACS buffer containing 5% Fetal bovine serum (FBS) for FACS analysis.

NOTE: At least 50,000 to 100,000 cells are expected from L4 /L5 DRG of one mouse.

2.9.1. Resuspend the mechanically isolated DRG cells (L4/L5) in 100 μL of PBS containing 5% Fetal Bovine Serum and then incubate with α-mouse CX3CR1-APC antibody (1:2,000) in the dark at 4 °C for 1 h.

2.9.2. Wash the cells with 5 mL of PBS once; centrifuge the cells 360 x *g* for 8 min at 4 °C.

2.9.3. Aspirate the supernatant, then resuspend the cell pellet in 300 μL of PBS for FCAS analysis. If cell sorting is planned, resuspend the cells in the FACS buffer instead.

REPRESENTATIVE RESULTS:

To validate the isolated cells, we first chose the Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice[17]. This line expresses a drug-inducible FK506-binding protein (FKBP)-Fas suicide fusion gene and green fluorescent protein (eGFP) under the control of the promoter of CSF1 receptor (CSF1R), which is specifically expressed in both macrophages and microglia. Systemic injection of FK-binding protein dimerizer, AP20187 (AP), induces the apoptosis of the cells expressing the transgene. The expression of EGFP also allows us to monitor the macrophages in the DRG. To deplete macrophages in the MAFIA mice, we began our studies with 3 daily intraperitoneal injections of AP (1 mg/kg). After the last injection, DRG were sectioned for immunostaining for GFP. We recorded a significant loss of GFP^+ cells in the DRG of the AP-treated mice compared to VEH-treated mice (**Figure 1A-B**). In a separate experiment, we used this protocol to mechanically dissociate the DRG macrophages after the treatment. Subsequent FACS analysis revealed a successful depletion of GFP^{hi} population in AP-treated mice (**Figure 1C-D**) and demonstrated the high quality of isolated cells.

We also characterized the isolated DRG cells from the wild-type mice. Mechanically isolated DRG cells (L4/L5) were stained with α-mouse CX3CR1-APC antibody. We found that 6% of the DRG cells were $CX3CR1^+$ macrophages (**Figure 2A-B**). Cell viability was also assessed with Propidium Iodide (final concentration of 2.5 μg/ml) which binds to the intracellular DNA of the nonviable cells, revealing that more than 80% of freshly isolated DRG cells were viable (**Figure 2C-D**).

FIGURE LEGENDS:

Figure 1: FACS analysis of macrophages in the DRG of MAFIA mice after AP treatment. MAFIA mice received daily intraperitoneal injection of 1 mg/kg of AP20187 (AP) or vehicle (VEH) for 3 days before the analysis. (**A, B**) Representative immunostaining images showing the AP-induced depletion of GFP^+ (green) macrophages in the L4/L5 DRG after the 3rd AP treatment. NF200 (blue) was used to label myelinated neurons. *Scale bar*: 50 μm. (**C, D**) The percent CSF1R-GFP^{hi} cells after mechanical dissociation of the L4/5 DRG was determined by FACS analysis, and a representative data set from three independent experiments is shown with the percentage of the gated cell population indicated. The result shows that 4% of total isolated cells from the DRG of VEH-treated animal were GFP^{hi} macrophages. In contrast, only 0.4% of total DRG cells were GFP^{hi} macrophages in AP-treated mouse.

Figure 2: FACS characterization of macrophages in the DRG of the wild-type mice. (**A, B**) Ipsilateral L4 and L5 DRG of naïve wild-type mouse were pooled for mechanical dissociation. The percent $CX3CR1^+$ macrophages were measured by FACS analysis (**A**). The gating for $CX3CR1^+$ cells was based on the background fluorescence in the cells incubated with APC-conjugated isotype control antibody (**B**). (**C, D**) Cell viability of freshly isolated DRG cells was assessed with Propidium Iodide (PI) staining. PI^+ cells (**C**) were gated based on the background fluorescence in the unstained cells (**D**). A representative data set from three independent experiments is shown with the percentage of the gated cell population indicated.

DISCUSSION

Here we introduce a new method to effectively enrich isolated macrophages from mouse DRG. The conventional approach to isolate DRG immune cells requires enzymatic digestion[15,18], which is now replaced with mechanical homogenization in our protocol to limit undesired cell damage and increase the yield. Therefore, the new protocol is far less time consuming. More importantly, enzyme digestion might stimulate the macrophages and change the molecular signature. In contrast, our mechanical approach greatly limits cellular stress.

Using FACS analysis, we further characterized the macrophages in the isolated DRG cells and demonstrated a great cellular recovery. More than 80% of isolated DRG cells under this protocol were found to be viable (**Figure 2D**). At least 50,000 to 100,000 cells are expected from L4 /L5 DRG of one mouse. Therefore, we can confidently conclude that the DRG cells can be further sorted into macrophage subpopulations[14] for either culture or RNA isolation. However, there are a few critical steps which may impact the yield. If the unsatisfying cell yield is noted, the insufficient or excessive homogenization in step 2.1 should be suspected. The extent of cell

death assessed with PI (**Figure 2**) or 7-AAD staining can be utilized to optimize the protocol. In addition, DRG dissection in step 1.4 may require repeated practice for beginners.

Currently, our lab mainly uses this protocol for studying the DRG macrophages. Likely, the application of this protocol can be expanded to study other non-neuronal cells in the DRG, such as satellite cells[19], and T cells[20]. Further studies are needed to confirm the effectiveness of cell isolation. Unfortunately, our current mechanical dissociation method is not ideal for isolation of DRG sensory neurons, and enzymatic digestion remains the most effective method for sensory neuron isolation[15].

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DISCLOSURES:
The authors declare no competing financial interests related to this manuscript.

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Figure

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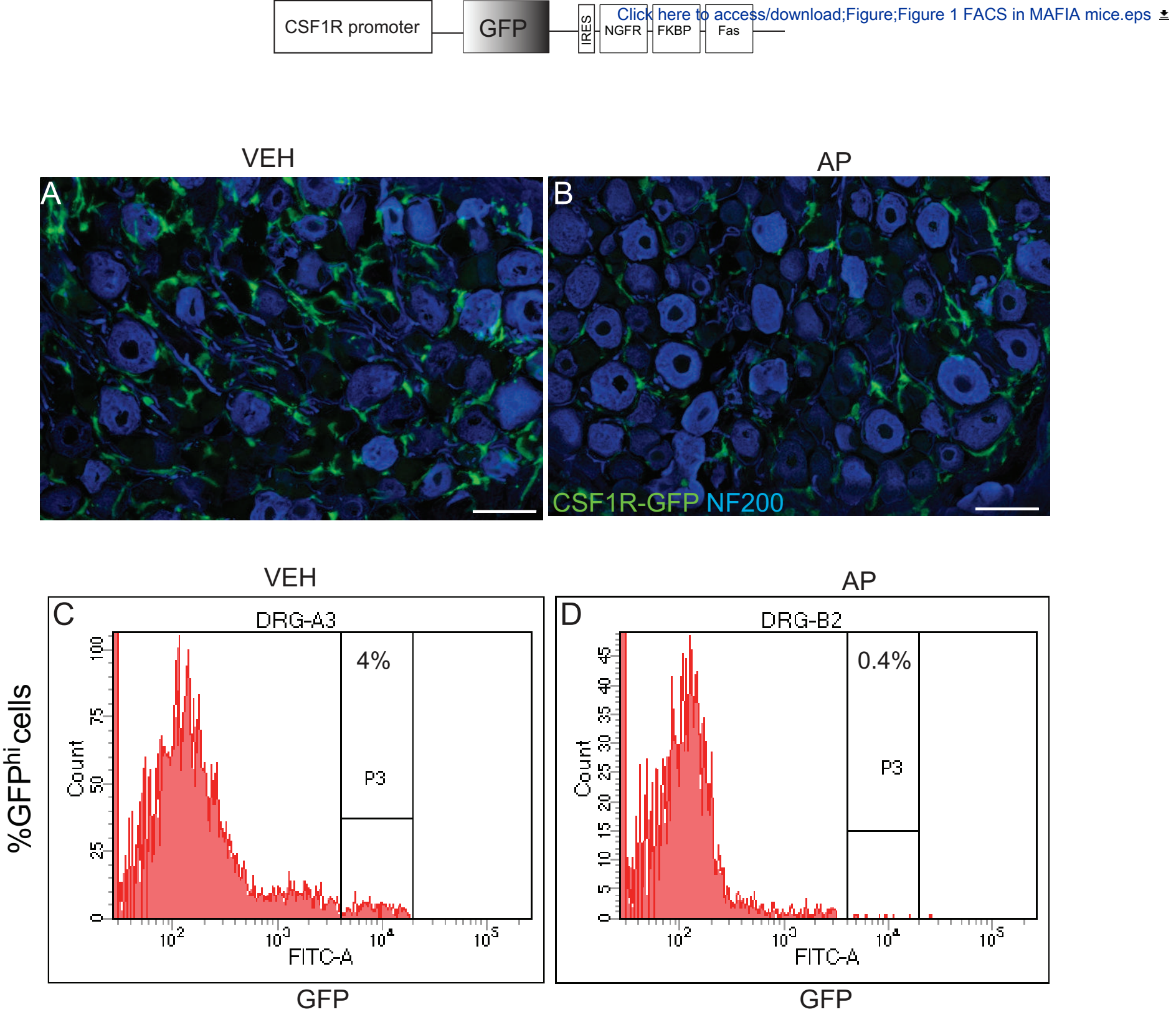

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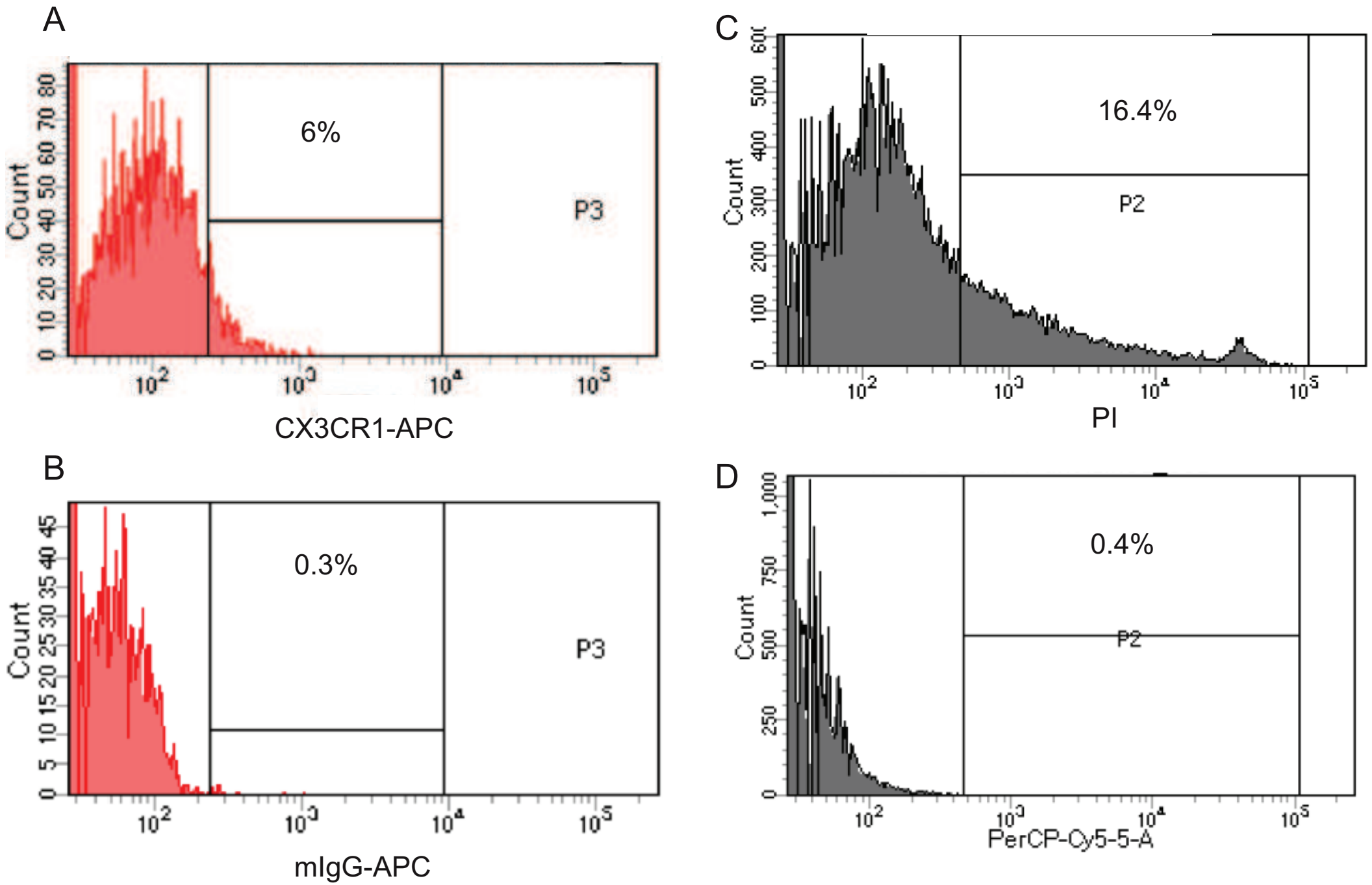

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Table of Materials and Reagents

Materials	Source	Identifier
AP20187	Clontech	635058
α-mouse CX3CR1-APC antibody	Biolegend	149007
Avertin	Sigma	T48402
Cell strainer (70 μm nylon)	Falcon	352350
Centrifuge	Eppendorf	5810R
Dounce tissue homogenizer	Wheaton	357538 (1ml)
FACS tubes (5ml)	Falcon	352052
Friedman-Pearson Rongeur	FST	16121-14
HBSS (10x, Ca^{++}/Mg^{++}-free)	Gibco	14185-052
Noyes Spring Scissor	FST	15012-12
Percoll	Sigma	P4937-500ml
Propidium iodide	Sigma	P4864-10ml

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Rebuttal Letter
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RE: Manuscript, JoVE60023 "Rapid isolation of dorsal root ganglion macrophages,"

Point-by-point responses.

Dear Dr. Vineeta Bajaj,

First, we want to thank you and two reviewers for your valuable comments and guidance. We were encouraged by comments such as "*the paper contains an interesting isolation method*" (Reviewer #1); *"describes a novel protocol of macrophage isolation"; "the manuscript is well-written"; "Data collected using this protocol and presented in the paper is convincing"* (Reviewer #2). With your permission to resubmission, we have diligently attempted to address each point to satisfy all reviewers. The changes are now marked in **RED** in the revision.

Editorial comments:

The manuscript has been revised to address the editorial comments. —Thank you!

- The figures are now submitted in eps format for higher resolution.
- **Table of Materials and Reagents** and a **Summary** are now included in the revised manuscript.
- It is now described how proper anesthetization is confirmed
- Brief explanation of laminectomy is included in the protocol.

Reviewers' Comments:

Reviewer #1:
Specific major points:
1) How will you stain/ do without transgenic mice- recommendation for antibodies-some gating strategies? It would be useful if this was accessible to everyone.

The data of CX3CR1-APC staining of the freshly isolated DRG cells from the wild-type mice are now included in Figure 2.

2) How many mice were used in this process? How many DRGs were pooled together?

Ipsilateral or contralateral L4 and L5 DRG from one mouse were combined for macrophage isolation and subsequent FACS analysis. About 50,000-100,000 cells are expected from two DRG.

3) Can you show the percentage of live and dead cells by flowcytometry using PI/7-AAD or any other dye such as Zombie (Biolegend)? Scatter plots.

Propidium iodide (PI) staining now reveals that more than 80% of the freshly isolated DRG cells are viable. The representative plots are included in Figure 2.

Minor points:
Correct the following lines:
--24 abstract FCAS should become FACS:

Revised. Thank you!

--35/36 $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6C^{hi}$ should become $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6C^{low/-}$

Revised.

--42 37C should become 37ºC

Revised.

--43 certain period of time - vague sentence

This sentence has been eliminated.

86 GFP should be eGFP

Revised.

103 Mild digestion with collagenase/ dispase- which collagenase? and any recommended concentration? Incubation time?

The technical details are now included in the text.

Reviewer #2:
Major Concerns:
None

Thank You!

Minor Concerns:
1. A little more information on steps for macrophage enrichment will help readers not familiar with FACS to better understand the technique. Is Percoll working solution served as a step for enrichment? What about the 800G centrifugation? How is it determined?

Percoll working solution is served for both myelin removal and macrophage enrichment. *800 g* centrifugation is determined based on the previous report in which 1000g was recommended and the recommendation from the manufacture (Sigma).

2. The L4/L5 DRG in rats are actually L3/L4 in mice as described originally by Quinn Hogan's group.

We are aware of the discrepancies in the literature (see References). We often trace the sciatic nerve to confirm the right DRG isolation. For the historic reason, we continue to use “L4/5” in this manuscript.

References:
Malin SA, Davis BM & Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nature Protocols.* 2007;**2** (1):152-160.
Shields SD, Eckert WA 3rd, Basbaum AI. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *The Journal of Pain*. 2003 Oct;4(8):465-70.
Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH. Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain*. 2008 May;136(1-2):188-201.

3. FACS in the Abstract was spelled wrong.

Revised. Thank you!

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Editorial comments.docx

Editorial comments:

- *Please include 6-12 keywords.*

-8 keywords now added

- *Please rephrase the Abstract to more clearly state the goal of the protocol within 100-300 words. Presently it is less than 100 words.*

-Expanded to 140 words.

- *Please expand the Introduction to include all of the following with citations wherever applicable:*
  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

-Revised.

- *Can this be 5-11 instead?*

-Revised

- *Please do not embed any table in the manuscript. Please combine this with the uploaded Table of Materials in .xlsx format and upload it individually to the editorial manager account.*

-Xlsx file is created.

- *For the protocol section please use imperative tense throughout.*
- *The numbering of the Protocol should follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.*
- *Please ensure you answer how question for each step- how is this step performed?*
- *Please use imperative tense throughout and provide all specific details in a stepwise manner. Each step presently can be expanded to show all the actions involved in it.*
- *Once all the changes are performed, please highlight 2.75 pages of the protocol including headings and spacings for filming purpose.*
- *We cannot have commercial terms in the manuscript. I have converted this to the generic term instead. Please check.*
- *Is there any age, sex, strain specific bias? What did you use in your experiment?*
- *How is this done? Do you place a catheter? Where is it placed? Please include details*
- *After placing the mouse, do you perform any shaving of the fur? How? Do you sterilize the surgical area? Please provide all specific details.*
- *Where do you perform the incision? How big is the cut? How do reach to the spinal area?*
- *How is this done? How do you visually identify the ipsilaterlateral and contralateral DRG?*
- *Before homogenization, do you perform a washing step? Do you mince it into small size?*
- *In the same tube?*
- *From step 1.1? Please bring out clarity.*

- *The same FACS tube from 2.4*
- *How are you sure that the cells obtained are single cells? Do you observe it under the microscope?*
- *What does the supernatant consist of? So the DRG is present in the pellet?*
- *What is meant by appropriate buffer? What kind of analysis is performed?*
- *How are you sure that the cells obtained are enriched DRG?*
- *Please expand on these steps. How are individual procedure performed with all specific details? Also please explain and provide the results for RNA studies as well.*
- *Some of these details should be moved to intro/protocol section. Also please bring out the relationship between macrophage, fas, apoptosis and DRG macrophage somewhere with citations to bring out clarity.*
- *Please use degree symbol from insert symbol feature of the word. Please do this throughout the manuscript and in the figure as well.*

-The protocol has been revised.

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-We chose the unpublished figures.

- *As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*
- *Critical steps within the protocol*
- *Any modifications and troubleshooting of the technique*
- *Any limitations of the technique*
- *The significance with respect to existing methods*
- *Any future applications of the technique*

-Revised.