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Video Article

Rapid Isolation of Dorsal Root Ganglion Macrophages

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/60023/

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[5,6,7,8,9,10,11]. 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. 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.
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.

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3. Perfuse the mouse transcardially with 10 mL of pre-chilled 1x PBS.
    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.
    2. Continue to use scissors to cut the diaphragm and the rib cage, open the pleural cavity to expose the beating heart.
    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.

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. Use a size 11 scalpel to make one 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.
    2. Use Friedman-Pearson Rongeur to peel off the connective tissues and muscles until the lumbosacral spine processes and bilateral transverse processes are exposed.
    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.

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

1. Homogenize the DRG tissue with a loose pestle in the Dounce homogenizer 20-25 times.
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.
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.
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.
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.
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.
7. Pellet the cells by centrifugation at 800 *x g* for 20 min at 4 °C.
8. Carefully aspirate the supernatant containing myelin in the medium without disturbing the cell pellet at the bottom of the FACS tube.
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.
    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. Wash the cells with 5 mL of PBS once; centrifuge the cells 360 x *g* for 8 min at 4 °C.
    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**).

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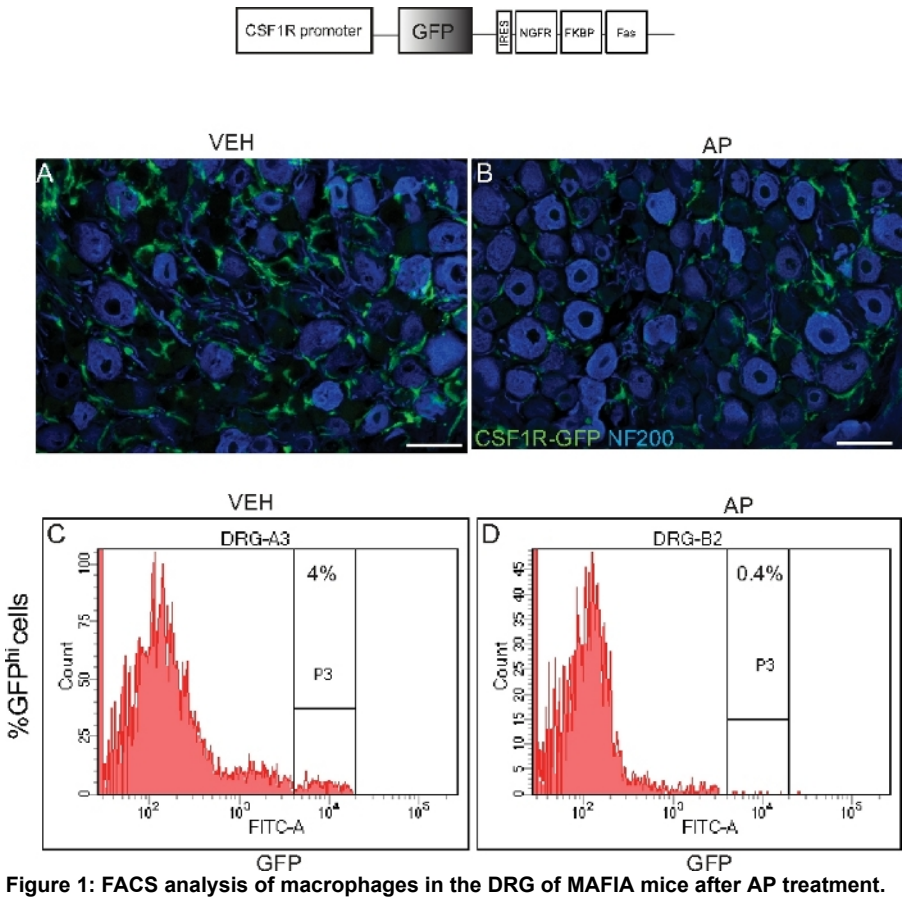

Figure 1: FACS analysis of macrophages in the DRG of MAFIA mice after AP treatment.
MAFIA mice received daily intraperitoneal injection of 1 mg/kg of AP20187 (AP) or vehicle (VEH) for 3 days before the analysis. (**A, B**) Representative immunostaining images showing the AP-induced depletion of GFP^{+} (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. Please click here to view a larger version of this figure.

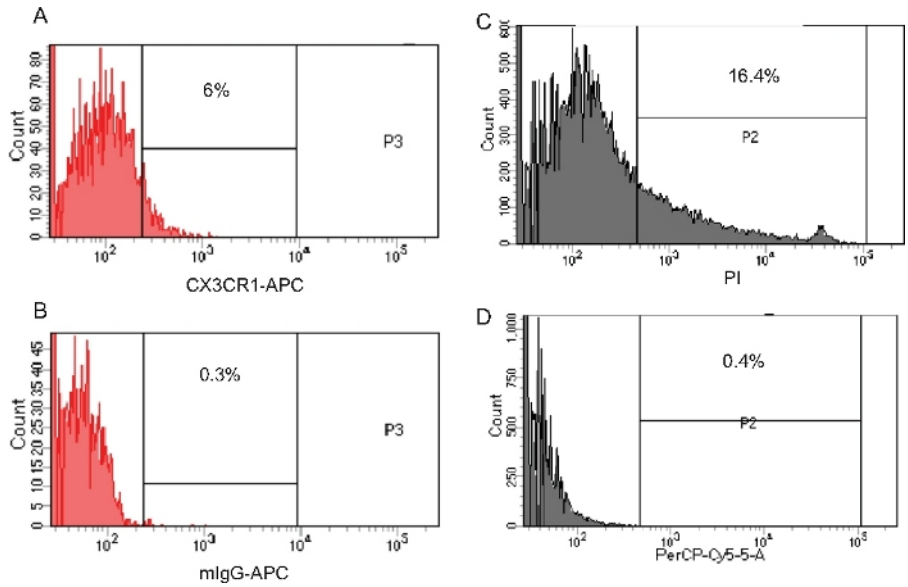

Figure 2: FACS characterization of macrophages in the DRG of the wild-type mice.
(**A, B**) Ipsilateral L4 and L5 DRG of naïve wild-type mouse were pooled for mechanical dissociation. The percent $CX3CR1^+$ macrophages were measured by FACS analysis (**A**). The gating for $CX3CR1^+$ cells was based 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. Please click here to view a larger version of this figure.

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].

Disclosures

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

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