Jun 27, 2025

Nuclei Isolation on Murine DRG for Single Nucleus RNA Sequencing

DOI
https://dx.doi.org/10.17504/protocols.io.6qpvrk1bzlmk/v1

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RE-JOIN

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Protocol Citation: Julia Younis, Ismail Yaman, Mira Jeong, Luis Tovias, Jiansen Yan, Brendan Lee, Nele Haelterman 2025. Nuclei Isolation on Murine DRG for Single Nucleus RNA Sequencing. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.6qpvrk1bzlmk/v1

Protocol status: Working
We use this protocol and it's working

Created: April 22, 2025

Last Modified: June 27, 2025

Protocol Integer ID: 138210

Keywords: Single Nuclei RNA Sequencing, Nuclei Isolation, Murine DRG, Dorsal Root Ganglia, downstream single nuclei rna, dorsal root ganglia sample, single nucleus rna, nuclei extraction buffer, fibrous composition of dorsal root ganglia sample, single cell rna, murine dorsal root ganglia, reproducible method for nuclei isolation, nuclei isolation, rna, sequencing, murine drg, fresh drg, nuclei, cell rna

Funders Acknowledgements:
Neuronal anatomy, connectivity, and phenotypic innervation of the knee joint
Grant ID: UC2 AR082200-01

Disclaimer

This protocol was optimized for 4-10 fresh DRGs per sample.

Abstract

This is a fast and reproducible method for nuclei isolation on murine dorsal root ganglia for downstream single nuclei RNA sequencing. This protocol utilizes the Nuclei Extraction Buffer, Anti-Nucleus Microbeads, gentleMACS Octo Dissociator, and Octo Coolers from Miltenyi Biotec for standardization and ease of use. Here, we adapted the Anti-Nucleus Microbeads protocol to be optimized for the limited tissue volume and fibrous composition of dorsal root ganglia samples.

With this method, 4 fresh DRGs can yield 140 K nuclei before anti-nucleus microbead cleanup, and 80 K nuclei after cleanup. Nuclei processed with this method yield data that pass quality-control analysis and contain all the cell populations that are captured by single cell RNA sequencing.

Materials

Materials

	A	B	C
	Name	**Vendor**	**Product Number**
	Nuclei Extraction Buffer	Miltenyi	# 130-128-024
	Anti-Nucleus MicroBeads	Miltenyi	# 130-132-997
	Murine RNase Inhibitor (3000 units)	New England BioLabs	# M0314S
	Corning Cell Culture Phosphate Buffered Saline (1X)	Fisher	# MT21040CV
	Bovine Serum Albumin	Sigma	# A9647-100G
	Sodium chloride, crystal	JT Baker	# 3624-07
	Potassium chloride, crystal	JT Baker	# 4001-01
	Sodium Phosphate	Omni Pur	# 8210
	Sodium Bicarbonate	EMD	# SX0320-1
	Sucrose	VWR	# M117
	Glucose	Amresco	# 0188
	Calcium chloride	Honeywell	# 10043-52-4
	Magnesium sulfate	Sigma	# 63139
	Trypan Blue Solution	Sigma	# 93595
	Others		
	gentleMACS C Tubes	Miltenyi	# 130-093-237

	A	B	C
	Falcon round bottom tubes, 5 mL	Fisher	# 352-054
	15 mL conical-bottom centrifuge tube	VWR	# 89039-666
	50 mL conical-bottom centifuge tube	VWR	# 89039-662
	LS Column	Miltenyi	# 130-042-401
	8-strip PCR tubes	Fisher	# AVSST-FL
	Pre-Separation Filters (30 um)	Miltenyi	# 130-041-407
	4-Chip Disposable Hemocytometer	Bulldog-Bio	# DHC-N420
	gentleMACS Octo Dissociator with Heaters	Miltenyi	# 130-096-427
	gentleMACS Octo Coolers	Miltenyi	# 130-130-533
	MACS MultiStand	Miltenyi	# 130-042-303
	QuadroMACS Separator	Milteny	# 130-091-051
	SoftFit-L Filtered Pipette Tips, 1000 uL	Fisher	# 2779-HR
	SoftFit-L Filtered Pipette Tips, 200 uL	Fisher	# 2769-HR
	SoftFit-L Filtered Pipette Tips, 20 uL	Fisher	# 2749-HR
	Pipet-lite xls lts, 1000 uL, single channel	Rainin	# L-1000xls
	Pipet-lite xls lts, 200 uL, single channel	Rainin	# L-200xls

	A	B	C
	Pipet-lite xls lts, 20 uL, single channel	Rainin	# L-20xls
	Small petri dish	Any	
	Razor blades	Any	
	Dissection tools (scissors, forceps, and hemostat)	Any	
	Dissection pins	Any	
	27 1/2 G needle	Any	
	5 mL syringe	Any	
	Dissection dish, large, black	Scintica	DD-90-S-BLK
	Centrifuge - Refridgerated	Eppendorf	

Recipes for Solutions

Artificial Cerebral Spinal Fluid (1 L):

- Add 500 mL of ddH_2O to a 1 L bottle
- Add the following reagents: 5.08 g NaCl, 0.186 g KCl, 0.173 NaH_2PO_4, 2.184 g $NaHCO_3$, 25.673 g sucrose, 3.6 g glucose, 0.111 g $CaCl_2$, and 0.843 g $MgSO_4$
- Adjust volume to 1 L with ddH_2O
- Filter with a 0.22 µm vacuum filter, aliquot, and store at 4°C

10% BSA Coating Solution (50 mL):

- Add 5 g of BSA to 30 mL of 1x PBS
- Adjust volume to 50 mL with 1x PBS
- Filter with a 0.22 µm vacuum filter, aliquot, and store at 4°C

Lysis Buffer (2 mL per sample):

Make fresh on ice

- Add 10 µL of RNase Inhibitor to 2 mL of nuclei extraction buffer

Wash Buffer (10.59 mL per sample):

Make fresh on ice

- Add 1.5 mL of nuclei extraction buffer to 9 mL of chilled 1x PBS
- Add 40 µL of 10% BSA solution

- Add 50 µL of RNase Inhibitor

Resuspension Buffer (2 mL per sample):
Make fresh on ice
- Add 10 µL RNase Inhibitor to 1.8 mL chilled 1x PBS
- Add 200 µL 10% BSA

Troubleshooting

Safety warnings

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Ethics statement

Procedures involving animal subjects were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

Background

1 This method was developed to isolate nuclei from murine dorsal root ganglia in preparation for single nucleus RNA sequencing. While this method works for frozen tissues, we recommend and have optimized each step for fresh tissue samples, as they yield higher nuclei counts and superior nuclei quality. Planning will be required to properly time the dissection, nuclei isolation, and sample loading at the core in the same day.

Cardiac Perfusion

2 Deeply anesthetize the mouse via inhalation of 4-5% isoflurane to induce anesthesia. Toe pinch to confirm animal is not receptive to painful stimuli.

3 On a Styrofoam board, fix the mouse in place by inserting 30-gauge syringes or needles into the paws and board.

4 Spray or wipe the ventral abdominal area of the mouse with 70% ethanol to sterilize the field and prevent fur from entering the chest cavity.

5 Use forceps to lift the skin, then make a lateral incision across the skin of the abdomen using surgical scissors.

6 Cut the skin down the midline of the mouse to the top of the thoracic cavity.

7 With forceps, pull back both flaps of skin to expose the peritoneum.

8 Make a lateral incision across the parietal peritoneum to expose the liver and diaphragm.

9 With small surgical scissors, cut the diaphragm (in a crescent shape, along the ribs, careful to not accidentally puncture the lungs or heart) to expose the thoracic cavity.

10 Make two upward cuts on the left and right side of the rib cage to create a flap that can be lifted to better access the heart. Using a hemostat, grip and secure the flap opening to provide access to the chest cavity.

11 With small surgical scissors, snip open the right atrium to allow for exsanguination in subsequent steps.

12 Using a 27.5-gauge needle, slowly inject 5 mL of room temperature 1x PBS to the left ventricle to remove the blood. Make sure the needle is angled towards the aorta to prevent lung inflation. When the fluid exiting the mouse is clear of blood, proceed to the next step.

Note: The volume of PBS can be increased to 10 mL if needed.

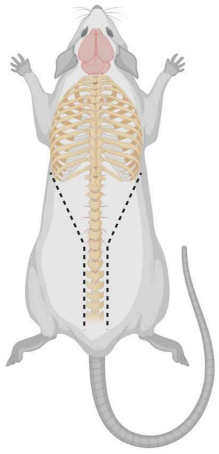
DRG Collection

13 Position the mouse so the dorsal side faces you. Cut and pull back the skin to expose the spine and back muscles.

14 Remove the tail by cutting at its base, using large surgical scissors.

15 Using large surgical scissors, start at the base of the tail and cut up alongside the spine to separate the spine from the ilium. Widen the angle of the scissors and cut until the scissors reach the outer ribcage. Repeat this cut on the other side.

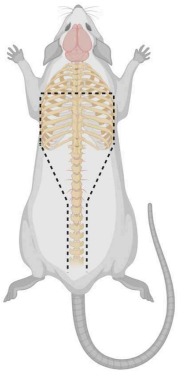
Important: Do not cut the ribs near the spine. The last floating ribs will be used as a land mark in future steps.



Note: Vertebrae are not anatomically accurate in diagram.

16 Using surgical scissors, remove the organs and trim the connective tissues on the ventral side of the spine until only muscle is left.

17 At the outer part of the ribcage, make an upwards cut on both sides. Then, cut across at the top of the ribcage to free the ribs and spine.



Note: Vertebrae are not anatomically accurate in diagram.

18 Using small surgical scissors, trim the muscles around the spine without cutting the floating ribs.

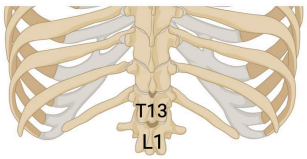
Note: On the ventral side, aggressively trim the muscles until the vertebrae are visible and easy to count.

Note: Leave a small amount of muscle on the dorsal side of the spine to help with orientation and pinning.

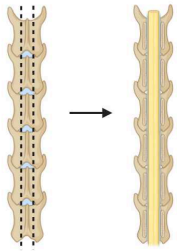
19 Locate the floating ribs attached to the top of T13- the vertebrae directly beneath is L1. To access and isolate the DRGs located at L3-L5: cut between L1 and L2, then cut after L6.

Note: If you only want to isolate the DRG at L4, you can cut before L3 and after L5 to ensure the DRG of interest is not damaged.

Note: The rostral side will have a larger opening for the spinal cord than the caudal side. Be mindful of the orientation and vertebrae collected.

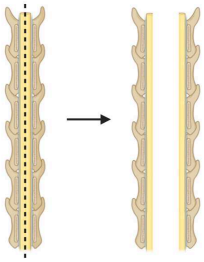


20 Have the ventral side of the spine piece face you. Using small scissors, remove the front plate of the spine by making a vertical cut to the right and left of the midline. The front plate should be around 1 mm thick.



21 Using small surgical scissors, cut the spine fragment in two along the midline, dividing it into equal halves.

Note: Cut as close to the midline as possible to prevent cutting the DRGs.



22 Take one half and place it underneath the dissection microscope. Pin the spine to a black rubber-bottomed petri dish.

23 Count the vertebrae to the DRG you need to isolate. Using fine forceps, remove the meninges around the DRG. Grab the DRG root (not the cell bodies) and pull to release the ganglion.

Note: L4 will be notably larger than the other DRGs.

24 Using small spring scissors, trim the DRG roots as close as possible to the DRG.

25 Collect the DRGs in chilled artificial cerebral spinal fluid on ice.

Note: Have one eppendorf collection tube per sequencing sample so that the DRGs will not have to be pooled in later steps.

Preparation

26 Turn on the GentleMACS Dissociator and wait 30 seconds for the instrument to start up. Click on the favorites folder, then select the program named 4C_nuclei_1.

27 Prepare multiple ice coolers as a workstation for the experiment and put materials and reagents on ice.

Per sample, you need:
- 1 petri dish
- 1 razor blade
- 1 C-tube
- 1 15mL conical (for first filter- don't coat with BSA)
- 1 FACS sorting tube (BSA coated, use for 2nd filtering step and after microbeads)
- 2 30µm cell strainers
- 1 LS separation column

28 Coat the FACS sorting tube with 10% BSA solution: pour in the BSA solution, swirl tubes, then pour out the BSA. The BSA solution can be re-used.

29 Prepare lysis, wash, and resuspension buffers.

Lysis Buffer (2 mL per sample):
- Add 10 µL RNase Inhibitor to 2 mL nuclei extraction buffer

Wash Buffer (10.59 mL per sample):
- Add 1.5 mL nuclei extraction buffer to 9 mL chilled 1x PBS
- Add 40 µL 10% BSA solution
- Add 50 µL RNase Inhibitor

Resuspension Buffer (2 mL per sample)
- Add 10 µL RNase Inhibitor to 1.8 mL chilled 1x PBS
- Add 200 µL 10% BSA

Nuclei Isolation

30 Using a P1000, transfer the DRGs to a petri dish on ice. Using a P200, remove any artificial cerebral spinal fluid that was transferred.

31 Add 200 µL of lysis buffer to DRGs. Using a razor blade, mince the DRGs as small as possible.

Note: If the DRGs are clumped together, nuclei yields will be lower. Mince the DRGs until they are in small, separate pieces.

Note: Work fast during this step, as timing is critical to not lyse the nuclei.

32 Wash the razor blade with 800 µL of lysis buffer over the petri dish.

33 Using a P1000, transfer the DRG pieces and lysis buffer to a chilled C-tube. Wash the petri dish with 0.5 mL of lysis buffer (x2), transfer contents to C-tube

34 Put C-tube on the GentleMACS Dissociator, ensure the correct dissociator slot is selected. Press Okay, then start. Place a cooler over the C-tube. Let the program run for 4 minutes, then abort the program with 1 minute remaining.

35 Pre-wet a 30µm cell strainer by pipetting 1x PBS on the strainer over a waste container, then place the strainer over a 15 mL conical.

36 Wash the plastic blades of the C-tube cap with 1 mL of wash buffer over the C-tube (x2).

37 Transfer the contents of the C-tube to the cell strainer to filter into the 15 mL conical tube.

38 Close the C-tube and gently tap the bottom against the bench to get out any remaining sample and filter contents.

39 Tap the cell strainer over the 15 mL conical and transfer any remaining sample from under the strainer with a pipette to the conical.

40 Centrifuge the 15mL conical at 500G at 4°C for 5 minutes.

41 Using a P1000, remove most of the supernatant. Then, using a P200, remove the rest of the supernatant. Be careful not to disturb the bottom of the tube where the nuclei are pelleted.

42 Add 450 µL of wash buffer to the collection tube and, using a P1000, mix the sample up and down 10 times to resuspend the pellet.

Note: Avoid creating bubbles when mixing

43 Pre-wet a new 30 µm cell strainer with 1x PBS and place over a coated FACS collection tube. Filter the sample.

Note: Remove any excess BSA in the FACS collection tube prior to filtering sample.

44 In a PCR tube, mix 3 µL of the sample with 3 µL trypan blue, then add to a hemocytometer. Check the nuclei count and morphology under a microscope.

45 To count: find big squares. Count all nuclei in 16 squares and write down number. To calculate: Nuclei number x dilution factor (2) x 10. This gives you the total nuclei count in 1 µL. Multiply this number by 450 µL to get the total number of nuclei in your suspension.

46 Add 50 µL anti-nucleus microbeads to sample. Slowly mix up and down with a P1000 2-3 times.

Note: Do not create bubbles when mixing

47 Place the sample upright in the 4°C fridge and let incubate for 15 minutes.

Note: Do not place the sample in the fridge door to avoid temperature fluctuations.

48 Place a LS separation column on the MACS stand. Place a waste collection container under the column.

Note: Ensure the separation column clicks into the stand.

49 3 minutes before the microbead incubation is complete: wash the column with 1 mL wash buffer, 3 times (for a total of 3 mL). Be careful not to let the column dry.

Note: Pipette into the center of the column for each step, as column walls are sticky.

50 Add 2 mL wash buffer to the sample. Slowly mix up and down with a P1000 2-3 times.

Note: Do not create bubbles when mixing

51 Add the sample to the center of the column. Let flow through.

Note: Be careful not to let the column dry.

52 Add 1 mL of wash buffer to the column. Let flow through.

53 Add 1 mL of wash buffer to the sample tube to wash walls, then transfer contents to column. Let flow through.

54 Place the (coated and washed) FACS collection tube under the column then transfer the column and tube to ice.

55 Add 1 mL of resuspension buffer to the center of the column, then quickly add the plunger and plunge until bubbles are seen (do not capture the bubbles).

Note: Avoid flow through of wash buffer without the plunger in this step, as pressure is needed to remove the microbeads from column.

56 Centrifuge the FACS collection tube at 500G at 4°C for 5 minutes.

57 Using a P1000, remove most of the supernatant. Then, using a P200, remove the rest of the supernatant until there is ~50 µL left.

Note: The supernatant can be collected in case the pellet is accidentally disturbed.

58 Resuspend the nuclei pellet in the ~50 µL of remaining supernatant.

59 In a PCR tube, mix 3 µL of the sample with 3 µL of trypan blue then add to a hemocytometer. Check the nuclei count and morphology under a microscope. For loading, you want to target 1000-1500 nuclei per µL.

60 Wash the sample by adding 500 µL of resuspension buffer and mixing up and down with a P1000 3-4 times. Then, centrifuge the sample at 500G at 4°C for 5 minutes.

61 Calculate the final volume needed to achieve 1000-1500 nuclei per µL. Remove the supernatant until this volume is left, then resuspend the nuclei.

Note: The supernatant can be collected in case the pellet is accidentally disturbed.

62 In a PCR tube, mix 1 µL of the sample with 5 µL of trypan blue then add to a hemocytometer. Check the nuclei count and morphology under a microscope.

To count: find big squares. Count all nuclei in 16 squares and write down number. To calculate: Nuclei number x dilution factor (5) x 10. This gives you the total nuclei count in

1 µL. Multiply this number by the volume that was used to resuspend the sample to get the total number of nuclei in your suspension.

63 If the sample quality looks good (intact nuclei with minimal debris at the targeted concentration), proceed to the core for sample loading. The left over 500 µL of resuspension buffer can be given to the core in case dilution of sample in necessary for loading.