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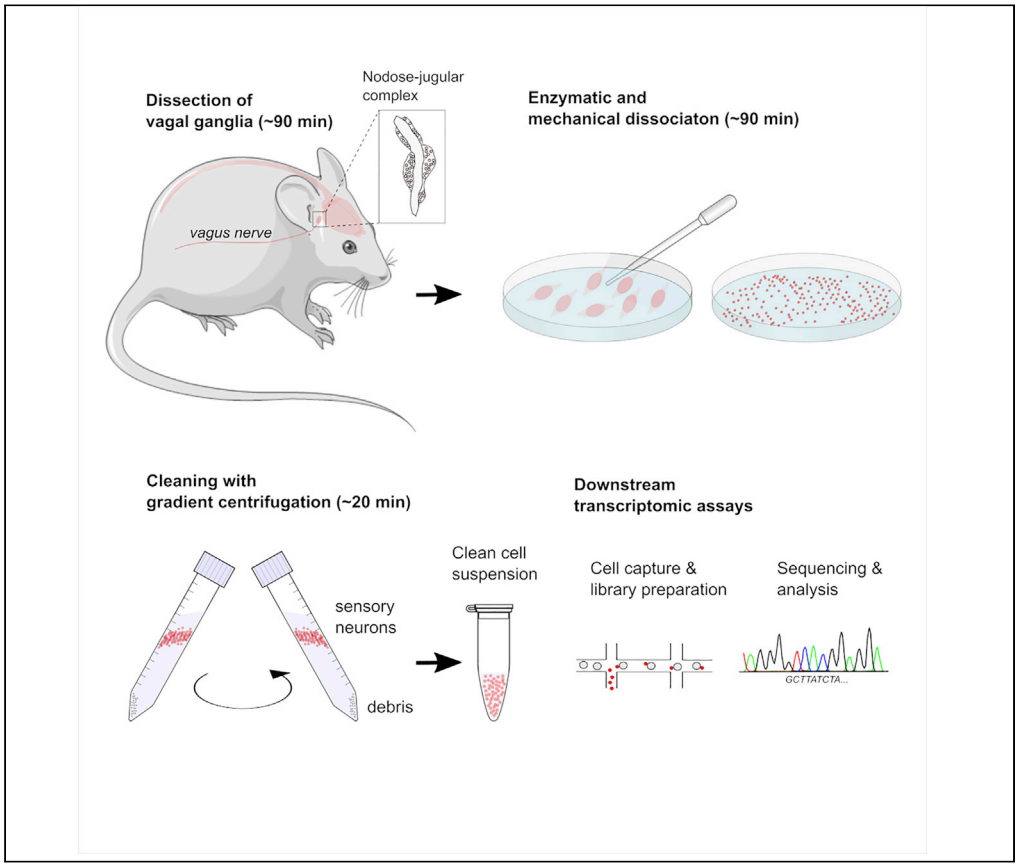
Protocol to Prepare Single-Cell Suspensions from Mouse Vagal Sensory Ganglia for Transcriptomic Studies



Vagal sensory neurons relay viscero- and somatosensory information from within the body and play a key role in maintaining physiological homeostasis. We recently characterized the diversity of vagal sensory neurons in the mouse using a single-cell transcriptomics approach. Here, we provide an in-depth protocol for the extraction of mouse vagal ganglia and the production of high-quality single-cell suspensions from this tissue. This effective protocol can also be applied for use with other peripheral and central neuron populations with few modifications.

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HIGHLIGHTS

An in-depth protocol for the extraction of mouse vagal ganglia

Production of high-quality single-cell suspensions of mouse vagal ganglia

This protocol can also be applied to other peripheral and central neuron populations

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Protocol to Prepare Single-Cell Suspensions from Mouse Vagal Sensory Ganglia for Transcriptomic Studies

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SUMMARY

Vagal sensory neurons relay viscero- and somatosensory information from within the body and play a key role in maintaining physiological homeostasis. We recently characterized the diversity of vagal sensory neurons in the mouse using a single-cell transcriptomics approach. Here, we provide an in-depth protocol for the extraction of mouse vagal ganglia and the production of high-quality single-cell suspensions from this tissue. This effective protocol can also be applied for use with other peripheral and central neuron populations with few modifications. For complete details on the use and execution of this protocol, please refer to Kupari et al. (2019).

BEFORE YOU BEGIN

⚠ CRITICAL: To ensure success, it is critical to move quickly between the steps. Have all essential reagents, equipment, and workstations prepared before starting the protocol.

Prepare the Following

1. Pasteur pipettes with decreasing outlet diameter
   a. Polish disposable glass Pasteur pipettes with a Bunsen burner to produce pipettes with gradually decreasing outer diameter: ~70% to 15% of the original opening.
   b. Coat the inner and outer pipette surfaces for minimum 10 minutes by aspirating ~2 ml 0.5% BSA in PBS and immersing the pipette in 1 ml of the same solution.
2. Set cell incubator to 37°C.
3. Cool a centrifuge for 15 ml tubes to 4°C.
4. Dissection tools
   a. For the extraction of ganglia have ready a pair of surgical scissors (Figure 1A), a pair of Standard Pattern Forceps with curved and serrated tips (Figure 1B), and two pairs of fine Dumont Forceps with 45° angled tips. (Figure 1C). Clean the tools with 70% ethanol before use.
   b. Prepare the stereo microscope and a Styrofoam (or alternative) platform that can be used to mount the tissue with needles.
   c. For the microdissection of ganglia, have ready a pair of Vannas spring scissors (Figure 1D), another pair of fine Dumont Forceps (45°) (Figure 1C) and a stereo microscope.

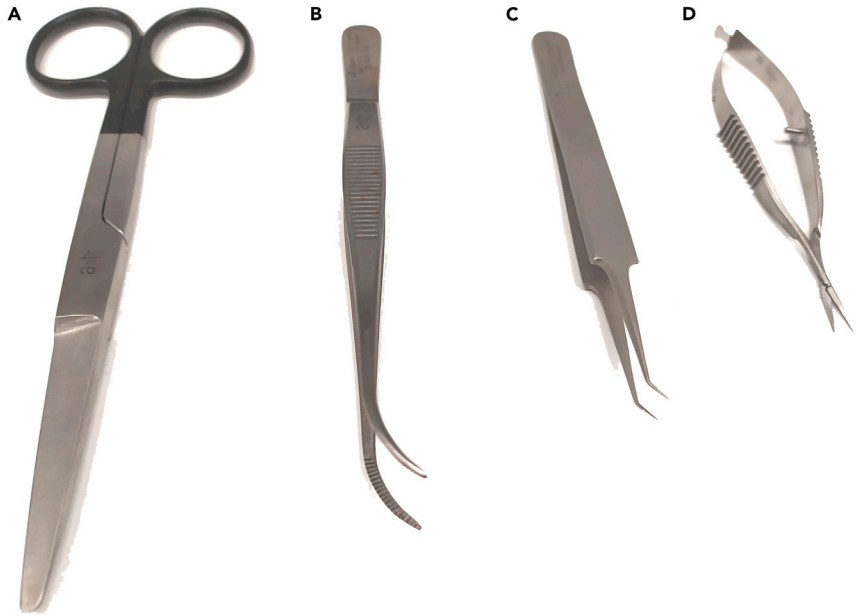

Figure 1. Dissection Tools Required for Ganglia Extraction and Microdissection

(A–D) (A) Surgical scissors, (B) standard pattern forceps with curved and serrated tips, (C) Fine Dumont forceps with angled tips, and (D) fine Vannas spring scissors.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
TrypLE™ Express	Life Technologies	Cat#12605-010
Papain	Worthington Biochemical	Cat#LK003172
Collagenase/Dispase	Roche	Cat#11097113001
Neurobasal-A	Gibco	Cat#10888
L-Glutamine	Gibco	Cat#25030-123
B27	Gibco	Cat#17504-044
Penicillin/Streptomycin	Sigma	Cat#P4458
Optiprep Density Gradient Medium	Sigma	Cat#D1556
DNase I	Worthington Biochemical	Cat#LK003172
Experimental Models: Organisms/Strains		
Mouse: C57BL/6JRj	JANVIER LABS	C57BL/6JRj
Mouse: Slc17a6$^{tm2(cre)Lowl}$	The Jackson Laboratory	Stock no: 016963
Mouse: B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J	The Jackson Laboratory	Stock no: 007914
Other	AgnTho's	Cat# 14130-17
Scissors, ToughCut, Straight Sharp-Blunt, 17,5 cm	AgnTho's	Cat# 11251-35
Dumont #5/45 Forceps Standard Tips, Dumoxel, 11 cm	AgnTho's	Cat# 11001-12

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Standard Pattern Forceps Curved, 2,5 mm Tip, 12 cm	AgnTho's	Cat# 15000-03
	Sarstedt	Cat# NC9531248
Conical tube 15ml	Corning	Cat#352340
40 µm Cell strainer	Corning	Cat#353801
35 mm Cell culture dish	Sigma-Aldrich	Cat#CLS7095B5X
Zeiss Stemi 2000 (stereo microscope)	Zeiss	n/a
CL 6000 LED (light source for the microscope)	Zeiss	n/a
Heraeus Megafuge 1.0 (centrifuge)	Heraeus	n/a
Scanlaf Mars (biosafety cabinet)	Scanlaf	n/a

MATERIALS AND EQUIPMENT

Artificial Cerebrospinal Fluid (ACSF) 4x Stock Solution

Stock solution can be stored for 1 month at 4°C

20.34g NaCl (348 mM)
0.75g KCl (10 mM)
0.6g NaH_2PO_4 (5 mM)
8.74g $NaHCO_3$ (104 mM)
102.7g Sucrose (300 mM)
27.4g Glucose (80mM)

Add all components to a glass bottle. Fill to 1L with ddH_2O. Stir to dissolve.

ACSF Working Solution

Prepare fresh and oxygenate for 20–30 min with carbogen (5% CO_2/95% O_2) gas on ice before use

25 ml of 4x ACSF (from 4°C)
74.55 ml of ddH_2O
400 µl of 1M $MgSO_4$
50 µl of 1M $CaCl_2$

Add ddH_2O to 4x ACSF and stir to combine. While stirring, add $MgSO_4$ and $CaCl_2$.

Worthington Kit (Papain + DNase Solution)

Prepare fresh

Add 4 ml of ACSF to the Papain bottle (25 U/ml) and 0.5 ml to the DNase bottle (2000 U/ml) and incubate 30 min at 37°C

Dissociation Working Solution

Incubate the solution at 37°C for >10 min before use

400 µl Tryp-LE
100 µl Collagenase/Dispase (20 mg/ml in ddH2O)
1900 µl Papain solution
100 µl DNAse I

Density Gradient Solution

450 µl ACSF
450 µl Neurobasal-A
100 µl Optiprep™ Density Gradient Medium

STEP-BY-STEP METHOD DETAILS

Sacrifice Animals

Timing: ~30 min

Vagal ganglia can be extracted from up to 10 animals in an hour by an experienced worker.

Note: Please see the Key Resources Table for the descriptions of the mouse models for which we have confirmed this protocol.

1. Sacrifice the animals using CO_2 inhalation, decapitate with sturdy surgical scissors, and rinse away excess blood in cold ACSF.
2. Transfer the heads to fresh cold ACSF and keep them on ice until ganglia extraction.

Note: We suggest using young animals (5 weeks of age). In our experience the need for more enzymatic and mechanical dissociation to disrupt the ganglia increases as the animals get older (this was evident at the age of 8 weeks). This effect is likely due to toughening of the connective tissue structures in the ganglia. If low cell yields are seen when using older animals, it might be necessary to use more animals to compensate for the lower output; however, this added stress can result in diminishing numbers of viable cells in the suspension. One should also keep in mind that with added stress, the cell survival could be biased towards certain cell populations. See the troubleshooting section for further advice.

Extract Vagal Sensory Ganglia

Timing: ~90 min

This section describes the extraction of vagal ganglia from freshly sacrificed animals.

Note: A person with some prior experience in microdissection should be able to learn the described protocol with a few tries. After the initial learning period it should be realistic to extract both ganglia from a single animal in 10 minutes or less.

⚠ CRITICAL: We suggest keeping the dissection time under two hours. In our experience the sensory neurons remain viable inside the ganglia for this time if the tissues are kept in cold ACSF; however, as more time passes the cell death processes within the first dissected ganglia will begin and hinder the collection of viable cells.

Optional: In highly vascularized tissue it is recommended to perform transcardial perfusion with cold ACSF. The clearing of blood and infiltrating with ACSF can make it easier to identify the ganglia when dissecting and can have a positive effect on cell viability in cases where the dissection takes a long time (>2h).

3. Cut open the scalp through the midline and peel skin to the sides.
4. Using surgical scissors, make a lateral cut on both sides of the skull and remove the top of the skull to expose the brain (Figure 2A).
5. Starting rostrally, use the standard pattern forceps to gently lift out the brain.

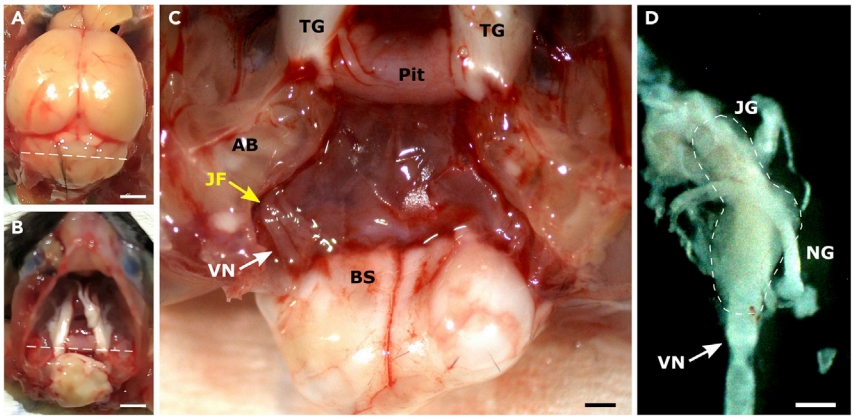

Figure 2. Extraction of Vagal Ganglia from a Mouse

(A) Exposed brain showing the approximate level of dissection (dashed line).

(B) View of the empty brain cavity. Dashed line shows the dissection level of trigeminal central roots.

(C) A closer view of the brain cavity showing important landmarks for dissection. TG = trigeminal ganglion, Pit = pituitary, TB = temporal bone, JF = jugular foramen, VN = vagus nerve, BS = brainstem.

(D) An extracted vagal ganglion complex. The dashed line approximates the area of the neuron tissue (grey opaque appearance). The lower voluminous part consists mostly of nodose neurons and the upper part of jugular neurons. The vagus nerve can be seen running through the ganglionic complex. Scale bars: 2 mm (A, B, C); 500 μm (D).

6. Cut the brain at the cerebellum and leave the brainstem in place (Figure 2A).
7. Discard the forebrain.
8. Pin the head dorsal side up on a platform and place it under the stereo microscope.
9. Use the Dumont forceps to cut the central roots of the trigeminal ganglia but leave the brainstem in place (Figure 2B). Gently pull back the brainstem until you can observe the vagus nerves (along with the glossopharyngeal and accessory nerves) coming out from the jugular foramina on both sides and connect to the brainstem (Figure 2C).

⚠ CRITICAL: Be careful not to pull on the brainstem too strongly as this could cause the vagus nerves to break. The nerves serve as a visual guide to the jugular foramen; therefore, it is very important to keep the nerves intact.

10. Focusing on one side, and being careful not to damage the nerve bundle, use the Dumont forceps to gently scrape the connective tissue adhesions around the jugular foramen so that the nerve can move more freely. Using the standard pattern forceps, place one tip close to the jugular foramen on the temporal bone and the other tip on the outside the skull. Grab the temporal bone and apply lateral pressure, twisting outwards until it starts separating from the occipital bone.
11. At the same time, using Dumont forceps, continue scraping away the connective tissue adhesions around the vagus nerve where it enters the jugular foramen. Continue to apply lateral pressure on the temporal bone as needed.

Note: It is acceptable if the temporal bone breaks during the procedure; however, this can make the extraction of ganglia more difficult. In this case, use the standard pattern forceps to gently remove the broken bone parts, while cutting any connective tissue adhesions to the vagus nerve with the Dumont forceps.

12. Using two sets of Dumont forceps, gently hold on to the vagus nerve bundle while continuing to remove connective tissue adhesions around the jugular foramen.

13. The vagal ganglion complex appears as a small translucent bulge quite close to the location of the former opening of the jugular foramen. It is commonly attached by adhesions to the side of the temporal bone inside the jugular foramen.
14. Using the Dumont forceps, grab the vagus nerve below the ganglion and gently pull to remove the ganglion.

⚠ CRITICAL: The sympathetic superior cervical ganglion is located around the same region in close connection with the carotid artery; do not mistake this ganglion as the vagal ganglion complex.

15. Place the isolated ganglion on a small petri dish with cold ACSF and observe it under the microscope to verify a successful extraction. You should see a small roundish gray mass of tissue connected to the stumps of the vagus nerve (see Figure 2D).
16. Proceed to extract the ganglion on the remaining side.

⚠ CRITICAL: While dissecting, remember to keep the already extracted ganglia in cold ACSF on ice until you are finished working. This will help minimize possible transcriptome changes and maintain viability of the cells.

Microdissect and Clean Ganglia

Timing: ~20 min

The step describes the removal of the remaining dura mater as well as nerve tissue from the ganglia, which is necessary to obtain a clean cell suspension.

17. Transfer ganglia into a 35 mm culture dish containing ACSF (4°C; work on ice).
18. Using the spring scissors and fine Dumont forceps, remove the nerve roots as completely as possible from the ganglia. This will reduce time to dissociate the tissue and minimize the amount of unwanted debris, increasing the quality and specificity of the cell suspension.
19. Transfer the ganglia to a 35 mm dish with 2.5 ml of warm (37°C) dissociation working solution.

Dissociate Ganglia

Timing: ~90 min

This step describes the dissociation of ganglia tissue by applying mechanical force as well as enzymatic treatment. Incubate for 20 minutes at 37°C in the dissociation working solution

20. Carefully pipette 10 times up and down using the Pasteur pipette with the widest bore; use the Dumont forceps to carefully tear open the ganglia
21. Incubate for 20 minutes at 37°C
22. Carefully pipette 10 times up and down using the Pasteur pipette with the second widest bore.
23. Incubate for 20 minutes at 37°C
24. Carefully pipette 10 times up and down with third Pasteur pipette; if bigger intact pieces remain use the forceps to carefully tear the tissue open
25. Incubate for 20–30 minutes at 37°C
26. Carefully pipette 10 times up and down with the fourth Pasteur pipette

⚠ CRITICAL: No large intact pieces of tissue should remain. If it is necessary to incubate longer, add Tryp-LE and repeat the last step; however, the incubation time for ganglia should not exceed 120 min as this can lead to a decrease in cell viability.

Note: Enzyme concentration, composition, and treatment duration may vary depending on different factors (e.g. age of animals or differences in enzyme activity). Thus, embryonic neuronal tissue requires shorter (~30min) digestion with papain, a mild protease. Mechanical separation is necessary for most tissues to prevent a prolonged enzymatic treatment which reduces cell viability. If possible, we recommend examining the dissociation progress regularly with a microscope.

Enrich Neurons

Timing: ~90 min

Here, we describe how to separate the neuronal fraction from the smaller non-neuronal cells and connective tissue debris. Using a cell strainer will remove bigger pieces of tissue that might remain in the suspension.

⚠ CRITICAL: It is important is to choose a suitable strainer depending on the cell size of interest. We chose a 40 μm strainer because in mouse the diameter of the biggest vagal sensory neurons is below this limit. The concentration of Optiprep can be adjusted as necessary (higher concentration will result in better debris removal but might lead to loss of cells).

Note: Remember to cool down the centrifuge chamber (4°C) in advance.

27. Stop the enzymatic reaction by transferring the cell suspension onto ice
28. Prepare a 15 ml tube containing 2 ml of cold ACSF and 1 ml of cold 1xPBS
29. Cover the tube with a 40 μm cell strainer and filter the cell suspension by carefully pipetting the suspension on top of the strainer mesh
30. Centrifuge the cell suspension at 100 g for 4 min at 4°C
31. During the centrifugation, prepare a density gradient in a 15 ml conical bottom tube. Following centrifugation, remove the supernatant from the sample but be careful not to disrupt the cell pellet
32. Resuspend the pellet in 450 μl ACSF and 450 μl Neurobasal-A
33. Very carefully transfer the suspension with a Pasteur pipette on top of the gradient by tilting the tube and pipetting along the side; at the end, two separate phases should be visible
34. Centrifuge at 700 g for 10 min at 4°C

Note: The neuronal cells will sink to the bottom. Most of the debris and smaller non-neuronal cells will remain at the border between the two phases of the gradient.

35. Remove most of the supernatant (leave the last 100ul) but be careful not to remove the cell pellet
36. Add 10 μl DNase I to avoid cell aggregation and gently resuspend the pellet with a pipette
37. Count the cells using a hemocytometer and adjust the volume to achieve the desired concentration; in our hands the final yield has routinely been around $1x10^3$ neurons per animal
38. Keep the cell suspension on ice and move to downstream steps without delay

EXPECTED OUTCOMES

A successful extraction process should routinely yield $1x10^3$ or more neurons per animal (from two ganglia) when counted with a hemocytometer. Good quality neuronal cell bodies appear roundish and bright under the microscope and are clearly distinguishable from the satellite glial cells by their bigger size (Figure 3). Trypan blue can be used to assess viability when counting the cells. A good quality suspension should have >70% viable cells.

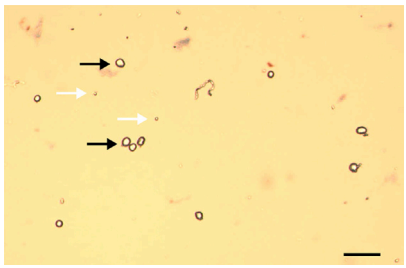

Figure 3. A Microscope View of a Vagal Ganglion Cell Suspension
Sensory neurons are identifiable by their roundish and large size (black arrows point to examples) from non-neuronal cells that are significantly smaller (white arrows). Scale bar: 100 μm.

LIMITATIONS

The protocol described here has been used in slightly modified versions to isolate cells from various neural tissues, such as sympathetic ganglia (Furlan et al., 2016), spinal cord (Häring et al., 2018), dorsal root ganglia (Zeisel et al., 2018), and now also vagal ganglia (Kupari et al., 2019). This broad spectrum of central and peripheral nervous tissue indicates that the protocol is useful in dissociating neural tissues in general. Age of the animals can be a major problem in obtaining a high-quality cell suspension. Specifically, isolating sensitive cells such as spinal cord neurons becomes increasingly problematic after only 3-4 weeks of age. Ganglionic neurons are less sensitive; however, cell isolation does become more difficult after 2-3 months of age.

TROUBLESHOOTING

Problem

Dissociation of ganglia takes too long.

Potential Solution

If this problem occurs, it is likely that the animals are too old, or the enzymatic digestion is not effective enough.

We advise using younger animals (5 weeks is preferred) for easier dissociation. Use a fresh batch of enzymes or adjust to higher concentrations. Limit the time enzymes are kept at 37°C before the dissociation to maximize activity.

Problem

Large amounts of debris in the suspension.

Potential Solution

This problem could originate from incomplete enzymatic and/or mechanical disruption, poor initial removal of connective/nerve tissue, filtering through the cell strainer with too much pressure (forcing debris through) or using an inappropriate Optiprep gradient.

Adjust the trituration steps to add more mechanical disruption. Also, check the openings of the Pasteur pipettes and use a slightly narrower bore. Try to be more thorough in the cleaning of unwanted connective tissue from the ganglia. Pipette the suspension gently on the cell strainer mesh to keep debris from seeping through. The mesh size can be adjusted for optimized yield/debris ratio. We recommend starting with a 40 μm mesh size and if possible, decrease to 20–30 μm. If needed, increase the Optiprep concentration to obtain a cleaner suspension. Large pieces of debris can be dense enough to end up in the cellular fraction so adjust the digestion protocol or filtering accordingly. Additionally, debris removal can be enhanced by different ways of cell sorting such as MACS (magnetic activated cell sorting) or FACS (fluorescence-activated cell sorting). MACS uses magnetic beads labeled with antibodies able to bind certain cell surface proteins. Thus, cell populations can be isolated using a magnetic field. If cells of interest can be labeled fluorescently (antibodies or transgenic proteins) FACS is another good option. FACS however, requires a certain amount of

cleanliness regarding the cell suspension; therefore, it should be used as an additional step after the density gradient.

Problem

Low cell yield in suspension.

Note: The authors routinely collected $1x10^3$ neurons/animal.

Potential Solution

Some potential reasons for this problem: too little starting material, enzymatic dissociation took too long and damaged the cells, dissociation from connective tissue was incomplete and many cells were lost with the debris, or trituration was too aggressive leading to cell death.

If the cell type of interest comprises only a small number of cells, try increasing the number of animals used for the suspensions. Optimize the enzymatic dissociation; namely, use higher enzyme concentrations if the digestion takes too long or lower if cells are sensitive. If feasible, use young animals. Ganglionic tissue from young animals is more easily dissociated and results in better final cell yields. Work to minimize the time spent between sacrificing the animals and beginning the dissociation protocol. Mechanical disruption of the tissue will also damage cells, so work to optimize the Pasteur pipette openings for your tissue and triturate with the lowest force required to progress in the dissociation. Even though cells are flexible when isolated, choose a cell strainer with mesh size that is not too small. In general, we recommend during the optimization experiments to confirm cell viability regularly during the procedure (e.g. after digestion and after density gradient).

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AUTHOR CONTRIBUTIONS

Conceptualization, M.H. and J.K.; Methodology, M.H. and J.K.; Investigation, M.H., J.K., and M.F.; Writing, M.H., J.K., and M.F.; Supervision, M.H. and J.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Furlan, A., La Manno, G., Lübke, M., Häring, M., Abdo, H., Hochgerner, H., Kupari, J., Usoskin, D., Airaksinen, M.S., Oliver, G., et al. (2016). Visceral motor neuron diversity delineates a cellular basis for nipple- and pilo-erection muscle control. Nat. Neurosci. *19*, 1331–1340.

Häring, M., Zeisel, A., Hochgerner, H., Rinwa, P., Jakobsson, J.E.T., Lönnerberg, P., La Manno, G., Sharma, N., Borgius, L., Kiehn, O., et al. (2018). Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. Nat. Neurosci. *21*, 869–880.

Kupari, J., Häring, M., Agirre, E., Castelo-Branco, G., and Ernfors, P. (2019). An Atlas of Vagal Sensory Neurons and Their Molecular Specialization. Cell Rep *27*, 2508–2523.e4.

Zeisel, A., Hochgerner, H., Lönnerberg, P., Johnsson, A., Memic, F., van der Zwan, J., Häring, M., Braun, E., Borm, L.E., La Manno, G., et al. (2018). Molecular Architecture of the Mouse Nervous System. Cell *174*, 999–1014.e22.