Protocol for pressure-clamped patch-clamp recording at the node of Ranvier of rat myelinated nerves

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Protocol for pressure-clamped patch-clamp recording at the node of Ranvier of rat myelinated nerves

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SUMMARY
The patch-clamp recording technique is indispensable for studying ion channel functions of cells but is challenging to apply to the node of Ranvier, a key site where action potentials are conducted along myelinated nerves. We have developed a pressure-clamped patch-clamp recording method applying to the node of Ranvier of rat myelinated nerves. The step-by-step protocol described here allows researchers to apply this approach to study mechanisms underlying saltatory conduction and information processing in myelinated nerves of mammals. For complete information on the generation and use of this protocol, please refer to Kanda et al. (2019).

BEFORE YOU BEGIN

© Timing: 30 min

Prepare all solutions listed in the Materials and Equipment section and prepare the equipment setup for patch-clamp recording (Figure 1) before starting this protocol.

Note: The high-speed pressure-clamp (HSPC) device is connected to the holder of the patch-clamp recording pipette to allow a fine control of the pressures within the patch-clamp recording pipette (Figure 1).

1. Prepare 2 mL ice-cold Leibovitz’s L-15 (L-15) medium in a 35 × 10-mm petri dish.
2. Oxygenate 400 mL of the Krebs bath solution with a mixture of 5% CO2 and 95% O2 before and during the experiment.
3. Pull recording pipettes that have pipette resistances ranging from 8 to 10 MΩ after filling approximately 15 μL recording electrode internal solution.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Isoflurane | Vet One | Cat#V1-502017 |
| NaCl | MilliporeSigma | Cat#S9625 |

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## MATERIALS AND EQUIPMENT

**Krebs bath solution (pH 7.35, 324 mOsm), 1,000 mL**

| Reagent               | Final concentration (mM) | Amount   |
|-----------------------|--------------------------|----------|
| NaCl                  | 117                      | 6.84 g   |
| KCl                   | 3.5                      | 0.26 g   |
| CaCl₂                 | 2.5                      | 0.36 g   |
| MgCl₂                 | 1.2                      | 0.24 g   |
| NaH₂PO₄               | 1.2                      | 0.19 g   |
| NaHCO₃                | 25                       | 2.1 g    |

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Alternatives: Alternative recording chambers are commercially available, for example, the Series 20 chamber, Warner Instruments, [www.warneronline.com](http://www.warneronline.com)
**Note:** The pH is adjusted to pH 7.35 with HCl.

**Note:** The osmolality is adjusted to 324 mOsm with sucrose.

**Note:** The Krebs bath solution is stored at 4 °C before it is used in experiments. The Krebs bath solution should be newly prepared after being stored in the refrigerator for 5 days.

**Note:** The Krebs bath solution is saturated by a mixture of 95% O₂ plus 5% of CO₂ at the room temperature of 24 °C when it is used during recordings.

**Note:** The pH is adjusted to pH 7.35 with KOH.

**Note:** The osmolality is adjusted to 330 mOsm with sucrose.

**Note:** Prepare the recording electrode internal solution on ice, aliquot it into 1-mL stock solution in 1.5-mL micro-centrifuge tubes, and store it in −20 °C freezer before using the solution. The recording electrode internal solution should be newly prepared after being stored in the freezer for one month.

**Note:** Prepare the enzyme solution on ice, aliquot it into 150 μL stock solution in 0.5-mL micro-centrifuge tubes, and store all aliquots at −20 °C.
Note: The enzyme stock solution should be discarded after being stored in the freezer for 1 month.

STEP-BY-STEP METHOD DETAILS

Surgery and nerve preparation

© Timing: 30 min

Note: Add 2 mL of ice-cold L-15 medium in a 35 × 10-mm petri dish.

1. Euthanize the rat with an overdose of isoflurane followed by decapitation with a pair of surgical scissors.
2. Quickly remove the nerves to be studied from rats.
   a. Infraorbital nerves (IONs) dissection
      i. Make an incision in the middle of head skin by a pair of surgical scissors.
      ii. Open the skulls with a pair of luer bone rongeur.
      iii. After exposing cortex, cut the brainstem at the transverse cerebral fissure by a pair of fine forceps, and remove the cortex to expose trigeminal ganglions (TGs) underneath the midbrain.
      iv. Open the round foramen and infraorbital foramen by a pair of luer bone rongeur forceps to expose infraorbital branches of the trigeminal nerves (infraorbital nerve, IONs) with TG (Figure 2B).
v. Carefully cut open the connective tissues that surround the IONs by using a micro scissors.
vi. Gently dissect out and harvest the IONs together with their TGs (Figure 2D).

b. Ventral nerves, spinal nerve, and sciatic nerves dissection
i. Remove the spinae muscles by using a pair of luer bone rongeur forceps.
ii. Open the spine through the Th1 to S1 levels and expose the spinal cord.
iii. Identify a L5 DRG and follow the L5 peripheral nerve down to the knee level (Figure 2C).
iv. Harvest the complex of the sciatic, spinal, and ventral nerves from the rat (Figure 2E).

△ CRITICAL: We recommend to keep nerve bundles moisturized by periodically applying drops of ice-cold L-15 medium on the nerves during the aforementioned nerve dissection procedures. During harvesting of the nerve bundles, avoid to stretch the nerve fibers since a strong stretch may lead to a mechanical damage of nerve fibers to potentially cause acute nerve demyelination.

3. Place the nerves into a 35 × 10-mm petri dish that contains 2 mL ice-cold L-15 medium and keep the petri dish on ice.

Note: At this point, when kept in the ice-cold (4°C) L-15 medium, the nerve preparations can be maintained healthy for up to 10 h.

4. Under a stereoscopic microscope, carefully peel off the connective tissues including blood vessels, fibrofatty tissue, and epineurium on the surface of the nerve bundles with a pair of fine forceps.
5. Add 1,350 μL of oxygenated Krebs bath solution into the recording chamber.

6. Affix one nerve preparation in a recording chamber with a tissue anchor and submerge it in the Krebs solution at the room temperature of 24°C.

7. Mount the recording chamber on the stage of the microscope, and adjust the nerve bundle orientation so that it is oriented perpendicular to the tip of the patch-clamp recording pipette.

Optional: If the conduction velocity of a single myelinated fiber is going to be measured, at this point, one can aspirate a peripheral cutting end of the nerve bundle into a suction electrode, and electrical stimulation can later be delivered through the suction electrode to evoke nerve impulses to allow to determine nerve conduction velocity.

8. Add 150 μL of the dispase-collagenase enzyme solution directly into the recording chamber and mix the enzymes with the Krebs solution well by pipetting up and down. Incubate the nerve bundle with the enzyme solution at the room temperature of 24°C for 5 min. The final concentrations of each enzyme are 0.07% in the Krebs solution.

9. Wash off the enzyme solution by continuously perfusing the nerve preparation with the oxygenated Krebs bath solution at 2 mL/min.

Node of Ranvier identification

© Timing: 5 min

Identify a healthy node of Ranvier for pressure-clamped patch-clamp recordings (Figure 3).

△ CRITICAL: A healthy myelinated nerve fiber has a narrow space between myelin sheaths at the node of Ranvier (Figure 3A) and we recommend to apply patch-clamp recordings to the healthy nodes of Ranvier in experiments. If a larger gap between myelin sheaths is shown at a node of Ranvier of a nerve fiber, it indicates that some degree of nerve damage may have occurred during nerve preparation (Figure 3B). If many fibers have this type of

Figure 3. Identifying a healthy node of Ranvier for pressure-clamped patch-clamp recordings

(A) Image shows a segment of an infraorbital nerve bundle viewed under a 40× objective. Arrows indicate the healthy nodes that shows very small gap at each node.

(B) Image shows a segment of a different infraorbital nerve bundle viewed under a 40× objective. Arrowheads indicate two unhealthy nodes that show a large gap at each node. Scale bar, 10 μm.
morphological changes at their nodes of Ranvier, we recommend to discard the nerve bundle and try to obtain a new and healthy nerve bundle.

Pressure-clamped patch-clamp recordings at nodes of Ranvier

Timing: 10 min

Procedures of the pressure-clamped patch-clamp recording at the node of Ranvier are shown in Figure 4.

Note: Prepare the patch-clamp recording pipettes that have electrode resistances ranging from 8 to 10 MΩ after filling the pipette with recording electrode internal solution.

10. Choose a healthy node of Ranvier in the nerve bundle (Figure 4A).
11. Fill a pipette with recording electrode internal solution by using a syringe that is attached with a 0.22-µm syringe filter.
12. Enter the pipette into the bath solution and adjust electrode offset at the holding potential of 0 mV.
13. Apply a high positive pressure of 200 mmHg into the recording pipette by using the HSPC and bring the pipette closer to the node of Ranvier within 5–10 µm (Figure 4B).

Note: It is difficult to insert the recording pipette tip into the nerve fiber layer if nerve fibers are still covered by some connective tissues. Return to step 4 and peel off connective tissues carefully with a pair of fine forceps.

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**Figure 4. Procedures of pressure-clamped patch-clamp recording at the node of Ranvier**

(A) Identify a healthy node for the pressure-clamped patch-clamp recording.
(B) The tip of the recording pipette approaches the node of Ranvier. The intra-pipette pressure is initially 200 mmHg and adjusted to 80–100 mmHg when the recording pipette tip reach the perineurium tissue at the node of Ranvier.
(C) The tip of the pipette penetrates the perineurium that surrounds the node of Ranvier. At this point, reduce the intra-pipette pressure to 5 mmHg.
(D) Adjust the position of the recording pipette tip and apply a negative pressure (~−2 to −10 mmHg) in the recording pipette to obtain gigahm seal. The asterisk in the image indicates the tip of the recording pipette.
(E) An example of membrane responses when the tip of the recording pipette approaches to Schwann cell membranes (middle panel) or to nodal axon membranes (right panel). The left panel shows the baseline holding current before the recording pipette tip approaches membranes. Scale bar, 10 µm.
△ CRITICAL: Pay attention and make sure that there is no air leakage in the pressure-clamped patch-clamp recording system and that the positive pressure is well maintained in the recording pipette. The pressure should stay stable, and there is a leak if the pressure drops. Also make sure that the tip of the recording pipette is not blocked by small particles that may be present in the recording electrode internal solution.

14. Reduce the positive pressure to 80–100 mmHg and advance the recording pipette tip to approach the node of Ranvier horizontally from one side of the node of Ranvier.

15. Slowly penetrate the perineurium that wraps around the nodal axon (Figure 4C).

**Note:** It may take several attempts with forward-backward-forward movement of the recording pipette tip to penetrate the perineurium at the node of Ranvier. After the pipette tip successfully penetrates the perineurium, one can see that there is an immediate expansion in the space between myelin and perineurium. This change may be due to the flowing of the recording electrode internal solution into the space.

16. Quickly reduce the positive pressure to 5 mmHg in the recording pipette using the high-speed pressure-clamp device and then change the command holding potential of the recording electrode to $-60 \text{ mV}$.

17. Slightly advance the tip of the recording pipette to approach and make a contact with the nodal axon (Figure 4D).

△ CRITICAL: Since axonal membrane is unable to be clearly differentiated from perineural tissues and Schwann cell membranes under the IR-DIC microscope, accessing axonal membranes with the recording pipette tip is mainly judged by the reduction of seal-test currents. Once touching axonal membranes, one can see a strong current oscillation accompanied with the reduction of seal-test currents in seal-test traces (Figure 4E). On the other hand, if the recording pipette tip touches Schwann cell membranes, current oscillation is usually very weak or absent, although seal-test currents become reduced as well (Figure 4E). After the penetration of perineurium, the tip of the recording pipette should be very close to the nodal axon (Figure 4E). To contact the nodal axon membranes, one only needs to slightly adjust the position of the recording pipette tip up and down in less than 1 µm until seeing the aforementioned changes of seal-test currents.

18. Gently push (1–2 µm) against the nodal axon with the tip of the recording electrode to allow the resistance of the seal test to increase to approximately 15 MΩ. This allows the recording pipette tip to optimally contact nodal membranes.

19. Gradually reduce the positive pressures in the recording pipette to negative pressures of $-2$ to $-10 \text{ mmHg}$ in approximately 10 s.

20. Wait for 30 s to 3 min until the formation of gigaohm seals (usually $>5 \text{ G}\Omega$) between the recording pipette and the axonal membranes at the node of Ranvier.

21. Compensate the pipette capacitance before rupturing nodal axon membranes to form the whole-cell recording configuration.

**Note:** At this point, one may perform single-channel recordings from the nodal axon membranes using the cell-attached recording configuration.

22. Rupture the nodal axon membranes by applying a negative pressure of $-30 \text{ mmHg}$ combined with a train of short electrical pulses ($\pm 200 \text{ mV}$, 20 ms each pulses) delivered to the nodal of Ranvier via the patch-clamp recording pipette.

**Note:** Axonal membranes at the node of Ranvier usually can be ruptured by the short electrical pulses in less than 100 pulses leading to the whole-cell patch-clamp recording configuration.
On the other hand, Schwann cells seem to have tougher cell membranes and require more than 300 times of electrical pulses to rapture their membranes to form the whole-cell patch-clamp recording configuration.

23. After establishing the whole-cell patch-clamp recording configuration, quickly adjust the negative pressure from −30 mmHg to −5 mmHg. Keeping the lower negative pressure of −5 mmHg in the recording pipette can prevent nodal axon membranes from reseal at the recording pipette tip during experiments.

24. Stable recordings usually can last for more than 1 h.

**Note:** The actual holding potential under the whole-cell patch-clamp recording configuration is −72 mV after the command holding potential of −60 mV is corrected for the junction potential of 12 mV.

⚠️ CRITICAL: If recording electrode access resistance is increased over time after establishing the whole-cell patch-clamp recording configuration, it could be due to the gradual membrane reseal at the tip of the recording pipette. Return to step 22 and rupture the membrane again.

**EXPECTED OUTCOMES**

We have described above in detail the experimental steps of applying pressure-clamped patch-clamp recording technique to the axon at a single node of Ranvier in rat myelinated fiber. After establishing the whole-cell patch-clamp configuration on the axonal membrane at the node of Ranvier, one can also trace and characterize the morphological properties of the myelinated axon including diameters and lengths of nodes of Ranvier and inter-nodal distances if recording electrode internal solution also contains the fluorescent dye Alexa 555 at the concentration of 85 μM (Figure 5A).

A high-quality recording from nodal axon membranes should show very negative resting potential at approximately −82 mV at the node of Ranvier. Input resistance is approximately 40 MΩ when recorded at the node of Ranvier of Aβ fibers of ION and approximately 35 MΩ when recorded at the node of Ranvier of Aα motor nerve fibers.

Under the voltage-clamp configuration, we have detected transient inward currents mediated by the voltage-gated Na⁺ channels and large non-inactivating outward currents mediated by TREK-1 and TRAAK channels in responses to depolarizing voltage steps (Figure 5B) (Kanda et al., 2019). Under the current-clamp configuration, we can elicit action potential firing at the node of Ranvier following the injection of depolarizing currents (Figure 5C) or following the electrical stimulation of the peripheral sites of the nerve bundle by a suction stimulation electrode (Kanda et al., 2019).

Some functional characteristics of nodal axon membrane at the node of Ranvier of ION have been described in our recent paper published in Neuron using this new recording technique (Kanda et al., 2019). The STAR protocol described here allows one to apply this new approach to investigate ion channels and their functions at nodes of Ranvier of different nerves. The pressure-clamped patch-clamp recording technique may also be used in conjunction with genetic and molecular biology approaches to advance our understanding of mechanisms underlying action potential conduction and information processing in different types of nervous systems.

**LIMITATIONS**

Our pressure-clamped patch-clamp recording method is applicable for the nodes of Ranvier of Aα motor nerve fibers and Aβ sensory nerve fibers in rats. Other types of myelinated fibers, including Aδ fibers in sensory nerves and myelinated fibers in the central nervous system, may be too thin in their
nodal axon diameters to be easily approached by the recording pipette. At present time, it is not certain if this new recording method is also applicable to the thinner nerve fibers. Recording pipettes with small tip sizes have to be used in order to access to and make patch-clamp recordings from the axonal membranes at the node of Ranvier. The resistance of these recording pipettes usually is in the range of 8 to 10 MΩ. The higher resistance of the recording pipettes will result in a larger voltage-clamp error. Therefore, one should take the consideration of the voltage-clamp error when interpreting voltage-clamp recording data obtained using our pressure-clamped patch-clamp recording technique. This protocol has only been applied to rat myelinated nerves. While it might be applicable to the myelinated nerves of other species including mice, this possibility remains to be tested. **TROUBLESHOOTING**

**Problem 1**
Nerve fibers are not healthy as manifested by a large gap at the node of Ranvier (step 10).

**Potential solution**
Make sure that nerve fibers are not over-stretched during making nerve preparations. In most cases, nerve fibers are damaged in the step 4 of removing connective tissues that surround nerve bundles. It is not necessary to completely remove all connective tissues that surround nerve bundles so that the potential nerve damage can be minimized.

**Problem 2**
Difficult to penetrate through perineurium that surround the axon at the node of Ranvier (step 15).
Problem 3
Recording pipettes seal onto Schwann cell membranes rather than onto nodal axon membranes (step 17).

Potential solution
Make sure that the tip of the recording pipette penetrates the perineurium right at the node of Ranvier. If the tip of the recording pipette is slightly off from the center of the node of Ranvier, the recording pipette tip will most likely seal onto Schwann cell membranes. It is also important to search nodal axon membranes very carefully by moving the pipette tip within the distance range of 1 μm from the original tip entering point. There is usually large current oscillation in seal test when the recording pipette tip touches axonal membranes. On the other hand, current oscillation in the seal test is not obvious when the recording pipette tip touches Schwann cell membranes.

Problem 4
Cannot form a gigaohm seal on the nodal axon membrane with the recording pipette (step 20).

Potential solution
Because the tip of the recording pipette needs to penetrate perineurium, it is essential to have positive pressures within the recording pipette before the tip of the pipette makes a close contact with nodal axon membranes to form a gigaohm seal. Air leakage in the pressure-clamp patch-clamp recording system is a common problem to prevent the recording electrode from forming a gigaohm seal onto axon membranes at the node of Ranvier. Make sure that there is no air leakage from the tubing, connectors, the recording pipette holder, and the HSPC system.

We also recommend to filter the recording electrode internal solution with a 0.22-μm syringe filter to remove the dust particles in the solution so that the tip of the recording pipette will not be clogged by the dust particles. Clogged recording pipette tip will not be able to form a gigaohm seal on nodal axon membranes and should be discarded.

Problem 5
Electrode access resistances become gradually increased over time during recordings (step 24).

Potential solution
One cause of the increase in the electrode access resistance is due to partial membrane reseal at the tip of the recording pipette. One will need to reopen the membrane seal in the manner described in step 22. Another common cause of the increase in the electrode access resistance is due to the drift of the tip of the recording pipette. To solve this problem, one needs to check the setups for holding (pipette holder, etc.) and controlling (micromanipulator, etc.) the recording pipette to improve the stability of the setups. If the pipette drift is mild, one can move the recording pipette tip back to the original position, which often can bring the access resistance back to the original level.

Optional: Optimizing enzyme treatment is needed for different types of nerves and also for the same type of nerves from animals at different ages. When nerves are optimally treated with the enzymes, it is relatively easy to have recording pipette penetrate through the perineurium and reach to the axonal membranes at the nodal of Ranvier. Insufficient treatment of nerve bundles with the enzymes makes it difficult to penetrate the perineurium. Over-treatment by the enzymes makes the targeted nerve fiber slip away from the tip of the positively pressured recording pipette. One should experiment with the time of the enzyme treatment or concentrations of the enzymes for different nerves to obtain optimal conditions.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be ful-
filled by the Lead Contact, Jianguo Gu [jianguogu@uabmc.edu].

Materials availability
There are no restrictions on any data or materials presented in this paper. This study did not
generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
J.G.G. and H.K. conceived and designed the project. J.G.G. supervised the project. H.K. conducted
the experiments. S.T. participated in the recordings of motor nerve fibers. Y.D. participated in the
discussion on the protocol.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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