# Immunostaining of Nav1.6 and Nav1.2 channels in cortical pyramidal neurons

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**Method Article**

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# Abstract

# Introduction

This protocol describes the immunohistochemical staining of $Na^+$ channel subtypes \($Na_v1.2$, $Na_v1.6$) and channel-associated protein ankyrin-G \(AnkG) in layer 5 pyramidal neurons of the rat neocortex. This specific staining requires a very light fixation of the brain tissue.

# Reagents

Sodium pentobarbital Phosphate buffer: 0.1 M phosphate buffer \(PB, pH =7.4), 0.01 M phosphate buffer saline \(PBS, pH = 7.4) Normal saline \(0.9% NaCl) Paraformaldehyde \(PFA; Shanghai Sangon) Triton X-100 \(Sigma) Sucrose \(Amresco) Embedding medium for frozen sectioning, O.C.T. compounds \(Sakura) Normal serum of goat, the host species of the secondary antibodies \(Invitrogen) Mounting medium for fluorescent sections \(H-1000, Vector) Nail varnish \(use those that dry quickly in a few seconds) Salts for artificial cerebrospinal fluid \(ACSF) and sucrose solution \(Amresco) Super glue \(Pattex) Primary antibodies 1) anti-$Na_v1.2$ IgG \(ASC-002, Alomone), use 1:200 dilution 2) anti-$Na_v1.6$ IgG \(ASC-009, Alomone), use 1:200 dilution 3) anti-Pan-$Na_v$ IgG \(ASC-003, Alomone), use 1:200 dilution 4) anti-AnkG IgG \(SC-12719, Santa Cruz), use 1:200 dilution Secondary antibodies 1) Anti-rabbit IgG: Alexa Fluor 488 goat anti-rabbit IgG \(A31627, Invitrogen), use 1:1,000 dilution 2) Anti-mouse IgG: Alexa Fluor 594 goat anti-mouse IgG \(A31624, Invitrogen), use 1:1,000 dilution **Reagent setup** **Fixative** Dissolve 1 g PFA in 100 ml 0.1 M PB in 60 °C water bath. Allow to cool at room temperature, add 1 g sucrose and then filter. This solution can be stored at 4 °C for several days. **5% Triton X-100 stock solution** Add 5 ml of Triton X-100 into 95 ml of 0.01 M PBS, stir gently until dissolved, and store at 4 °C. **0.3% Triton X-100** Add 3 ml of 5% Triton X-100 into 47 ml PBS, stir gently until dissolved, and store at 4 °C. **0.1% Triton X-100** Add 15 ml of 0.3% Triton X-100 into 30 ml PBS, stir gently until dissolved, and store at 4 °C. **Blocking and permeabilizing solution** 5% normal goat serum in 0.3% Triton X-100. **CRITICAL** Make up fresh before use. **Antibody dilution solution** 5% normal goat serum in 0.1% Triton X-100. **CRITICAL** Make up fresh before use. **ACSF** containing \(in mM) NaCl 126, KCl 2.5, $MgSO_4$ 2, $CaCl_2$, 2, $NaHCO_3$ 26, $NaH_2PO_4$ 1.25, dextrose 25 \(315 mOsm, pH 7.4) **Ice-cold aerated slicing solution \(sucrose solution)** sucrose-substituted ACSF in which equiosmolar sucrose is used as a substitute for NaCl, containing \(in mM) KCl 2.5, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, $NaH_2PO_4$ 1.25, dextrose 10, sucrose 213. **Cryoprotectant** Dissolve 30 g sucrose in 0.1 M PB to a final volume of 100 ml, and store at 4 °C.

# Equipment

Microscope slides \(poly-L-Lysin-coated) and coverslips ImmEdge Hydrophobic Barrier Pen \(PAP pen; H-4000, vector) Opaque humid box \(with vertical racks on which slides can lie horizontally) Rotary shaker \(TY-20, Shanghai SIBAS) 24-well plates \(Costar) Slice incubation chamber Vibratome for brain slices \

(VT 1000S, Leica) Freezing microtome for cryostat sections \(CM1900, Leica) Gas tank \(95% $O_2$ and 5% $CO_2$) Cut-off glass Pasteur pipette \(Corning) with the thin end removed and then attached with a rubber teat, used for living slice transfer Curving glass Pasteur pipette \(Corning) with the thin end heated and bended to make an L-shape, used for fixed slice transfer Scissors, forceps and blades Confocal microscope \(LSM 510, Zeiss) Upright microscope \(BX51, Olympus)

# Procedure

**Part 1 Perfuse the animal and obtain the cryostat sections** 1. Deeply anesthetize Sprague-Dawley rats \(P16-20) with 1% sodium pentobarbital. 2. Briefly perfuse rats through left ventricle with 12-15 ml normal saline and then perfuse with 8-10 ml 1% PFA and 1% sucrose in 0.1 M PB \(Perfusion speed: 7.5 ml per min). **CRITICAL** Make sure that the rat is only lightly fixed. 3. Dissect out the brain, then cut the region of interest with a scalpel, fix the block in the same fixative for 2 hours and then immerse in 30% sucrose in 0.1 M PB till it sinks to the bottom of the container. 4. Obtain cryostat coronal sections \(thickness of 16 or 100 μm) using the freezing microtome. 5. Let sections dry at room temperature. **CRITICAL** Do not store slices for future use. 6. Use the PAP pen to draw a hydrophobic barrier on each glass slide to confine the flow of reagents to the defined area. **Part 2 Prepare and fix the living brain slices** 1. Prepare the ACSF and ice-cold sucrose solution \(bubble the solution with 95% $O_2$ and 5% $CO_2$ for ~30 min). 2. Anesthetize the animal deeply with 1% sodium pentobarbital. 3. Dissect out the brain and place in the ice-cold sucrose solution. 4. Mount the brain on the vibratome stage with super glue and keep it submerged in the ice-cold sucrose solution. 5. Cut coronal cortical slices \(thickness of 300-350 μm) with the vibratome \(at a high frequency and low speed) and let them recover for at least 45 min in the aerated ACSF at ~35 °C. 6. Transfer the slices with the cut-off glass Pasteur pipette into the fixative \(1% PFA and 1% sucrose in 0.1 M PB) and fix for 2 hours. **CRITICAL** Living slices should be used freshly. 7. Rinse slices completely \(3x10 min) with 0.01 M PBS in 24-well plate. **TIPS** For floating slice transfer, use the L-shape Pasteur pipette which may cause less damage to the tissue. **Part 3 Perform immunostaining** 1. Incubate the sections in a blocking and permeabilizing solution \(5% normal goat serum, 0.3% Triton X-100 in PBS) for 2 hours at room temperature \(20-22 °C). **CRITICAL** For the staining of axon blebs, omit the blocking and permeabilizing step and directly incubate the slices in the primary antibodies. 2. Wash the sections in 0.01 M PBS \(3x10 min). 3. Incubate the sections overnight at 4 °C with primary antibodies against AnkG \(1:200, Santa Cruz) and either $Na_v1.2$, $Na_v1.6$ or Pan-$Na_v$ \(1:200, Alomone) in antibody dilution solution. 4. Wash the sections in 0.01 M PBS \(3x10 min). 5. Incubate sections at room temperature for 2 hours in Alexa 488 conjugated goat anti-rabbit IgG and Alexa 594 goat anti-mouse IgG in antibody dilution solution \(1:1000, Invitrogen). 6. Complete wash with 0.01 M PBS \(3x10 min). 7. Mount sections with the mounting media and take pictures with the confocal microscope.

# Timing

About 4 days. Day 1 Reagent preparation \(fixative and cryoprotectant), 2-3 hours; perfusion, 1 hour; post-fix 2 hours; then cryoprotection. Day 2 Cryoprotection. Day 3 A. For fixed tissue: sectioning with the freezing microtome \(30 min per rat brain). B. For living slices: making ACSF and slicing solutions; preparing living slices, 30 min; slice incubation in the aerated ACSF for at least 45 min; then fixation, 2 hours. C. For all sections from the fixed tissue and the living slices, blocking and permeabilizing for 2 hours, and then incubating in the primary antibodies overnight. Day 4 Incubating the sections in the secondary antibodies for 2 hours.

## Critical Steps

1. During perfusion, make sure that the rat is only lightly fixed. Use 1% PFA and 1% sucrose instead of 4% PFA; perfuse the animal with as little fixative as possible. We generally used ~10 ml fixative for each animal. 2. For immunostaining of axon blebs, skip the permeabilizing step with 0.3% Triton X-100 and directly incubate the sections in the primary antibodies in 0.1% Triton.

## Anticipated Results

AnkG staining labels the axon initial segments \(AIS) of cortical pyramidal neurons. Pan-$Na_v$ staining is present along the entire length of the AIS. $Na_v1.2$ and $Na_v1.6$ immunosignals preferentially accumulate at the proximal AIS and the distal AIS, respectively.