Ultrafast Sodium Imaging of the Axon Initial Segment of Neurons in Mouse Brain Slices

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Monitoring Na^+ influx in the axon initial segment (AIS) at high spatial and temporal resolution is fundamental to understanding the generation of an action potential (AP). Here, we present protocols to obtain this measurement, focusing on the AIS of layer 5 (L5) somatosensory cortex pyramidal neurons in mouse brain slices. We first outline how to prepare slices for this application, how to select and patch neurons, and how to optimize the image acquisition. Specifically, we describe the preparation of optimal slices, patching and loading of L5 pyramidal neurons with the Na^+ indicator ING-2, and Na^+ imaging at 100 µs temporal resolution with a pixel resolution of half a micron. Then, we present a data analysis strategy in order to extract information on the kinetics of activated voltage-gated Na^+ channels by determining the change in Na^+ by compensating for bleaching and calculating the time derivative of the resulting fit. In sum, this approach can be widely applied when investigating the function of Na^+ channels during initiation of an AP and propagation under physiological or pathological conditions in neuronal subtypes. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Preparation of cortical slices
Basic Protocol 2: Selection, patching, and Na^+ fluorescence recording of a neuron
Support Protocol: Calibrating Na^+ fluorescence
Basic Protocol 3: Data analysis

Keywords: action potential • axon • pyramidal neuron • sodium imaging • somatosensory cortex • voltage-gated sodium channels

How to cite this article:
Blömer, L. A., Canepari, M., & Filipis, L. (2021). Ultrafast sodium imaging of the axon initial segment of neurons in mouse brain slices. *Current Protocols*, *1,* e64. doi: 10.1002/cpz1.64

INTRODUCTION

Since the milestone work on the giant axon of *Loligo* (Hodgkin & Huxley, 1952), the Na^+ inward current has been recognized as the main determinant of the onset and upstroke of the action potential (AP). In polarized neurons of the mammalian central nervous system, the AP is initiated in the axon initial segment (AIS)(Bean, 2007). More specifically, APs are generated in the distal part of the AIS (Palmer & Stuart 2006), where voltage-gated Na^+ channels (VGNCs) are highly expressed (Kole et al., 2008). Investigating VGNCs in the AIS is therefore crucial to understand neuronal communication, either under healthy conditions or in the case of various diseases caused by

CURRENT PROTOCOLS
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Current Protocols e64, Volume 1
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.64

dysfunctions in these channels (Wimmer, Reid, So, Berkovic, & Petrou, 2010). Standard electrode techniques have been used to record fast Na^+ currents mediated by VGNCs (Alle, Roth, & Geiger, 2009; Astman, Gutnick, & Fleidervish, 2006; Yue, Remy, Su, Beck, & Yaari, 2005). This experimental approach, however, gives no information on the origin and distribution profile of the Na^+ influx, which can be instead obtained by Na^+ fluorescence imaging (Baranauskas, David, & Fleidervish, 2013; Fleidervish, Lasser-Ross, Gutnick, & Ross, 2010; Kole et al., 2008). Na^+ fluorescence imaging allows one to record the Na^+ influx associated with APs along the AIS at single-neuron resolution in brain slices. The most commonly Na^+ indicator used has been Na^+-binding benzofuran isophthalate (SBFI) (Minta &Tsien, 1989). However, more recently, the Na^+ indicators Asante NaTRIUM Green-2 (ANG-2) (Miyazaki & Ross, 2015; Miyazaki, Lisman, & Ross, 2019) and ION NaTRIUM Green-2 (ING-2; commercially available from Ion Indicators) have been shown to provide a significant improvement in the signal-to-noise ratio (SNR) compared to SBFI. Combined with the latest technology in acquisition systems and lasers (Filipis et al., 2018), we recently measured Na^+ signals in the AIS at 10 kHz (Filipis & Canepari, 2020), i.e., at a temporal resolution comparable to that of optical AP recordings using voltage-sensitive dyes (Popovic et al., 2015). Similar to the optical measurement of dendritic Ca^{2+} currents (Jaafari & Canepari, 2016; Jaafari, De Waard, & Canepari, 2014; Jaafari, Marret, & Canepari, 2015), the Na^+ current was extracted by calculating the time derivative of the Na^+ transient.

Here, we present protocols for recording Na^+ transients in the AIS at the highest temporal resolution achievable thus far, i.e., at a temporal resolution of 100 µs, and at a spatial resolution of half a micron. Although this article presents the case of the AIS of layer 5 (L5) somatosensory cortex pyramidal neurons in mouse brain slices, the same technique can be used to record Na^+ transients in any neuron type by adapting the protocols to the desired preparation. Basic Protocol 1 describes the procedure for preparing brain slices, optimized for this measurement, and Basic Protocol 2 explains how to optimally select and patch a cell and finally perform the fluorescence measurements. The Support Protocol is included to describe the calibration procedure used to express fluorescence changes as changes in Na^+ concentration. Lastly, Basic Protocol 3 presents a data analysis procedure that ultimately leads to the extraction of the Na^+ current.

NOTE: Experiments described here were ethically carried out in accordance with European Directives 2010/63/UE on the care, welfare, and treatment of animals. Procedures were reviewed by the ethics committee affiliated with the animal facility of the university (D3842110001). We used mice (C57BL/6J) at 21 to 35 postnatal days old purchased from Janvier Labs. These animals were housed with their mother with ad libitum access to food and water. All experiments performed by others must be approved by their specific animal care and use committee.

BASIC PROTOCOL 1

PREPARATION OF CORTICAL SLICES

Some general considerations in preparing healthy brain slices have been published in other Current Protocols articles (see, for example, Madison & Edson, 2001). To be able to record Na^+ currents in L5 pyramidal cells (Basic Protocol 2), it is important to prepare cortical slices in a way that leaves these neurons unblemished. In addition, it is important that patched cells are parallel to the slice so that their extremities lie in the same focal plane as the soma. As the pyramidal cells in L5 have their dendrite extended all the way into the first layer and the cortex is curved around the midbrain, 2 to 3 sagittal slices per hemisphere, cut at a 15° angle from the horizontal plane, contain unscathed pyramidal cells.

NOTE: For the dissection, follow the protocol approved by your institution and by local authorities.

Materials

Carbogen: 95% O_2/5% CO_2 gas mixture
Mouse (C57BL/6J strain, 21 to 35 postnatal days old; Janvier Labs)
Cyanoacrylate instant glue (e.g., KRYLEX KB0624)
Deionized water

Spatula (Chattaway spatula, 15 cm; King Scientific, cat. no. SH285-15)
Slice holder (e.g., Campden Instruments, model CL.7450-2A) or materials for custom-made slice holder:
  Plastic bottle or jar with plastic cap
  Stretchable nylon net
  PVC tube pieces (two)
  Glass beaker
  Lid
37°C water bath (Thermo Fisher Scientific)
Stainless steel blades (Campden Instruments, cat. no. 7550-1-SS)
Vibratome (VT 1200, Leica) with custom-made holder with 15° inclination
Instruments for brain dissection:
  Operating scissors (14-cm length; World Precision Instruments, cat. no. 501218-G)
  Fine scissors (10.5-cm length; Fine Science Tools GmbH, cat. no. 14094-11)
  Spring scissors (5-mm cutting edge; Fine Science Tools GmbH, cat. no. 91500-09)
  Adson forceps (12 cm; World Precision Instruments, cat. no. 14226-G)
Filter paper
Glass petri dishes
Razor blade (Fisher Scientific, cat. no. S65921)
3-ml plastic balloon pipet with end cut off (to have an ~0.5-cm-diameter opening)
Syringe with bent needle (90° angle)

Additional reagents and equipment for preparing slicing solution (see recipe) and 1× artificial cerebrospinal fluid (ACSF; see recipe) and for mouse anesthesia (see Current Protocols article; Davis, 2008)

Preparation for dissection and slicing

1. Prepare 150 ml slicing solution and keep at −20°C for 90 to 120 min.
2. Prepare 800 ml of 1× ACSF and bubble with carbogen for ≥20 min before starting dissection.
3. Take slicing solution from step 1 out of the freezer and break formed ice with a spatula. Bubble solution with carbogen for ≥20 min before starting the dissection.

   No need to form a slurry, but avoid having big chunks of ice.

4. Build a slice holder to place brain slices in and keep them below surface of the ACSF (see step 15) or use a purchased slice holder. For the custom-made slice holder, create a plastic ring that can fit inside a glass beaker by cutting out middle of a big cap of a plastic bottle or jar and create another ring by cutting off neck of the bottle or jar (Fig. 1A). Then, stretch out and screw a stretchable nylon net between cap and neck rings to hold the slices (Fig. 1B). Use two PVC tube pieces to fix rings inside the glass beaker and leave space for carbogen tube at the edge (Fig. 1C).

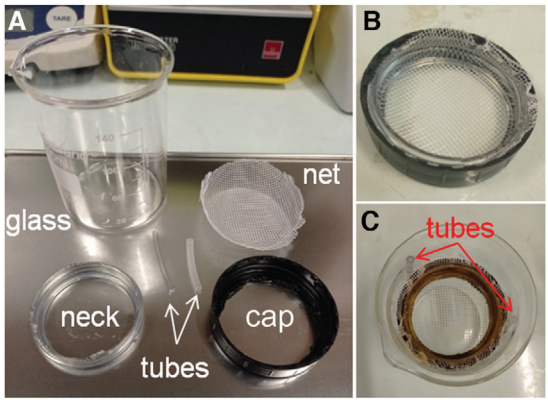

Figure 1 Construction of the recovery chamber. (**A**) Tools to construct the recovery chamber to hold slices as described in step 4 of Basic Protocol 1. (**B**) Stretched net screwed between the cap and the neck of a plastic bottle or jar to create the slice holder. (**C**) Slice holder fixed inside the glass beaker using two PVC tube pieces.

A slice holder can be easily constructed in this way. Avoid gluing the ring inside the beaker so that you can easily remove it to clean it. Alternatively, a slice holder can be purchased in advance.

5. Fill slice holder with the oxygenated ACSF from step 2 and place in a 37°C water bath. Place a lid on top of slice holder to prevent evaporation and bubble with carbogen, letting solution's temperature equilibrate.

6. Mount a fresh stainless steel blade on a vibratome with a custom-made holder with 15° inclination and calibrate instrument according to the manufacturer's instructions. Take out necessary instruments for brain dissection (Fig. 2A and 2B).

A holder with 15° inclination can be made in the laboratory using a metal piece if the necessary equipment to cut metal is available. Cut the metal piece to be similar to the flat holder provided with the vibratome, but instead of having the top part flat, cut it at a 15° inclination following the schematic given in Figure 2C.

Brain dissection

7. Anesthetize mouse (see Current Protocols article; Davis, 2008).

We use isoflurane injected in a custom-made airtight chamber installed in a fume hood, but any commercial system equipped with an anesthetic vaporizer can be used. The dimensions of our chamber (length × width × height) are 16 × 16 × 12 cm. We use ∼200 µl isoflurane (1 ml/ml; Axience) delivered through both sides of a cotton swab, and we wait ∼1 min, until the mouse is immobile, before decapitating it using operating scissors (see step 8).

8. Decapitate mouse using operating scissors and dissect out brain quickly:

   a. Place head of the mouse on a paper towel and use fingers to peel back the skin from the back and over the head, toward the nose.
   b. Use fine scissors to cut away any brainstem tissue and surrounding bone.
   c. Use spring scissors to carefully cut along midline of the brain. First cut through any remaining skin layers and then through skull. Cut past the intersection of bregma.
   d. Place Adson forceps under one half of skull and move slightly to force upward, breaking the bone and exposing the brain. Do this on both sides of cut.
   e. Move ice in the slicing solution (see step 3) so that a small space of clear solution is created. Using the spatula, carefully lift brain from the base of the skull and

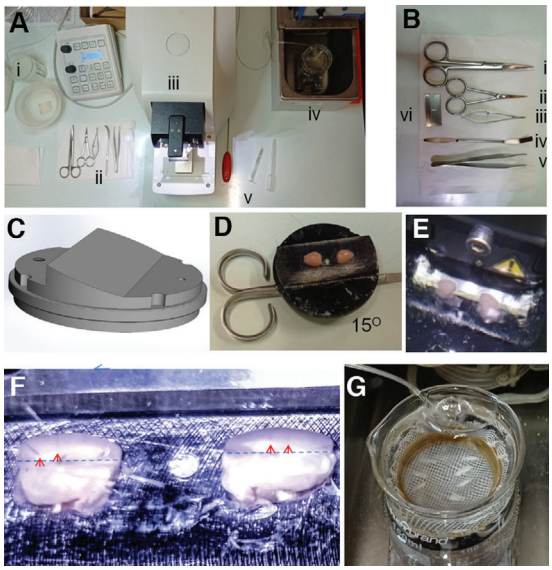

Figure 2 Dissection and slicing preparation. (**A**) Preparation of tools and instruments for slicing: i) oxygenated slicing solution; ii) instruments for brain dissection; iii) vibratome; iv) slice holder with oxygenated ACSF in a 37°C water bath covered with a lid; v) vibratome screwdriver (left), syringe with a bent needle for cutting slices (middle), and plastic balloon pipet with its end cut off for transferring slices (right). (**B**) Instruments for brain dissection: i) operating scissors used for decapitation; ii) fine scissors used for removing the skin from the skull; iii) spring scissors used for cutting through the skull; iv) Chattaway spatula used to move the brain; v) forceps used to remove the skull; vi) blade used to cut the cortices. (**C**) Design of the slice holder with 15° inclination. (**D**) Cortices glued on the holder. The scissors are used to prevent slices from sliding by counteracting the holder slope. (**E**) Vibratome blade that has cut halfway through both cortices. Using the syringe with a bent needle, slices are cut at this point and moved to the slice holder. (**F**) Cortices inside the vibratome right before cutting a slice. Visible blood vessels running throughout layers 1 to 6 are marked with red arrows, indicating that these slices have axons and dendrites that run parallel to the slice surface. The blue dashed lines indicate the position where the slices will be cut with the bent syringe needle. (**G**) All 350-µm slices are placed in the slice holder inside the 37°C water bath for 30 min.

let brain sink in the slicing solution, all the while bubbling with carbogen. Leave brain to rest for 3 to 4 min.

9. While the brain is resting in the slicing solution, spread a thin layer of cyanoacrylate instant glue at two locations next to one another on disk of the vibratome holder with 15° inclination (see step 6). Place an object under disk to counteract the 15° inclination and make it horizontal, so the glue does not run.

10. Dissect out cortices:

    a. Carefully take brain out of the icy slicing solution (see step 8e) and place it, dorsal side up, on a piece of filter paper in a glass petri dish to allow excessive fluids to be removed.
    b. Use a razor blade to make a coronal section straight down from lambda to separate any remaining cerebellum and hindbrain. Similarly, cut away olfactory bulbs if still attached.

11. Make a sagittal cut, straight down midline of the brain, dorsal to ventral, separating the two hemispheres. Mount both hemispheres on vibratome holder:

    a. Using the forceps and spatula, carefully lift one of the hemispheres and place it on one of the two layers of glue on the holder, in such a way that the midsagittal surface is glued to the holder and the dorsal part of the brain is facing down the

slope of the holder. Repeat this for second hemisphere, again making sure that the dorsal side faces downhill and the cut side is glued. Very gently press tops of the two hemispheres down to make sure that they are well connected to the holder (Fig. 2D).

b. Install holder in the vibratome basin.

c. Carefully pour all slicing solution in vibratome basin, making sure that no ice touches the hemispheres. Bubble with carbogen.

Brain slicing

12. Position a fresh stainless steel blade in vibratome clamp and move holder up ~14,000 µm. Set speed to 0.14 mm/s, make sure that device is in "manual" mode, and make a slice.

    The following procedure (see steps 13 to 15) is dependent on your tissue slicer model, so modify accordingly.

13. When the blade has sliced completely through the two cortices, press "stop" and move holder down 100 µm so that the blade is not touching the top of the brain tissue. Using a 3-ml plastic balloon pipet with its end cut off, suck up slices and discard them. Move blade back to its starting point.

14. Make thin slices (350 µm), discarding them until hippocampus becomes visible. After every slice, remember to move holder down 100 µm before moving the blade back to the starting point. Once the hippocampus is visible, change speed to 0.06 mm/s and set cutting thickness to 350 µm.

    Once you clearly see the hippocampus, you have reached the area with optimal slices to collect. Do not cut the hemispheres in half before slicing because the exact position of the hippocampus is unknown without seeing it, and you might cut part of the cortex.

    From this point, stop cutting through the end of the cortices because the steel blade is not long enough to go all the way through them (part of the vibratome would pass through, too, pushing the brain) and because the slice would be too big to fit inside the balloon pipet without folding. Instead, use a syringe with a bent needle (90° angle) as a mini-blade to cut around halfway through the cortices and save only the part with the cortex (see step 15d).

15. Discard first slice made at this speed and start saving next ones. Keep slicing until hippocampus is no longer visible. Make 4 to 5 slices of 350 µm per hemisphere as outlined below:

    a. Make sure that there are no bubbles under net in the slice holder. Use balloon pipet to suck away any formed bubbles that would disturb the slices.

    b. Start to cut a slice of 350 µm. Press "stop" once blade has cut through the cortex of both hemispheres and partly through the hippocampus, but do not let it cut through end of the cortices (Fig. 2E and 2F).

    c. Move vibratome holder down 100 µm.

       Do this after every slice to avoid pressing down on the rest of the brain in step 15d.

    d. Use a syringe with a bent needle (90° angle) as a mini-blade and gently press down on slice on top of the stainless steel blade to separate the sliced cortex from the rest of the brain. Suck up cortical slice with the balloon pipet and deposit slice in the slice holder (Fig. 2G).

    e. Keep information about collected slices.

       Visible blood vessels running from layer 1 to layer 6 are an indication that the axon and the dendrites are parallel to the slice surface in all the different layers of the cortex (see Basic Protocol 2, step 6). Note this information for every slice (Fig. 2F).

f. Move blade to the starting point. Move vibratome holder up 450 µm and make another 350-µm-thick slice.

16. Clean vibratome, rinsing with deionized water and drying fully. Leave slices at 37°C for 30 min while bubbling with carbogen.

    The vibratome should be cleaned because the salts in the slicing solution tend to crystallize, damaging the machine.

17. Take slice holder out of the 37°C water bath while continuing to bubble with carbogen and leave to cool down at room temperature (22° to 24°C).

    After ~1 hr, the slices are ready for the experiment (see Basic Protocol 2).

SELECTION, PATCHING, AND Na^+ FLUORESCENCE RECORDING OF A NEURON

BASIC PROTOCOL 2

To perform patch-clamp recordings in brain slices, one should see other Current Protocols articles that have addressed the general technical aspects in detail, including the setup for electrophysiological recordings (Finkel & Bookman, 2001), the fabrication of electrodes (Rae & Levis, 2004), and the careful selection of the tissue for establishing patch-clamp recordings (Poolos & Jones, 2004). Briefly, brain slices (Basic Protocol 1) are secured in a recording chamber installed in the electrophysiology system and are continuously perfused with oxygenated ACSF, and the temperature is controlled between 32°C and 34°C. Pyramidal neurons are selected to ensure that only healthy cells with an axon on the slice surface are patched. This selection process requires some practice, but it is necessary, as it avoids patching neurons in which the axon either is cut halfway or goes down into the slice bulk, preventing the possibility of having it in the focal plane. A schematic of the microscope is shown in Figure 3.

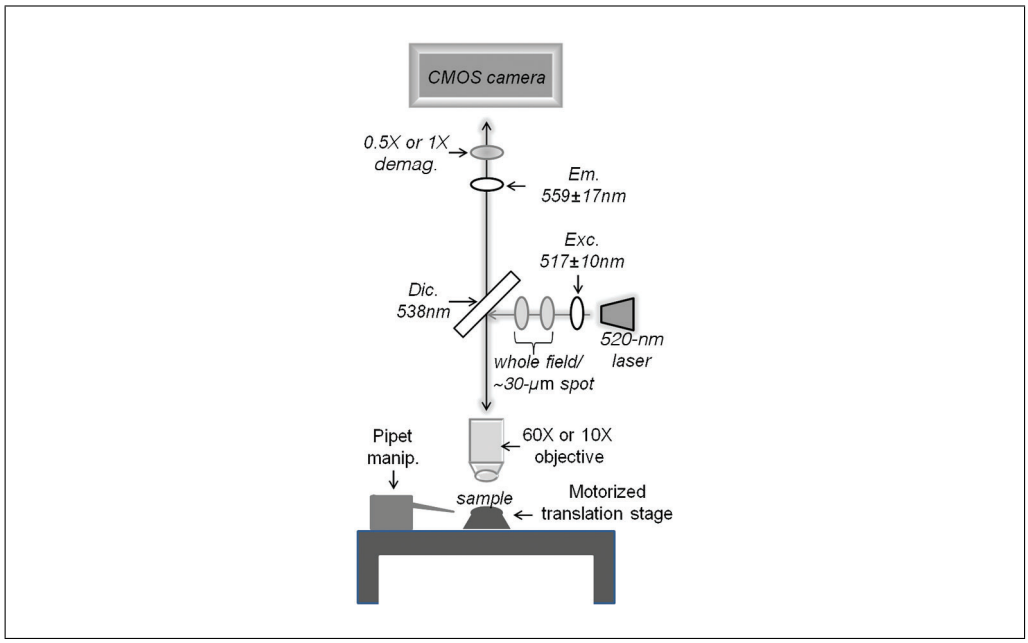

Figure 3 Schematic of the setup for high-resolution Na^+ imaging. The 520-nm laser beam is band-pass-filtered at 517 ± 10 nm. A telescope allows illumination of the whole field uniformly or a spot of ~30 µm. Alternating between a 60× objective and a 10× objective is allowed. The emitted light, passing through a 538-nm dichroic mirror, is band-pass-filtered at 559 ± 17 nm. It is possible to change lenses to switch between 0.5× and 1× demagnification to obtain a pixel resolution of 500 or 250 nm, respectively, on the CMOS sensor.

Materials

ACSF (see Basic Protocol 1, step 2)
Intracellular solution (see recipe)
ION NaTRIUM Green-2 (ING-2; Ion Indicators) Na^+ dye (see Support Protocol for calibration)
Brain slices (see Basic Protocol 1)

DMZ puller (Martinsried)
Borosilicate glass pipets (OD = 1.5 mm, ID = 1.1 mm; Harvard Apparatus)
Electrophysiology setup:
- Electrophysiology system, including recording chamber, microscope, motorized XY translation stage, and micromanipulator (PatchStar manipulator mounted on TMC anti-vibrant table) (SliceScope Pro system, Scientifica)
- Perfusion system (with temperature control; Scientifica)
- Camera (DaVinci 2K CMOS Camera, SciMeasure) and camera software
- 1×/0.5× variable demagnification (Cairn Research)
- Standard 10× objective (air objective; for initial broad visualization only; Olympus) and 60× objective (water immersion objective, NA = 1, WD = 2 mm; Olympus)
- IR-DIC system (Scientifica)
- Manometer (World Precision Instruments)
- Patch clamp amplifier (MultiClamp 700A, Molecular Devices)
- A/D acquisition board (USB-6221 board, controlled by Matlab; National Instruments)

0.2-µm nylon filters
Clear 0.5-ml tubes
Vortex mixer
Mini-centrifuge (e.g., Thermo Fisher Scientific, cat. no. 75004061)
1-ml syringes without needle
Forceps (Fine Science Tools GmbH, cat. no. 11251-20)
Harp slice grids (ALA Scientific)
PVC tubing (various sizes; Cole-Parmer)
20-ml syringes (Cole-Parmer)
20-µl microloader tips (Eppendorf, cat. no. 5242956003)
Illumination tools:
- Telescope (with two lenses, for wide-field or ∼30-µm-spot illumination at output of fiber; Cairn Research)
- Filter cube, comprising excitation (517 ± 10 nm) and emission (559 ± 17 nm) filters and 538-nm dichroic mirror (FF01-517/20, FF01-559/34, and FF538-Di01 from Semrock recommended)
- Laser [Tri-Line Laser-Bank (Cairn Research) through Ø550 µm, 0.22 NA, SMA-SMA Fiber Patch Cable (Thorlabs), with 520 nm/0.5 W multimode diode head (Ushio)]
- Master-9 pulse stimulator (A.M.P.I.)
- Turbo-SM data acquisition software (RedShirt Imaging)

Preparation

1. Using a DMZ puller, pull borosilicate glass pipets to form patch pipets of 4 to 5 MΩ resistance when filled with intracellular solution.

   When using the DMZ puller, we advise to start from program "P06" and change the parameter "H" of the second pull to obtain the right tip size. A typical starting value is H = 150, increasing gradually with use until the filament needs to be changed at around H = 400.

2. Use ACSF from Basic Protocol 1, step 2, to continuously perfuse the recording chamber at a rate of ∼2 ml/min. Use same rate for vacuuming away the ACSF. Heat perfusion system to keep the temperature in the recording chamber at 32° to 34°C (nearly physiological temperature).

3. Dilute ING-2 Na^+ dye:

   a. Thaw 1 ml intracellular solution and use a 0.2-µm nylon filter to clean solution.
   b. Dilute ING-2 Na^+ dye to 0.5 mM in intracellular solution in a clear 0.5-ml tube. Protect dye from light.
   c. To remove impurities, vortex thoroughly and then briefly centrifuge tube in a mini-centrifuge. Carefully, so as not to disturb the pellet, transfer supernatant to a clean 0.5-ml tube. Repeat this process until no pellet is visible after centrifuging.
   d. Keep tube on ice to prevent degradation of the intracellular solution. Keep the rest of the intracellular solution in a 1-ml syringe without a needle and with a 0.2-µm nylon filter on ice.

Slice selection

4. Transfer a single brain slice to recording chamber under the microscope of the electrophysiology system. Adjust slice position using forceps.

5. Set camera software to focus mode and use a 1024 × 1024 configuration.

6. Using 1× demagnification and a standard 10× objective, focus on slice surface. Ignore prefrontal part of the cortex and the subiculum and look at widest part of the cortex, the somatosensory areas. Identify if there are blood vessels running in parallel to slice surface all the way from the dorsal end toward the hippocampus (Fig. 4A).

   When a blood vessel is parallel to the slice surface, this normally indicates that the axons will be parallel, too, as axons are known to follow blood vessels to the brain surface (Andreone, Lacoste, & Gu, 2015).

7. Before rejecting a slice, flip it to check if cells on the other side are more parallel to the focal plane.

8. Once a slice with parallel blood vessels is selected, place a harp slice grid to hold slice. Try to place grid wires parallel to the blood vessels and the *x*-plane of the camera.

Cell selection

9. Move to a region with parallel blood vessels. Switch to 60× objective and focus on slice surface. Use IR-DIC system.

10. Move to dorsal end and save position of the motorized XY translation stage.

    The cell bodies of L5 pyramidal neurons are located in the middle part of the cortex, at about 500 to 900 µm from the dorsal end of the brain, given that the whole cortex is ∼1500 µm at its widest part.

11. Locate pyramidal neuron cell bodies and axons.

    It is useful to find dead pyramidal neurons and examine whether their axons run parallel to the focal plane. Dead neurons have shrunken and show high contrast under DIC. Because of its increased contrast, it is easy to follow the axon of a dead cell up through the cortical layers and determine whether the area of the slice is indeed parallel.

12. Once a suitable region is identified, select a healthy neuron to patch.

    An example is shown in Figure 4B. A healthy pyramidal neuron can be recognized by its typical large triangular shape. These cells are symmetrical and show little contrast

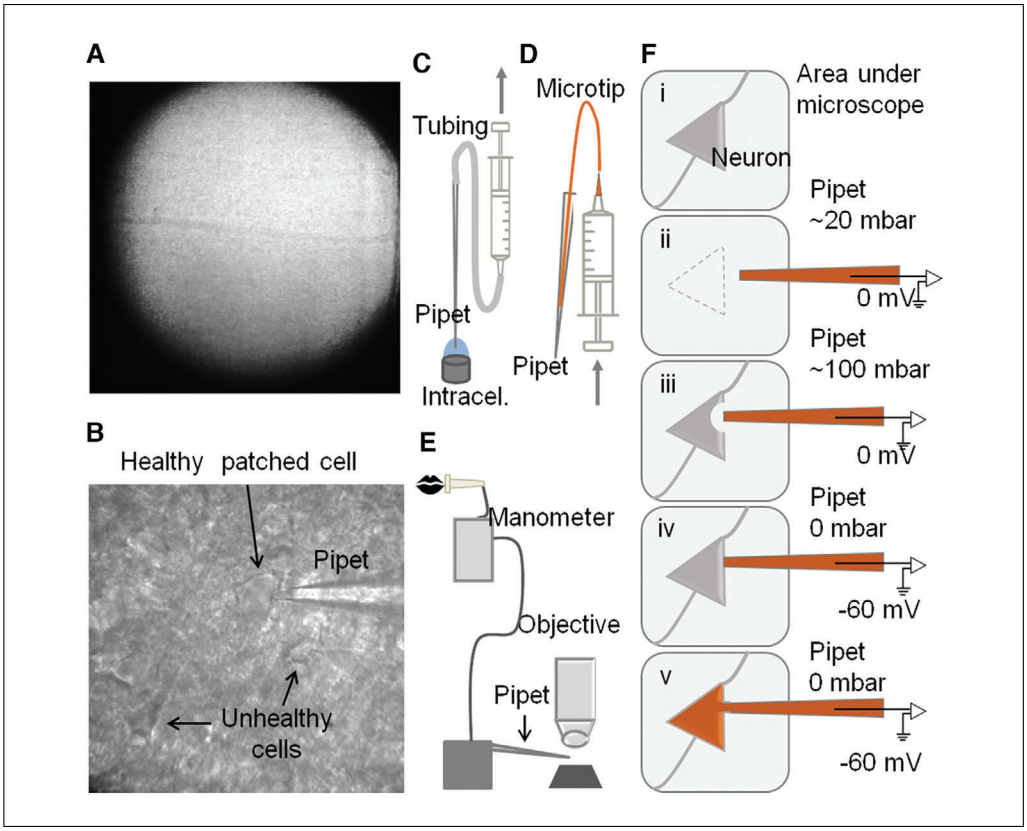

Figure 4 Pipet filling, positioning, and patching. (**A**) Example of blood vessels running in parallel to the slice surface all the way from the dorsal end toward the hippocampus, as seen with the 60× objective and 1× demagnification. (**B**) Example of a healthy patched cell. Unhealthy cells are indicated; their contrast is higher, and their membranes appear damaged. (**C**) Front-filling of a patch pipet. Position the tip of the pipet in a droplet of intracellular solution and provide slight negative pressure from the top of the pipet using some tubing and a syringe. (**D**) Back-filling of a patch pipet. Using a syringe with a microloader, fill the pipet with the intracellular solution containing the dye. (**E**) When mounted on the holder, the pipet is connected to a manometer to measure the pressure applied. Provide a pressure of ~20 mbar by blowing into the end of a syringe without a plunger. (**F**) Patching a cell (in voltage clamp). After selecting a healthy cell (i), position the tip of the pipet just above the cell membrane while holding the voltage at 0 mV (ii). Apply a strong pressure (~100 mbar) and move the pipet down quickly, slightly blowing the tissue out of the way. Position the pipet in front of the selected neuron (iii). Release the pressure to form a seal. Change the voltage to –60 mV (iv). Enter into the whole-cell configuration by gently sucking the mouthpiece connected to the manometer and pipet and allow the cell to fill with dye (v).

with their surroundings, and the nucleus is difficult to identify. Unhealthy cells are usually swollen, and their enlarged round nucleus is clearly visible, indicating that the cell is about to release its contents and die. Select cells located 20 to 35 µm below the slice surface (by measuring the depth with the Z-translator). With superficial cells, there is a higher chance that the axon runs out of the slice and is cut off, whereas cells 35 µm below the surface are more difficult to image, as the larger scattering prevents good measurements.

13. Save position of the slice surface above the cell and move objective up, out of the solution.

Patching a pyramidal neuron

14. To avoid spilling of the dye, front-fill a patch pipet (see step 1) with clear intracellular solution. Position tip of the pipet in a droplet of intracellular solution and provide slight negative pressure from top of the pipet using some PVC tubing and a 20-ml syringe. To only fill the tip, apply this negative pressure for ~20 s (Fig. 4C).

15. Using a 20-ml syringe with a 20-µl microloader tip, back-fill pipet with the dye diluted in intracellular solution (see step 3; Fig. 4D). Make sure that there are no bubbles in tip before mounting the pipet on the holder.

16. Move patch pipet down to the slice surface:

    a. Apply ~20 mbar positive pressure (measured with a manometer) to pipet (Fig. 4E). Submerge pipet in the ACSF surrounding the slice.

       The moderate pressure keeps the tip of the pipet clean and avoids dye leakage while approaching the slice surface. The pressure can be maintained by placing a stopcock (Cole-Parmer) between the mouthpiece and the manometer.

    b. Keep electrode in voltage clamp–testing mode, compensating the junction potential to 0 mV and applying continuous pulses of 0.5 ms duration and –5 mV amplitude.
    c. Find tip of the pipet with the objective and move objective and pipet down together in fast mode, until the pipet tip is ~200 µm above the slice surface.
    d. Change to slow mode for both the micromanipulator and the objective and keep moving them until the pipet is hovering just above the slice surface.

17. Move objective down to identify the pyramidal cell to patch (Fig. 4F, i) and zoom in once or twice to have a better view of cell. Mark a spot on screen and position edge of the soma below the electrode tip. Patch soma at its thinner part, toward dendrite, to stay as far as possible from the nucleus. Focus again on pipet tip and position tip slightly away (5 µm) from the spot (Fig. 4F, ii).

18. Patch neuron:

    a. Focus on cell of interest and apply a large positive pressure on patch pipet (~100 mbar).
    b. Quickly move pipet straight down until the tip of the pipet is at the same focus as the cell.

       You might need to adjust the objective at the same time if the cell moves to a different focal plane. Be aware that if this higher pressure is maintained for too long, a substantial leak of the dye will be unavoidable, preventing high-quality imaging recordings.

    c. Once the pipet and soma are on the same focal plane, move pipet toward the cell until the pipet can be seen pushing the cell (Fig. 4E, iii). Compensate junction potential.
    d. Immediately release pressure to form a seal in voltage clamp mode and set voltage at –60 mV (Fig. 4F, iv).

       Ideally, steps 18a to 18d should be achieved in <30 s.

19. Move pipet back 1 to 2 µm so that the tip does not push the cell. To achieve a whole-cell configuration, apply a short, robust suction (Fig. 4F, v). Estimate series resistance, which ideally should be ~10 MΩ or below.

20. Immediately check if dye spillage occurred while patching neuron. If this is the case, discard patch, move ≥100 µm away from that cell, and patch another neuron. If there is no dye spillage, continue experiment.

Fluorescence imaging

21. Wait 20 to 30 min for dye to equilibrate through the cytosol.

    After patching a cell and allowing the Na^+ indicator to equilibrate, the recording can start (see below). The data acquisition frame rate is 10 kHz, with 500 nm pixel resolution, using a DaVinci 2K CMOS camera. The somatic V_m is simultaneously recorded by the A/D acquisition board connected to the patch clamp amplifier in order to monitor the somatic AP. For the V_m acquisition, we recommend a sampling rate of 20 kHz,

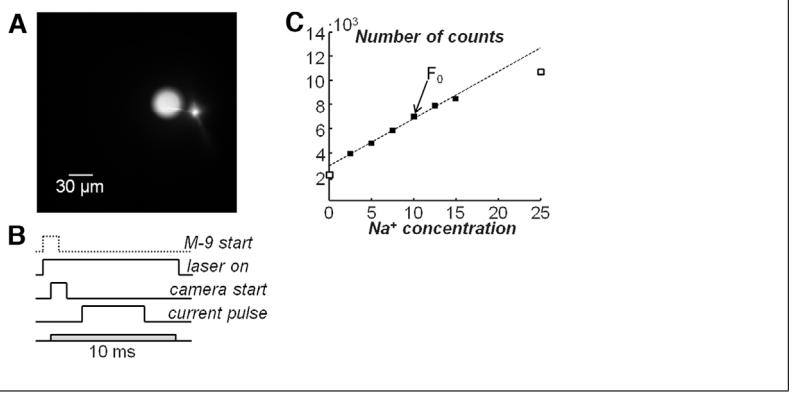

Figure 5 Imaging procedure and calibration of ING-2. (**A**) L5 pyramidal neuron loaded with ING-2 viewed in the 512 × 512 configuration and illuminated by the laser spot on the AIS. (**B**) Timing of the Master-9 (M-9) outputs after the starting pulse (dotted line) controlling the laser illumination, the start of the camera, and the pulse of somatic current injection eliciting the AP. In total, this corresponds to an imaging acquisition of 8 ms. (**C**) Calibration of ING-2 $\Delta F/F_0$ signals in terms of $\Delta[Na^+]$ for an internal set of solutions with different NaCl concentrations and 80 µM ING-2. The plot shows the number of counts at the different Na^+ concentrations, and the dashed line is the linear fit of the points between the 2.5 and 15 mM Na^+ concentrations.

i.e., twice the sampling rate of the imaging. Dye excitation is performed with a 520-nm Tri-Line laser, and a telescope is used to illuminate the AIS only. It is strongly recommended to control the timings of the recording with an accurate pulse stimulator (Master-9), with the delay and duration of pulses set with ≥10 µs precision. The Master-9 should be started by an output of the same A/D board as that recording the electrophysiology.

22. Switch to 0.5× demagnification and set camera configuration to 512 × 512.

    The 1× demagnification is used for patching (see step 6), as the better contrast allows you to see when the pipet is touching the cell. The 0.5× demagnification is used for imaging because it increases the number of photons detected by each pixel by a factor of 4.

23. Set telescope to achieve wide-field illumination and place filter cube on the microscope pathway. Illuminate with low light, where laser is essentially still off and the light comes only from the diode, and check fluorescence over the cell. Center AIS in the area that will be illuminated by the 30-µm spot and turn off laser (Fig. 5A).

24. Change camera configuration to 128 × 30 pixels, which allows acquisition at 10K frames/s. Turn on laser again at a low intensity to optimize the position of the AIS in the spot and bring it into focus.

    This configuration can be obtained from the manufacturer.

    It is very important to keep the soma out of the illuminated spot because strong light on the cell body produces rapid phototoxicity.

25. Set a laser to trigger mode and set intensity to its maximum.

26. Switch patch clamp amplifier from voltage clamp to current clamp mode and set steady current so that the V_m is between –60 and –80 mV.

    In healthy cells, the resting V_m (when the steady current is zero) is within this range.

27. Program three channels of Master-9 pulse stimulator to let it control the laser, start the camera, and set the timing of occurrence of the somatically injected current

triggering the AP. Prepare for acquisition of a series of images for 8 ms, as illustrated in Figure 5B:

a. Trigger start of the Master-9 with a TTL output of the electrophysiology A/D acquisition board.
b. With no delay, trigger laser on with a TTL pulse of 8.7 ms.
c. Start camera acquisition with a delay of 0.5 ms from the laser trigger.

This will prevent acquisition of images while the laser is turning on.

d. Trigger primary output of the patch clamp amplifier so that it provides an output pulse of 3 to 5 ms duration and 1 to 2 nA amplitude delayed by ∼2 ms from the camera start.

This intensity normally elicits an AP.

28. Prepare Turbo-SM data acquisition software to control the camera acquisition speed and number of frames:

a. Set recording time to 0.1 ms and number of frames to 80.
b. Record a "dark frame" that is used to subtract levels corresponding to no light from the camera signals.
c. Start recording by pressing "Record" button in Turbo-SM.

The camera should start recording only after being triggered by the Master-9.

29. Start an electrophysiology recording lasting 10 ms that will also start the Master-9.

30. Wait 1 min before performing next trial to let the bleached indicator equilibrate again.

The highly precise timing of the Master-9 allows repeating the protocol with no jitter. Thus, in healthy and stable cells, the AP of the sequential recordings superimpose with a sample precision (50 µs). In this case only, sequential series of images (trials) can be averaged in order to increase the SNR.

31. Continue recording until AP starts widening.

This is a sign that photodamage is beginning, and at this stage, AP recordings should be terminated. In our experience, photodamage starts after 10 recordings.

32. After finishing the AP recordings, set current pulse to zero and record one series without stimulus.

This recording will quantify the kinetics of the indicator bleaching.

33. Take wide-field images of cell, which is particularly important to measure the distance from the soma for the different AIS regions, before destroying the patch:

a. Set back wide-field illumination in the telescope.
b. Set exposure time of the frames in Turbo-SM to ≥20 ms.
c. Increase laser duration in the Master-9 to acquire longer sequences.
d. Decrease laser intensity to reduce bleaching and prevent saturation.
e. Take different stacks at different focal planes.

We recommend using steps of 2 µm in the z-plane.

f. Optional: Move position of the cell to reconstruct the apical dendrite and the secondary dendrites that are still intact.

34. If another cell in the same slice is used, move ≥100 µm away from previous cell to avoid its fluorescence.

If the previous axon was not perfectly parallel, we advise changing the slice.

CALIBRATING Na^+ FLUORESCENCE

In contrast to Ca^{2+} imaging, where a calibration of total Ca^{2+} influx has to consider the binding to endogenous buffers (see, for example, Canepari & Mammano, 1999), in Na^+ imaging, the fluorescence change can be directly calibrated in terms of total Na^+. In this case, indeed, a linear relation between the fluorescence change and the total Na^+ transient occurs, with only a small fraction of Na^+ binding to the indicator (Naumann, Lippmann, & Eilers, 2018). Thus, we report here how the Na^+ dye (Basic Protocol 2) can be calibrated using intracellular solutions with different Na^+ concentrations, from 0 to 25 mM. Subsequently, the number of light counts is plotted against the Na^+ concentration. The calibration needs to be performed once independently (or several times for statistical accuracy), and not after every experiment.

Additional Materials *(also see Basic Protocol 2)*

1 M NaCl
Na^+-free intracellular solution (see recipe; make fresh)
1.6 mM ING-2 (Ion Indicators) Na^+ dye
Deionized water

1. Prepare a serial Na^+ dilution, from 0 to 25 mM Na^+ concentration, in steps of 2.5 mM, with 38 µl per dilution:
   a. Stock solution: Add 25 µl of 1 M NaCl to 475 µl Na^+-free intracellular solution to obtain a 50 mM Na^+ concentration.
   b. For 25 mM Na^+: Dilute 20 µl stock with 18 µl Na^+-free intracellular solution.
   c. For 15 mM Na^+: Dilute 12 µl stock with 26 µl Na^+-free intracellular solution.
   d. For 12.5 mM Na^+: Dilute 10 µl stock with 28 µl Na^+-free intracellular solution.
   e. For 10 mM Na^+: Dilute 8 µl stock with 30 µl Na^+-free intracellular solution.
   f. For 7.5 mM Na^+: Dilute 6 µl stock with 32 µl Na^+-free intracellular solution.
   g. For 5 mM Na^+: Dilute 4 µl stock with 34 µl Na^+-free intracellular solution.
   h. For 2.5 mM Na^+: Dilute 2 µl stock with 36 µl Na^+-free intracellular solution.
   i. For 0 mM Na^+: Prepare 38 µl Na^+-free intracellular solution.
   j. Add 2 µl of 1.6 mM ING-2 Na^+ dye to each dilution (80 µM final concentration). Mix well by vortexing and protect from light.

   We have found that ∼40 µl is the minimal volume that allows dipping the 60× objective while focusing on a surface.

2. Perform calibration:
   a. Start with 0 mM Na^+. Pipet 40 µl into microscope recording chamber and lower 60× objective into the solution. Do not change position of the objective after this initial positioning. Measure fluorescence with the camera.
   b. Remove solution, rinse with deionized water, and pipet next concentration (2.5 mM Na^+) into the recording chamber. Measure fluorescence again.
   c. Repeat this process until fluorescence has been measured at all concentrations.

3. Analyze results:
   a. Plot number of light counts against Na^+ concentration in mM, as shown in Figure 5C.
   b. Fit linear part (usually between 2.5 and 15 mM) of the plot to obtain the linear relationship between light counts and Na^+ concentration:

$$F\left(\left[Na^+\right]\right) = a \cdot \left[Na^+\right] + b$$

4. Considering that the intracellular Na^+ concentration at rest is 10 mM, or $F_0 = F([10$ mM$])$, calculate concentration at 1% change in fluorescence (1% $\Delta F/F_0$, or $[Na]_\alpha$):

a. Calculate light count for F_0 by simply solving the equation for F([10 mM]).
b. Solve $\Delta F/F_0 = 1\%$, which can also be written as $(F1 - F_0)/F_0 = 0.01$.

As b is 10 mM, this gives $[Na]_\alpha = 0.01 \cdot F_{0a} + 10$.

DATA ANALYSIS

BASIC PROTOCOL 3

In this protocol, it is explained how to process the recordings from Basic Protocol 2 in order to extract the Na^+ fluorescence change and calculate the Na^+ current. Data analysis is performed using custom-written Matlab scripts in Windows 7 or 10. All files can be found at *https://www.mathworks.com/matlabcentral/fileexchange/83708-sodium-imaging*.

Materials

Recordings (see Basic Protocol 2)
Matlab (see step 1 for version) and custom Matlab scripts (see above)
Calibration data (see Support Protocol)

1. *Convert ".tsm" files and open files:* Convert recordings (".tsm" files) into Matlab files with a version of Matlab prior to 2011. Alternatively, translate ".tsm" files into standard ".tif" files and then open with any version of Matlab. Finally, save image sequences as ".mat" files.

   The custom-made software of the DaVinci 2K CMOS camera automatically saves image sequences in ".tsm" format.

2. *Averaging trials:* Average aligned recordings to increase SNR:

   a. To check if recordings are temporally aligned, superimpose their electrically recorded APs.

      We average up to eight recordings not affected by photodamage. The recordings must be temporally aligned in order to average sequences, i.e., the APs of individual trials must perfectly superimpose. If this is not the case, the analysis can still be performed on individual trials.

      If the duration or the peak of an AP differs by >0.1 ms (one frame), the recording is not suitable for averaging.

   b. To check if recordings are spatially aligned, superimpose images. If needed, move them on *x/y*-plane in order to compensate for possible small lateral movements of the preparation during the recording.
   c. Check bleach recording for lateral movements and align if necessary.
   d. Average all temporally and spatially aligned trials and use any of their APs as a reference.

3. *Extracting Na^+ signals:* Perform pre-processing to simplify procedure and correct the signal for photobleaching:

   a. Load ".mat" files.

      The image sequence is a three-dimensional matrix that represents the fluorescence signal $F_{in}(x,y,t)$*, corresponding either to an average or to a single recording.*

   b. To subtract the autofluorescence, choose an unstained region U(x,y,t) in first image and subtract mean fluorescence intensity from the raw recording to remove the effect of the background on the fractional fluorescence change ($\Delta F/F_0$):

$$F(x, y, t) = F_{in}(x, y, t) - mean(U(x, y, t))$$

      As an example, see Figures 6A and 6B.

   c. Choose a single pixel or a region of interest to average (Fig. 6A).

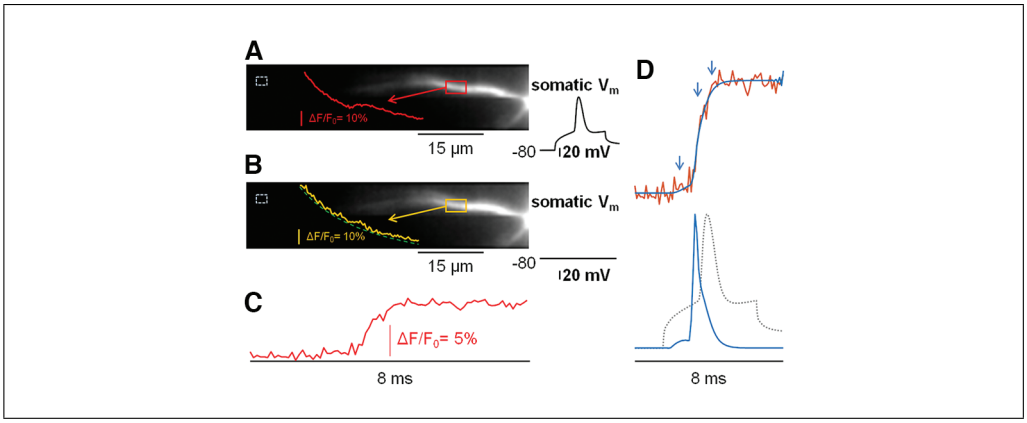

Figure 6 Extraction of Na^+ currents. (**A**) AIS of an L5 pyramidal neuron loaded with ING-2 in the recording position. The background region is indicated with the gray rectangle. A region of interest is outlined by the red rectangle. The trace on the right is the somatic AP elicited by a current pulse. The red trace is the ING-2 $\Delta F/F_0$ from the region of interest recorded at 10 kHz and associated with the AP. (**B**) Bleach recording. The yellow trace is a recording from the same region without stimulation. The green dashed trace is the tri-exponential fit of the yellow trace. (**C**) ING-2 $\Delta F/F_0$ after bleach correction. (**D**) Top, Na^+ signal fitted with the model function reported in Basic Protocol 3, step 4. The three arrows point to different phases of the δC increase, corresponding to a subthreshold component, a fast increase, and a slower increase. Below, the Na^+ current calculated as the time derivative of the fit. The $\Delta F/F_0$ trace is from an average of five trials.

d. Calculate F_0 by averaging the mean fluorescence over some of the first frames without stimulus (for instance, the first six frames):

$$F_0(x, y) = mean(F(x, y, 1:6))$$

e. Calculate $\Delta F/F_0$ for each pixel (Fig. 6C):

$$\Delta F/F_0 = \frac{F - F_0}{F_0}$$

f. Apply steps 3a to 3e in parallel with the bleach recording to get its fractional fluorescence change ($\Delta B/B_0$) (Fig. 6C).

g. Fit $\Delta B/B_0$ with a tri-exponential function Tr(y,t) to get a smooth bleach trace that will not introduce additional noise and/or artifacts into Na^+ signal (Roder & Hille, 2014) (Fig. 6D):

$$Tr(y, t) = C_1 e^{C_2 \cdot t} + C_3 e^{C_4 \cdot t} + C_5 e^{C_6 \cdot t} + C_7$$

h. To estimate the difference in the light level between the signal and the bleach recording, for each pixel, divide standard deviation of the first eight frames of the raw signal recording by that of the raw bleach recording:

$$fr = \frac{std(G(x, y, 1:8))}{std(Bl(x, y, 1:8))}$$

This is only a first estimate since this value cannot be precisely calculated because of the noise in both recordings.

i. Finally, to get the corrected $\Delta F/F_0$ signal for photobleaching, subtract from it the fitted bleach trace multiplied by the light-difference factor (Fig. 6D):

$$S = \frac{\Delta F}{F_0} - fr \cdot Tr$$

As mentioned, fr is only a first estimation, and so if any trace does not have mean zero up until the frame when the current stimulation begins, change its fr value and repeat step 3i.

4. *Translating signal into number of ions:* Use the calibration described in the Support Protocol to convert the *S* signal into an Na^+ concentration (in mM) and then convert signal into a number of ions by estimating the volume of the axon:

   a. Use calibration to convert the fluorescence change into concentration of Na^+ in mM:

   $$\left[Na^+\right] = S \cdot 17.4\ mM$$

   b. Estimate radius of the axon, at each distance from the soma, considering that the size of a pixel is 0.5 µm. Apply a simple mask to fluorescence image so that the pixels below a certain threshold are set to zero. Then, obtain radius by using the number of consecutive positive pixels at the line x, and fit the following equation against the *x*-axis to obtain a smooth, realistic profile:

   $$r = A \cdot x^B + C$$

   Direct measurement of the radius is impossible with our spatial resolution.

   c. Calculate volume (V) and lateral surface (A), where h = 0.5 µm, r_1 = r(y), and r_2 = r(y + 1):

   $$V = \frac{\pi}{3} \cdot \left(r_1^2 + r_2^2 + r_1 \cdot r_2\right) \cdot h$$

   $$A = \pi \cdot (r_1 + r_2) \cdot \sqrt{(r_1 - r_2)^2 + h^2}$$

   This calculation stems from the fact that each compartment of the axon has the shape of a truncated cone.

   d. Convert Na^+ concentration into the number of Na^+ ions per unit volume using Avogadro's number, $N_{av} = 6 \times 10^{23}$ mol^{-1}:

   $$N_i = \left[Na^+\right] \cdot N_{av}$$

   e. Evaluate kinetics of the S signal, which depends on Na^+ influx and diffusion (Zylbertal, Kahan, Ben-Shaul, Yarom, & Wagner, 2015).

   In a previous report, we estimated the contribution of longitudinal diffusion to the kinetics of the Na^+ transient in the AIS of L5 pyramidal neurons (Filipis & Canepari, 2020). We found that in the case of one AP, diffusion can be neglected while quantifying the rising slope of the Na^+ signal, which typically lasts 1 to 2 ms. We predict, however, that this might not be the case in other systems, where longitudinal diffusion must therefore be considered before calculating the time derivative of the Na^+ transient.

   f. Convert Na^+ concentration into charge surface density using the charge constant $C = 1.6 \times 10^{-19}$ Coulombs:

   $$\delta C = \left[Na^+\right] \cdot N_{av} \cdot V \cdot C/A$$

   This process (see steps 4a to 4f) is necessary to calculate the longitudinal diffusion, and then the Na^+ current, which is expressed in Amperes, is finally calculated (see step 5).

5. *Calculating Na^+ currents:* The noise of the optical recordings is too large to extract a kinetically faithful curve by directly calculating the time derivative. A way is to apply a smoothing filter that improves the SNR while minimally affecting the time course of the signal, but this procedure is generally insufficient. Fitting the δC signal with an appropriate function is therefore the best solution. The choice of the model function to fit is not unique, and the more complex the function, the more accurate the fit will be. However, we recommend choosing a "minimal" function, where the fit is performed on the lowest possible parameter that can be used to quantify a component of the Na^+ current. The model that we used was built according to the characteristics of

the signal, which shows two components, a subthreshold signal and a suprathreshold signal. The subthreshold signal (first arrow, Fig. 6D) starts after the stimulation at the soma but before the beginning of the somatic AP. The suprathreshold signal is a steep increase starting at the beginning of the somatic AP (second arrow, Fig. 6D) followed by a slower increase (third arrow, Fig. 6D). Considering these characteristics, the model used to fit the signal is a two-component function:

$$F_x(t) = F_x^{sub}(t) + F_x^{supra}(t)$$

where the subthreshold component $F_x{}^{sub}(t)$ is a function with $dF_x{}^{sub}(t)/dt = 0$ before $\tau = t$(current injection), slowly increasing for $t > \tau$:

$$F_x^{sub} = \begin{cases} 0 \text{ for } t \leq \tau \\ \alpha \cdot (1 - e^{-\beta \cdot (t-\tau)^2}) \end{cases}$$

The suprathreshold component $F_x{}^{supra}(t)$ is the product of the three sigmoid functions, with the first two matching the fast δC increase and the third sigmoid matching the slow δC increase:

$$F_x^{supra}(t) = \gamma \cdot \left(\frac{1}{1+e^{(\eta_1 - t)\cdot \nu_1}}\right) \cdot \left(\frac{1}{1+e^{(\eta_2 - t)\cdot \nu_2}}\right) \cdot \left(\frac{1}{1+e^{(\eta_3 - t)\cdot \nu_3}}\right)$$

Use function above to maximize the likeliness between the model and the experimental trace with the following steps:

a. Set parameters η_1 and η_2 equal to the exact time of the fast δC increase and parameter ν_1 equal to the half sampling time of the fluorescence acquisition.
b. To find the parameters α and β, use Expectation-Maximization (EM) algorithm in Matlab to fit the signal up to η_1-2 with $F_x{}^{sub}$.
c. To find the parameters γ, η_3, ν_2, and ν_3, use EM algorithm on the entire signal to fit F_x.
d. Calculate Na^+ current at position y as

$$I_{Na}(t) = \frac{F_x(t) - F_x(t-1)}{\delta t}$$

where $\delta t = 0.1$ ms is the sampling time (Fig. 6D).

REAGENTS AND SOLUTIONS

Artificial cerebrospinal fluid (ACSF), 1×

Combine 125 mM NaCl, 26 mM $NaHCO_3$, 1 mM $MgSO_4$, 3 mM KCl, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, and 23 mM glucose. Prepare fresh immediately before use. Alternatively, prepare a 5× stock solution without $CaCl_2$ and glucose to prevent microorganism growth and store ≤2 months at 4°C. After diluting the stock solution, add glucose and calcium on the day of the experiment. We do not recommend preparing a 10× stock solution because it easily precipitates.

This solution, when oxygenized, is used to maintain brain slices, and it is constantly perfused around the slices when performing combined electrophysiology and imaging experiments (see Basic Protocol 2).

Intracellular solution

125 mM $KMeSO_4$
5 mM KCl
8 mM $MgSO_4$
5 mM Na_2-ATP

0.3 mM Tris-GTP
12 mM Tris-phosphocreatine
20 mM HEPES
Adjusted to pH 7.35 with KOH and to 295 mOsm with H_2O
Aliquot in vials of 1 ml
Store ≤6 months at −20°C to prevent ATP/GTP degradation

Na^+-free intracellular solution

125 mM $KMeSO_4$
5 mM KCl
8 mM $MgSO_4$
0.3 mM Tris-GTP
12 mM Tris-phosphocreatine
20 mM HEPES
Prepare fresh immediately before use

Slicing solution

125 mM NaCl
26 mM $NaHCO_3$
2.5 mM $MgSO_4$
3 mM KCl
1 mM NaH_2PO_4
0.5 mM $CaCl_2$
23 mM glucose
Prepare fresh immediately before use

COMMENTARY

Background Information

The sensitivity of Na^+ fluorescence imaging is highly dependent on the indicator. Optical measurements of Na^+ using the indicator SBFI allowed the reconstruction of a spatial profile of Na^+ along the axon of L5 somatosensory cortex neurons (Baranauskas et al., 2013; Fleidervish et al., 2010; Katz et al., 2018; Kole et al., 2008), but with insufficient temporal resolution to reconstruct the kinetics of the Na^+ current. Improved dyes such as the ING-2 reported here (Basic Protocol 2 and Support Protocol) allow higher acquisition rates that enable reconstructing Na^+ currents during physiological APs along the AIS. Although there is also a 4-fold improvement in the spatial resolution due to the optimized optical system developed in our laboratory (Filipis et al., 2018), the real spatial resolution is, however, limited by light scattering to a couple of microns. The improvements in terms of temporal resolution described in this article depend on the indicator, and we expect that better Na^+ dyes that may be available in the near future will allow higher acquisition rates with a better SNR. For ING-2, we established that a critical limitation is its maximal tolerated concentration, which is 500 μM. This parameter also limits the rate and the number of trials. We wrote this article considering the prospect of using the described protocol details with future indicators that have sensitivity similar to ING-2 but that can be used at higher concentrations, permitting larger numbers of trials at higher acquisition rates.

Critical Parameters

Diffusion

A striking difference between Ca^{2+} and Na^+ dynamics in the cell is that the former is dominated by binding to mobile and immobile endogenous Ca^{2+} buffers (Ait Ouares, Filipis, Tzilivaki, Poirazi, & Canepari, 2019; Ait Ouares, Jaafari, & Canepari, 2016; Canepari & Mammano, 1999). In contrast, Na^+ remains mostly free in the cytosol, and it spatially equilibrates by diffusion before being extruded by the cell. In the case of the AIS of L5 pyramidal neurons, during a single AP, the contribution of longitudinal diffusion to the fast Na^+ kinetics is marginal (Filipis & Canepari 2020). However, in principle, diffusion cannot be ignored in other systems. An example might be the AIS of other neurons, where a different shape of the axon hillock might cause Na^+ to diffuse more rapidly in the direction of the soma. In the case in which diffusion

Table 1 Troubleshooting Guide for Sodium Imaging of the Axon Initial Segment in Brain Slices

Protocol	Problem	Possible cause	Evaluation	Solution
1	Brain hemisphere unglued while slicing	Glue dried	Ensure that the other hemisphere is well glued	Set the unglued hemisphere aside, out of reach of the steel blade but inside the slicing solution. Continue slicing the other hemisphere and, when finished, glue the unglued hemisphere again and slice.
2	Spilled dye	Too slow of an approach to the cell before patching, insufficient front-filling of the pipet with intracellular solution, or too-strong pressure during the fast approach phase	Check whether a high fluorescence background is observed after establishing the whole-cell configuration	Patch another cell
2	Axon moved out of focus in between recordings	Mechanical instability	Make sure that the table is pumped and mechanically uncoupled from the external environment. Check under transmitted light for potential drift of the pipet.	During recordings, manually return the AIS to its original position using low light. After termination of the experiment, improve the stability of your system.
3	Mean signal before stimulation is not 0 after the bleach correction	This happens sometimes in parts that are in different focus during the signal and the bleach recording	Ensure that neighboring regions have similar signals	Fit the bleach data with a different function such as the bi-exponential or the power 2

contributes substantially to the Na^+ dynamics, correcting for diffusion is necessary before calculating the time derivative of the Na^+ concentration (Basic Protocol 3).

Resources

In this article, we have described in detail the equipment used in our laboratory. This includes a dedicated set of optical tools that we have found optimal for the technique (Basic Protocol 2). We use 10× and 60× objectives and two demagnifications (1× and 0.5×) to change the field of view and the equivalent pixel size, adapting for higher resolution (250 nm per pixel) under transmitted light during the patch and lower resolution (500 nm per pixel) to collect more photons during Na^+ imaging. However, alternative optical settings can be used according to what is available in the laboratory. It must be said that the CMOS camera that we use allows 2 × 2 binning. This arrangement, however, is not equivalent to changing from 1× to 0.5× demagnification from the point of SNR improvement. Indeed, CMOS binning changes the well capacity of the larger pixel, but the digits corresponding to the number of photons also change accordingly, and a gain cannot be set in this device. Therefore, we recommend optimizing the demagnification using hard optics.

Troubleshooting

A list of problems that can be encountered during these protocols and their possible solutions is shown in Table 1.

Understanding Results

These protocols describe how to acquire dynamic Na^+ optical measurements with unprecedented spatiotemporal resolution, allowing reconstruction of the Na^+ currents along the AIS of neurons in mouse brain slices. Obtaining signals with a high SNR along the whole AIS requires careful preparation of slices (Basic Protocol 1), selection of neurons (Basic Protocol 2), optimal imaging steps (Basic Protocol 2), and robust analysis (Basic Protocol 3), as described in this article. The sample data given in Figure 6D demonstrate a slope change before the peak of the signal and a subthreshold signal. Therefore, the product of three sigmoid functions is used to fit these signals. The first sigmoid sets the baseline before the onset of the signal, whereas the other two sigmoids set the shapes of a first and second component. An additional sigmoid is used to reproduce the subthreshold component. The derivative of the fit at the bottom of Figure 6D shows a first sharp current with fast kinetics and a second one with slower kinetics. It is recommended to quantitatively analyze the time course of the current with respect to the parameters of the somatic AP. In this example, the peak of the AP shows a delay from the current peak because the driving force for Na^+ is much larger at the onset of the AP with respect to the time to peak. The second component, attributed to slowly inactivating VGNCs, coincides with the AP repolarization. These results demonstrate the possibility of optically measuring the physiological Na^+ current underlying the generation of a neuronal AP, enabling a plethora of future studies that can unravel the role of specific VGNCs in initiating and propagating the AP under physiological conditions or pathological conditions linked to several brain disorders.

Time Considerations

Performing the measurements described in this article requires 1 day of experiments. In detail, preparing the solutions in the morning requires 30 min, and slice preparation (Basic Protocol 1) requires ∼40 min, with ∼2 hr of preliminary work. Slices can be used up to ∼6 hr, after which the tissue starts decaying. The time needed to choose the optimal cell to patch can vary from 2 min to 1 hr (Basic Protocol 2). A single optical recording requires ∼1 hr, including 30 min to load the cell with the dye and 30 min to perform optical measurements (Basic Protocol 2). Calibration of the Na^+ fluorescence requires ∼2 hr (Support Protocol). Analyzing a recording to extract the Na^+ fluorescence change and calculate the Na^+ current requires ∼30 min (Basic Protocol 3).

Acknowledgments

This work was supported by the Agence Nationale de la Recherche through three grants (ANR-18-CE19-0024 - OptChemCom; Labex *Ion Channel Science & Therapeutics*, program number ANR-11-LABX-0015; and National Infrastructure France Life Imaging "NoeudGrenoblois") and by the *Federation pour la recherché sur le Cerveau* (FRC–Grant *Espoir en tête*, Rotary France).

Author Contributions

Laila Blomer: Investigation; validation; visualization; writing-original draft; writing-review & editing. **Marco Canepari:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review

& editing **Luiza Filipis:** Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing-original draft; writing-review & editing.

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