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

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Article Title

Ultrafast sodium imaging from the axon initial segment of neurons in mouse brain slices

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SIGNIFICANCE STATEMENT:

Voltage-gated Na^+ channels are transmembrane proteins which facilitate the generation of the action potential, i.e. the most fundamental physiological process in neuronal cells. Measuring the Na^+ current that initiates the action potential in the initial segment of the axon is therefore crucial to understand this mechanism. In this unit we describe a method for ultrafast Na^+ imaging of neurons in brain slices that allows the first optical measurement of this current. The significant improvement in the temporal resolution of Na^+ imaging enables the direct measurement of Na^+ currents under physiological and pathological conditions associated with dysfunctions of voltage-activated Na^+ channels.

ABSTRACT:

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 a protocol to obtain this measurement, focussing 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 described the preparation of optimal slices, patching and loading L5 pyramidal neurons with the Na^+ indicator ING-2 and then achieving 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.

KEYWORDS:

Sodium imaging, voltage-gated sodium channels, axon, action potential, pyramidal neuron, somatosensory cortex.

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 dysfunctions in these channels (Wimmer et al., 2010).Standard electrode techniques have been used to record fast Na^+ currents mediated by VGNCs (Yue et al., 2005; Astman et al., 2006; Alle et al., 2009). 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 (Kole et al., 2008; Fleidervish et al., 2010; Baranauskas et al., 2013). 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 benzofuran isophthalate (SBFI, Minta &Tsien, 1989). However, more recently, the Na^+ indicators Asante NaTRIUM Green-2 (ANG-2, Miyazaki & Ross, 2015; Miyazaki et al., 2019) and the commercially available IonNaTRIUM Green-2 (ING-2, Ion Indicators, Austin, TX) have been shown to providea 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 optical AP recordings using voltage sensitive dyes (Popovic et al., 2015). Similar to the optical measurement of dendritic Ca^{2+} currents (Jaafari et al., 2014; Jaafari et al., 2015; Jaafari & Canepari, 2016), 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 temporal resolution of 100 µs and spatial resolution of half a micron. Although this unit presents the case of the AIS of L5 somatosensory cortex pyramidal neuron in mouse brain slices, the same technique can be used to record Na^+ transients in any neuron type by adapting the protocol 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. A Support Protocol is included to describe the calibration procedure used to express fluorescence changes into changes of sodium concentration. Lastly, Basic protocol 3 presents a data analysis procedure that finally leads to the extraction of the Na^+ current.

NOTE: Experiments described here are 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 to the animal facility of the university (D3842110001). We used 21-35 postnatal days old mice (C57BL/6j) purchased from Janvier Labs (Le Genest-Saint-Isle, France). These animals are 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

BASIC PROTOCOL TITLE

CORTICAL SLICES PREPARATION

Introductory paragraph:

Some general considerations about preparing healthy brain slices have been published in other protocol units (see for example Madison & Edson, 2001). To be able to record Na^+ currents in L5 pyramidal cells, it is important to prepare cortical slices that leave these neurons unblemished. In addition, it is

important that patched cells are parallel to the slice, so 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 1st layer, and the cortex is curved around the midbrain, 2-3 sagittal slices per hemisphere, cut with a 15 degree angle from the horizontal plane, contain unscathed pyramidal cells.

Materials:

Mouse (C57Bl6 strain, P21-35, JanvierLabs, France)
Freezer
Isoflurane (Isoflurin 1 ml/ml, Axiance, France)
Airtight chamber (used for anesthesia)
O_2(95%)/CO_2(5%) gas mixture (carbogen).
PVC tubing and stopcocks to supply the carbogen at three different points simultaneously (Cole-Parmer, France)
Vibratome (VT 1200, Leica, Wetzlar, Germany) with custom made holder with 15 degrees inclination
Stainless steelblades (Item 7550-1-SS, Campden Instruments, Loughborough, UK)
37 degrees water bath (Thermo Fisher Scientific, USA)
Operating scissors 14 cm length (Item 501218-G, World Precision Instruments, USA)
Fine scissors 10.5 cm length (Item 14094-11, Fine Science Tools GmbH, Germany)
Spring scissors 5 mm cutting edge (Item 91500-09, Science Tools GmbH, Germany)
Adson forceps 12 cm (Item 14226-G, World Precision Instruments, USA)
Chattaway spatula 15 cm (Item SH285-15, King Scientific, UK)
Razor blade (Item S65921, Fisher Scientific, Canada)
Slice holder: beaker with mesh to keep slices (either custom made in the lab or purchased, e.g. model CL.7450-2A from Campten Instruments, UK)
Artificial cerebrospinal fluid (ACSF) 1X (see Reagents & Solutions for recipe)
Slicing solution (see Reagents & Solutions for recipe)
Glue (for instance KRYLEX KB0624)
Filter paper
Glass petri dishes
Paper towels
Glass beakers
Plastic balloon pipette, the end cut off to have a ~0.5 cm opening (diameter) to transport brain slices
Syringe with bended needle, so the tip has a 90-degree angle (used as a mini-blade)

Protocol steps—*Step annotations:*

Preparation of slices

1. Prepare 150 mL of slicing solution and keep at -20°C for 90-120 minutes.
2. Prepare 800 mL of ACSF and bubble with carbogen for at least 20 minutes before starting the dissection.

3. Take the slicing solution out of the freezer and break the formed ice with a spatula (no need to form a slurry, but you should avoid having big chunks of ice). Bubble the solution with carbogen for at least 20 minutes before starting the dissection.
4. Build or purchase a slice holder to place the brain slices in and keep them below the surface of the ACSF. A slice holder can be easily constructed by using a stretchable net, a glass beaker, a plastic ring and PVC tubes. Create a plastic ring cutting the midle of a big tap and the neck from a bottle or jar that can fit inside the glass beaker (Figure 1A). Then, screw a stretched nylon hose between the tap and the neck to hold the slices (Figure 1B). Use two pieces of PVC tubes to fix the ring inside the beaker glass and leave space for the carbogen tube to fit in the edge (Figure 1C). Avoid gluing the ring inside the beaker glass so that you can easily remove it to clean it.
5. Dissection and slicing preparations. Figure 2A
    a. Fill the slice holder with oxygenated ACSF and place in the 37°C water bath. Place a cover on top of the slice holder to prevent evaporation and bubble with carbogen letting the solution equilibrate in temperature.
    b. Mount a fresh blade on the Vibratome and calibrate the instrument according to the manufacturer's instructions.
    c. Take out necessary instruments for the brain dissection. Figure 2B

Brain dissection

Follow the protocol approved by your institution and by local authorities

6. To anaesthetize the mouse we use isoflurane injected in a custom-made airtight chamber installed in a fume hood, but any commercial system equipped with anaesthetics vaporizer can be used. The dimensions of our chamber (lengthXwidthXheight) in cm are: 16X16X12. We use ~200 µL of isoflurane delivered through both sides of a cotton swab and we wait around 1 minute until the mouse is immobile before decapitating it using operating scissors.
7. Dissect out the brain quickly.
    a. Take the head of the mouse, place on a paper towel, and, using your fingers, peel back the skin from the back over the head towards the nose.
    b. Take the fine scissors and cut away any brainstem tissue and surrounding bone.
    c. Take the spring scissors and carefully cut along the midline of the brain. First cut through any remaining skin layers, and then through the skull. Cut past the intersection of Bregma.
    d. Use forceps slightly move under one half of the skull, lift the bone and force it to move upward, breaking the bone and exposing the brain. Do this on both sides of the cut.
    e. Move the ice in the slicing solution so that a small space of clear solution is created. Take the chattaway spatula and carefully lift the brain from the base of the skull and let the brain sink in the slicing solution, all the while bubbling with carbogen. Leave the brain to rest for 3 – 4 minutes.
8. While the brain is resting in the slicing solution, take the Vibratome holder with the 15 degrees inclination and spread a thin layer of glue on two locations next to one another. Place an object under the disk to counteract the 15 degrees inclination and make it horizontal, so the glue does not run. A 15 degrees inclination holder can be made in the laboratory using a metal piece (if

the necessary equipment to cut metal is available). Cut the metal piece similar to the flat holder provided by the Vibratome but instead of having the top part flat, cut a 15 degree inclination, following the schematic given in Figure2C.

9. Dissect out the cortices.
    a. Carefully take the brain from the icy slicing solution and place, dorsal side up, on a piece of filter paper to allow excessive fluids to be removed.
    b. Take a razor blade and make a coronal section straight down from lambda, to separate any remaining cerebellum and hindbrain.
    c. Similarly, cut away the olfactory bulbs if still attached.
    d. Make a sagittal cut, straight down the midline of the brain, dorsal to ventral, separating the two hemispheres.
10. Mount both hemispheres on the Vibratome holder.
    a. Using the forceps and spatula, carefully lift one of the hemispheres and place on the layer of glue on the Vibratome 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 the second hemisphere, again making sure the dorsal side faces down-hill, and the cut side is glued. Very gently press the top of the two hemispheres down to make sure they are well connected to the holder. Figure 2D
    b. Install the holder in the Vibratome basin.
    c. Take the slicing solution and carefully pour everything in the Vibratome basin, making sure that no ice touches the hemispheres. Bubble with carbogen.

Brain slicing

11. Position a clean blade in the vibratome clamp, and move the holder up ~14000 µm. Set the speed to 0.14 mm/s, make sure the device is in 'manual' mode and make a slice. The following described procedure is dependent on your tissue slicer model, so modify accordingly.
12. When the blade is completely through the two cortices, press stop and move the holder down 100 µm, so that the blade is not touching the top of the brain tissue. Using a 3 mL plastic balloon pipette with its end cut off, suck up the slices and discard them. Move the blade back to its starting point.
13. Take thin slices (350 µm), discarding them until the hippocampus becomes visible. After every slice, remember to move the holder down 100 µm before moving the blade back to the starting point.
14. Once you clearly see the hippocampus, you have reached the area with optimal slices to collect. Change the speed to 0.06 mm/s and set the cutting thickness to 350 µm. 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 the slice would be too big to fit inside the balloon pipette without folding. Instead use the syringe with the bended point as a mini-blade to cut around halfway through the cortices and save only the part with the cortex. Do not cut the hemispheres at half before slicing because the exact position of the hippocampus is unknown without seeing it and you might cut part of the cortex.

After every slice move the holder down 100 µm. Discard the first slice made with this speed and start saving the next ones. Make 4 to 5 slices of 350 µm per hemisphere as outlined below.

a. Make sure there are no bubbles under the net in the slice holder. Use the balloon pipette to suck away any formed bubbles that would disturb the slices.
b. Start to cut a slice of 350 µm. Press 'stop' once the blade has cut through the cortex of both hemispheres and partly through the hippocampus, but do not let it cut through the end of the cortices (Figure 2E,F).
c. Each time, move the holder down 100 µm. This is to avoid pressing down on the rest of the brain in step d.
d. Take the syringe with the bended point (to use as a mini-blade) and gently press down on the slice on top of the steel blade to separate the sliced cortex from the rest of the brain. Suck up the cortical slice with the balloon pipette, and deposit the slice in the slice holder (Figure 2G).
e. Move the blade to the starting point. Move the holder up 450 µm and make another 350 µm thick slice.
f. Keep slicing until the hippocampus is no longer visible.
g. Keep information about the slices you collect. 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 at all the different layers of the cortex (see below).Note this information for every slice. (Figure 2E)

15. Clean the Vibratome, rinsing with DI water and dry fully, as the salts in the slicing solution tend to crystallize, damaging the machine.
16. Leave the slices at 37°C for 30 minutes while bubbling with carbogen.
17. Take the slice holder from the water bath while continuing bubbling with carbogen, and leave to cool down at room temperature (22-24°C). After ~1 hour the slices are ready for the experiment.

BASIC PROTOCOL 2

BASIC PROTOCOL TITLE

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

Introductory paragraph:

To perform patch clamp recordings in brain slices, one should see other units that have addressed in detail the general technical aspects that involve the set-up 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 are secured in a recording chamber installed in the electrophysiology system and 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 is either cut halfway or it 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.

Materials:

DMZ puller (Martinsried, Germany)
Borosilicate glass pipettes (OD = 1.5 mm, ID = 1.1 mm, Harvard Apparatus)
Syringes (20 mL), stopcocks and PVC tubing of various sizes to apply pressure (Cole-Parmer, France)
Syringes (1 mL)
Nylon filters (0.2 µm diameter)
0.5 mL tubes
Mini-centrifuge for 1.5 mL and 0.5 mL tubes (such as 75004061 from Thermo Fisher Scientific, USA)
Microloader tips 20µL (Item 5242956003, eppendorf, France)
Forceps (Item 11251-20, Fine Science Tools GmbH, Germany)
Harp slice grids to keep the slice in place (from ALA Scientific, Farmingdale, NY)
Diluted ING-2

Electrophysiology set-up

- Slicescope Pro system (Scientifica, Uckfield, UK), including microscope, motorized XY translation stage and PatchStar manipulators mounted on a TMC anti-vibrant table.
- Olympus 60X objective (water immersion, NA = 1, WD = 2 mm) and a standard 10X air objective (for initial broad visualization only).
- System for IR DIC also provided by Scientifica.
- CMOS DaVinci-2K camera (SciMeasure, Decatur, GA).
- Data acquisition software Turbo-SM provided by RedShirtImaging (Decatur, GA).
- 1X/0.5X variable demagnification before the camera (Cairn Research, Faversham, UK).
- Multiclamp amplifier 700A (MolecularDevices, Sunnyvale, California).
- A/D acquisition board USB-6221 board (National Instruments, Austin, Texas) controlled by Matlab.
- Scientifica perfusion system with temperature control.
- Manometer to measure the pressure given to the patch electrode (World Precision Instruments,USA).

Illumination and Imaging tools

- Tri-Line Laser-Bank (Cairn Research) through a Ø550 µm, 0.22 NA, SMA-SMA Fiber Patch Cable (Thorlabs, Newton, NJ), with multimode diode head 520 nm / 0.5W (Ushio).
- Telescope with two lenses (Cairn Research) for wide-field or ~30 µm illumination spot at the output of the fibre.

- Filter cube comprising excitation (517 ± 10 nm) and emission (559 ± 17 nm) filters and a 538 nm dichroic mirror. We recommend the FF01-517/20, FF01-559/34 and the FF538-Di01 from Semrock (Rochester, NY).
- Pulse stimulator Master-9 (A.M.P.I., Jerusalem, Israel)

Protocol steps—*Step annotations:*

Preliminary work

1. Pull patch pipettes of 4-5 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 the ACSF from protocol 1 to continuously perfuse the recording chamber at a rate of ~2 mL/min. Use the same rate for vacuuming away the ACSF. Heat the perfusion pathway to keep the temperature in the recording chamber at 32-34°C (nearly physiological temperature).
3. Dilute the sodium dye.
    a. Thaw 1 mL of intracellular solution and use the 0.2 µm nylon filter to clean the solution.
    b. Dilute the sodium dye to 0.5 mM in intracellular solution in a clear 0.5 mL tube. Pay attention to protect the dye from light.
    c. The ING-2 indicator can contain some impurities. Thus, vortex thoroughly and centrifuge the tube. Carefully not to disturb the pellet, take the supernatant and deposit in a clean 0.5 mL. Repeat this process until no pellet is visible after centrifuging.
    d. Keep the tube on ice to prevent degradation of the intracellular solution.
    Keep the rest of the intracellular solution in a syringe without needle, with a nylon filter, on ice.

Slice selection

4. Transfer a single brain slice to the recording chamber. Adjust the slice position using forceps.
5. Set the camera software to focus mode and use a 1024X1024 configuration.
6. Using 1X demagnification and the 10X objective, focus on the slice surface. Ignore the prefrontal part of the cortex and the subiculum. Look at the widest part of the cortex, the somatosensory areas. Identify if there are blood vessels running in parallel with the slice surface all the way from the dorsal end towards the hippocampus (Figure 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 et al., 2015).
7. Before rejecting a slice, it is recommended to flip it to check if the cells on the other side are more parallel with the focal plane.
8. Once a slice with parallel blood vessels is selected, place the harp grid to hold the slice. Try to place the 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 the 60X objective and focus on the slice surface. Use IR light and DIC.
10. Move to the dorsal end and save the position of the XY stage. The cell bodies of L5 pyramidal neuron are located in the middle part of the cortex at ~500 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 their 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, show little contrast with their surrounding and the nucleus is difficult to identify. Unhealthy cells are usually swollen and their enlarged round nucleus is clearly visible, indicating the cell is about to release its contents and die. Select cells located 20-35 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, while cells below 35 µm are more difficult to image as the larger scattering prevents good measurements.
13. Save the position of the slice surface above the cell and move the objective up, out of the solution.

Patching a pyramidal neuron

14. To avoid spilling of the dye, front-fill a patch pipette with clear intracellular solution. Position the tip of the pipette in a droplet of intracellular solution and provide slight negative pressure from the top of the pipette using some tubing and a syringe. To only fill the tip, apply this negative pressure for ~20 seconds (Figure 4C).
15. Using a syringe with a micro-loader, back-fill the pipette with the dye diluted in intracellular solution (Figure 4D). Make sure there are no bubbles in the tip before mounting the pipette on the holder.
16. Move the patch pipette down to the slice surface.
    a. Apply ~20 mbar positive pressure (measured with the manometer) on the pipette (Figure 4E). This moderate pressure keeps the tip of the pipette clean andavoidsdye-leakage while approaching the slice surface. Submerge the pipette in the ACSF surrounding the slice.
    b. Keep the 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 the tip of the pipette with the objective and move the objective and pipette down together with the fast mode, until the pipette tip is ~200 µm above the slice surface.

d. Change to slow mode for both the manipulator and objective and keep moving them until the pipette is hovering just above the slice surface.

17. Move the objective down to identify the pyramidal cell to patch (Figure 4Fi) and zoom in once or twice to have a better view of the cell. Have a marked spot on the screen and position the edge of the soma below the electrode tip. Patch the soma at its thinner part, towards the dendrite, to stay as far as possible from the nucleus. Focus again on the pipette tip and position the tip slightly away (5 µm) from the spot (Figure 4Fii).
18. Patch the neuron.
    a. Focus on the cell of interest and apply a large positive pressure on the patch pipette (~100 mbar).
    b. Quickly move the pipette straight down until the tip of the pipette is at the same focus with 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 pipette and soma are on the same focal plane, move the pipette towards the cell until you see the pipette pushing the cell (Figure 4Eiii). Compensate for the last time the junction potential.
    d. Immediately release the pressure to form a seal in voltage clamp mode and set the voltage at -60 mV (Figure 4Fiv). Ideally, steps a-d should be achieved in less than 30 seconds.
19. Move the pipette back1-2 µm so that the tip does not push the cell. To achieve the whole-cell configuration, apply a short robust suction (Figure 4Fv). Estimate the series resistance that, ideally, should be around 10 MΩor below.
20. It is useful to check immediately if dye spillage occurred while patching the neuron. If this is the case, it is advisable to discard the patch, move at least 100 µm away from that cell and patch another neuron. If there is no dye spillage, you can continue the experiment.

Fluorescence imaging

After patching a cell and allowing the Na^+ indicator to equilibrate, the recording can start. The data acquisition frame rate is 10 kHz, with 500 nm pixel resolution, using a CMOS DaVinci-2K camera. The somatic V_m is simultaneously recorded by the A/D board connected to the patch clamp amplifier in order to monitor thesomatic AP. We recommend for the V_m acquisition a sampling rate of 20 kHz, 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), where the delay and duration of pulses are set with at least 10 µs precision. The Master-9 should be started by an output of the same A/D board recording the electrophysiology.

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

22. Switch to 0.5X demagnification and set the camera configuration to 512X512. The 1X demagnification is used for patching as the better contrast allows you to see when the pipette is touching the cell. The 0.5X demagnification is used for imaging since it increases by a factor of 4 the number of photons detected by each pixel.
23. Set the telescope to achieve wide-field illumination and place the filter cube on the microscope pathway. Illuminate with low light where the laser is essentially still off and the light comes only from the diode and check the fluorescence over the cell. Centre the AIS in the area that will be illuminated by the 30 µm spot and turn off the laser (Figure 5A).
24. Change the camera configuration into 128X30 pixels that allows acquiring at 10k frames/s. This configuration can be obtained by the manufacturer. Turn on again the laser at a low intensity to optimize the position of the AIS in the spot and bring it into focus. It is very important to keep the soma out of the illuminated spot since strong light on the cell body produces rapid phototoxicity.
25. Set a laser to trigger mode and the intensity to its maximum.
26. Switch the patch clamp amplifier from voltage clamp to current clamp mode and set the steady current so that the V_m is between -60 mV and -80 mV. In healthy cells the resting V_m (when the steady current is zero) is within this range.
27. It is important to program three channels of the Master-9 pulse stimulator to let it controlling the laser, the camera start and the timing of occurrence of the somatically injected current triggering the AP. Prepare for an acquisition of a series of images for 8 ms as illustrated in Figure 5B.
    1. Trigger the start of the Master-9 (M-9) with a TTL output of the electrophysiology A/D board.
    2. With no delay, trigger the laser on with a TTL pulse of 8.7 ms.
    3. Start the camera acquisition with a delay of 0.5 ms from the laser trigger. This will prevent acquiring images while the laser is turning on.
    4. Trigger the primary output of the patch clamp amplifier so that it provides an output pulse of 3-5 ms duration and 1-2 nA amplitude delayed by ~2 ms from the camera start. This intensity normally elicits an AP.
28. Turbo-SM controls the camera acquisition speed and number of frames.
    a. Set the recording time to 0.1 ms, the number of frames to 80.
    b. Record a “dark frame” that is used to subtract the levels corresponding to no light to the camera signals.
    c. The camera should start recording only after being triggered by the Master-9. Thus, start recording by first pressing the ‘Record’ button in Turbo-SM.
29. Start an electrophysiology recording lasting 10 ms that will also start the Master-9.
30. It is important to wait a minute before performing the 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 the AP starts widening. This is the 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 the current pulse to zero and record one series without stimulus. This recording will quantify the kinetics of the indicator bleaching.
33. Take a wide-field images of the cell before destroying the patch. This is particularly important to measure the distance from the soma of the different AIS regions. To do it we recommend the following steps.
    a. Set back the wide field illumination in the telescope.
    b. Set the exposure time of the frames in Turbo-SM to 20 ms or more.
    c. Increase the laser Duration in the Master-9 to acquire longer sequences.
    d. Decrease the 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. Optionally, move the position of the cell to reconstruct also the apical dendrite and the secondary dendrites that are still intact.
34. If another cell in the same slice is used, move at least 100 µm away from the previous cell to avoid its fluorescence. If the previous axon was not perfectly parallel, we advise to change the slice.

SUPPORT PROTOCOL 1

SUPPORT PROTOCOL TITLE

CALIBRATING Na^+ FLUORESCENCE

Introductory paragraph:

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 et al., 2018). Thus, we report here how the Na^+ dye can be calibrated using intracellular solutions with different sodium 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.

Materials:

Na^+ free intracellular solution (see Reagents &Solutions for recipe)
NaCl (1M)
ING-2

Illumination and Imaging tools

- Tri-Line Laser-Bank (Cairn Research) through a Ø550 µm, 0.22 NA, SMA-SMA Fiber Patch Cable (Thorlabs, Newton, NJ), with multimode diode head 520 nm / 0.5W (Ushio).
- Telescope with two lenses (Cairn Research) for wide-field or ~30 µm illumination spot at the output of the fibre.
- Filter cube comprising excitation (517 ± 10 nm) and emission (559 ± 17 nm) filters and a 538 nm dichroic mirror. We recommend the FF01-517/20, FF01-559/34 and the FF538-Di01 from Semrock (Rochester, NY).
- CMOS camera (see protocol 2).
- Turbo-SM software (see protocol 2)
- Pulse stimulator Master-9 (A.M.P.I., Jerusalem, Israel)

Protocol steps—*Step annotations:*

Calibration of ING-2

1. Prepare a serial Na^+ dilution, from 0 to 25 mM Na^+ concentration, in steps of 2.5 mM. Use a 38 µL per dilution and add 2 µL of ING-2 (at 1.6 mM) for each dilution (80 µM is the final concentration). We found that 40 µL is the minimal volume that allows dipping the 60X objective while focussing on a surface.
    a. Take 25 µL 1M NaCl and add to 475uL intracellular without Na^+ to obtain a 50 mM Na concentration. This is your stock solution.
    b. For 25 mM Na^+: dilute 20 µl of stock with 18 µL intracellular without Na^+.
    c. For 15 mM Na^+: dilute 12 µL of stock with 26 µL intracellular without Na^+.
    d. For 12.5 mM N^a: dilute 10 µL of stock with 28 µL intracellular without Na^+.
    e. For 10 mM Na:dilute 8 µL of stock with 30 µL intracellular without Na^+.
    f. For 7.5 mM $Na^·$ dilute 6 µL of stock with 32 µL intracellular without Na^+.
    g. For 5 mM Na: dilute 4 µL of stock with 34 µL intracellular without Na^+.
    h. For 2.5 mM $Na^·$ dilute 2 µL of stock with 36 µL intracellular without Na^+.
    i. For 0 mM Na: take 38 µL intracellular without Na^+.
    j. Add 2 µL ING-2 to each dilution. Mix well and protect from light.
2. Perform the calibration.
    a. Start with 0 mM Na^+. Pipette 40 µL in the microscope recording chamber and lower the objective into the solution. Do not change the position of the objective after this initial position. Measure the fluorescence with the CMOS camera.
    b. Remove the solution, rinse and pipette the next concentration (2.5 mM Na^+), into the recording chamber. Measure the fluorescence again.
    c. Repeat this process until all concentrations have been measured.
3. Analysing the results.
    a. Plot the number of light counts against the mM sodium concentration as shown in Figure 5C.

b. Fit the linear part (usually between 2.5 and 15 mM) of the plot to obtain the linear relationship between light counts and sodium concentration (F([Na+]) = a * [Na+] + b).

4. Considering that the intracellular Na^+ concentration at rest is 10 mM, or F_0 = F([10 mM]), calculate the concentration at 1% change in fluorescence (1% $\Delta F/F_0$ or $[Na]_\alpha$).
    a. Calculate the light count for F_0 by simply solving the equation for F([10mM]).
    b. Solve $\Delta F/F_0$ = 1%, which you can also write as $(F1-F_0)/F_0 = 0.01$. As b is 10 mM, this gives: $[Na]_\alpha = 0.01 * F_{0a} + 10$

BASIC PROTOCOL 3

BASIC PROTOCOL TITLE

DATA ANALYSIS

Introductory paragraph:

In this protocol it is explained how to process the recordings in order to extract the Na^+ fluorescent 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 in

https://www.mathworks.com/matlabcentral/fileexchange/83708-sodium-imaging

Protocol steps—*Step annotations:*

1. *Convert ".tsm" files and open files*: The custom-made software of the DaVinci2k CMOS camera automatically saves image sequences in a ".tsm" format. Convert".tsm" files into matlab files with versions of Matlab prior to 2011. Alternatively, translate ".tsm" files into standard ".tif" files and then open with any version of Matlab. Finally, save the image sequences as ".mat" files.

2. *Averaging trials:* Average aligned recordings to increase the SNR. We average up to 8 recordings not affected by photo damage. 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 be still performed on individual trials.

    a. To check if recordings are temporally aligned, superimpose their electrically recorded APs. If the duration or the peak of an AP differs by more than 0.1 ms (one frame), the recording is not suitable for averaging.
    b. To check if recordings are spatially aligned, superimpose the images. If needed, move them on the x-y plane in order to compensate for possible small lateral movements of the preparation during the recording.
    c. The bleach recording must also be checked for lateral movements and aligned if necessary.
    d. Average all the temporally and spatially aligned trials and use any of their APs as a reference.

3. Extracting Na^+ signals: To extract the Na^+ signals, some pre-processing is recommended to simplify the procedure and correct the signal for photobleaching.

a. Load ".mat" files. The image sequence is a 3-dimentional 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 the first image and subtract the mean fluorescence intensity from the raw recording to remove the effect of the background on the fractional fluorescence change ($\Delta F/F_0$). As an example, see Figure 6A,B.

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

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

d. Calculate the F_0 by averaging the mean fluorescence over some of the first frames without stimulus (for instance the first 6 frames).

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

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

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

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

g. Fit the $\Delta B/B_0$ with a tri-exponential function Tr(y,t) to get a smooth bleach trace that will not introduce additional noise and/or artefacts to the Na^+ signal (Roder & Hille, 2014). (Figure 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 the standard deviation of the first 8 frames of the raw signal recording, with 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.

j. 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 (Figure 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 a mean zero up until the frame when the current stimulation begins, change its fr value and repeat step j.

4. Translating signal into number of ions: Use the calibration described in Support protocol 1to convert the *S* signal into a Na^+ concentration (in mM concentration). After that, the signal can be further converted into a number of ions by estimating the volume of the axon. This process is necessary, first, to calculate the longitudinal diffusion, and then, in a second step, to finally calculate the Na^+ current that is expressed in Amperes.

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

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

b. Estimate the 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 the fluorescence image so that the pixels below a certain threshold are set to zero. Then, the radius can be obtained by using the number of consecutive positive pixels at the line *x* and an equation $r = A \cdot x^B + C$ is fitted against the *x* axis to obtain a smooth realistic profile. The direct measurement of the radius is impossible with our spatial resolution.

c. Each compartment of the axon has the shape of a truncated cone, thus calculate the volume (V) and the lateral surface (A) as:

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

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

Where h=0.5 µm, r_1=r(y) and r_2=r(y+1).

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

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

e. At this stage, you should evaluate the kinetics of the S signal, which depends on Na^+ influx and diffusion (Zylbertal et al., 2017). In a previous report we have 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 1-2 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 the Na^+ concentration into charge surface density using the charge constant $C=1.6 \cdot 10^{-19}$ Coulomb

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

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 the function is complex 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 sub and a supra threshold signal. The subthreshold signal (first arrow figure 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 figure 6D) followed by a slower increase (third arrow figure 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:

$$F_x^{supra}(t) = \gamma \cdot \left(\frac{1}{1+e^{(\eta_1 - t)\cdot v_1}}\right) \cdot \left(\frac{1}{1+e^{(\eta_2 - t)\cdot v_2}}\right) \cdot \left(\frac{1}{1+e^{(\eta_3 - t)\cdot v_3}}\right)$$

The function above allows maximizing the likeliness between the model and the experimental trace with the following steps:

a. Set the parameters η_1 and η_2 equal to the exact time of the fast δC increase and the parameter v_1 equal to the half sampling time of the fluorescence acquisition.
b. To find the parameters α and β, use the 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, v_2 and v_3, use the EM algorithm on the entire signal to fit F_x.
d. Calculate the Na^+ current at position γ as

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

Where δt= 0.1 ms is the sampling time (Figure 6D).

REAGENTS AND SOLUTIONS:

Artificial cerebrospinal fluid ACSF

This solution, when oxygenized, is used to maintain the brain slices, and is constantly perfused around the slice when performing combined electrophysiology and imaging experiments. ACSF 1X contains:

125 mM NaCl
26 mM $NaHCO_3$
1 mM $MgSO_4$
3 mM KCl
1 mM NaH_2PO_4
2 mM $CaCl_2$
23 mM glucose

Prepare this solution fresh every day. You can prepare a 5X stock solution, without $CaCl_2$ and glucose to prevent microorganism growth, and store it at 4°C for up to two months. We do not recommend preparing a 10X stock solution since it easily precipitates. After diluting the stock solutions, add glucose and calcium on the day of the experiment.

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

It can be prepared from the stock solution described above.

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_20

Aliquot stocks of intracellular solution in vials of 1 mL and keep at -20°C before use to prevent ATP/GTP degradation. It is recommended to use the solution within six months from its preparation.

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

Diluted ING-2

0.5 mM ING-2
Intracellular solution

COMMENTARY

BACKGROUND INFORMATION:

The sensitivity of Na^+ fluorescent 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 (Kole et al., 2008; Fleidervish et al., 2010; Baranauskas et al., 2013; Katz et al., 2018;), but with insufficient temporal resolution to reconstruct the kinetics of the Na^+ current. Improved dyes such the ING-2 reported here allow higher acquisition rates that enable reconstructing Na^+ currents during physiological APs along the AIS. While 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 unit 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 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 unit with the prospective of using the described protocol details with future indicators having 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 the binding to mobile and immobile endogenous Ca^{2+} buffers (Canepari & Mammano, 1999; Ait Ouares et al., 2016; Ait Ouares et al., 2019). 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 contributes

substantially to the Na^+ dynamics, correcting for diffusion is necessary before calculating the time derivative of the Na^+ concentration.

Resources

In this unit we have described in detail the equipment used in our laboratory. This includes a dedicated set of optical tools that we found optimal for this technique. We used 10X and 60X objectives and two magnifications (1X and 0.5X) 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 we used allows 2X2 binning. This arrangement, however, is not equivalent to changing from 1X to 0.5 magnification 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 changes accordingly and a gain cannot be set in this device. Therefore, we recommend to optimize the magnification using hard optics.

TROUBLESHOOTING:

A list of problems can be encountered during these protocols and their possible solutions are listed in Table 1.

UNDERSTANDING RESULTS:

These protocols describe how to acquire dynamic Na^+ optical measurements with unprecedented spatiotemporal resolution allowing the reconstruction of the Na^+ currents along the AIS of neurons in mouse brain slices. Obtaining signals with high SNR along the whole AIS requires careful preparation of slices, selection of neurons, optimal imaging steps and robust analysis as described in this unit. The sample data given in Figure 5D demonstrates 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 while 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 in the bottom of Figure 5D shows a first sharp current with fast kinetics and a second one with slower kinetics. It is recommended to quantitatively analyse 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 repolarisation. These results demonstrate the possibility to optically measure the physiological Na^+ current underlying the generation of a neuronal AP, enabling a plethora of future studies that can unravel the role of specific VGNC in initiating and propagating the AP under physiological or pathological conditions linked to several brain disorders.

TIME CONSIDERATIONS:

Performing the measurements described in this unit requires a day of experiments. In detail, preparing the solutions in the morning requires 30 minutes, while slices preparation (see Basic Protocol 1) requires ~40 minutes with ~2 additional hours of preliminary work. Slices can be used up to ~6 hours. The time to choose the optimal cell to patch can vary from 2 minutes to 1 hour (see Basic Protocol 2). A single optical recording requires ~1 hour, including 30 minutes to load the cell with the dye and 30 minutes to perform optical measurements (see Basic Protocol 2).

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FIGURE LEGENDS:

Figure 1. Construction of 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 tap and the neck of a plastic bottle to create the slice holder. **C** Slice holder fixed inside the glass beaker using two PVC tube pieces.

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 37^{o}C water bath covered with lid. V) Left, vibratome screwdriver. Middle, bended syringe for cutting slices. Right, plastic balloon pipette with its end cut off, used to transfer slices. **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 scull. iv) Chattaway spatula used to move the brain. v) Forceps used to remove the scull. vi) Blade used to cut the cortices. **C** Design of the 15 degrees inclination slice holder. **D** Cortices glued on the 15 degrees holder. The scissors

are used to prevent slices from sliding by counteracting the holder slope. **E** Vibratome blade has cut halfway through both cortices. Using the bended syringe, 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 layer 1-6 pointed with red arrows, indication that these slices have axons and dendrites that run parallel to the slice surface. The blue dotted line indicates the position where the slice is cut with the bended syringe. **G** All 350 µm slices are placed in the slice holder inside the 37°C water bath for 30 minutes.

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 illuminating the whole field uniformly or a spot of ~30 µm. Alternating between a 60X and a 10X 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 a 0.5X or 1X demagnification to obtain a pixel resolution of 500 nm or 250 nm respectively on the CMOS sensor.

Figure 4. Pipette filling, positioning and patching. **A** Example of blood vessels running in parallel with the slice surface all the way from the dorsal end towards the hippocampus as seen with the 60X objective and 1X magnification. **B** Example of healthy patched cell. Unhealthy cells are indicated; their contrast is higher and their membrane appears damaged. **C** Front filling of a patch pipette. Position the tip of the pipette in a droplet of intracellular solution and provide slight negative pressure from the top of the pipette using some tubing and a syringe. **D** Backfilling of a patch pipette. Using a syringe with micro-loader, fill the pipette with the intracellular solution containing the dye. **E** When mounted on the holder the pipette 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 plunger. **F** Patching a cell (in voltage clamp). After selecting a healthy cell (i), position the tip of the pipette just above the cell membrane while holding the voltage at 0 mV (ii). Apply a strong pressure (~100 mbar) and move the pipette down quickly, slightly blowing the tissue out of the way. Position the pipette 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 pipette and allow the cell to fill with dye (v).

Figure 5. Imaging procedure and calibration of ING-2. **A** L5 pyramidal neuron loaded with ING-2 viewed in the 512X512 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, it corresponds to an imaging acquisition of 8 ms. **C** Calibration of ING-2 $\Delta F/F_0$ signals in terms of $\Delta[Na^+]$ 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 straight line is the linear fit of the points between 2.5 mM and 15 mM Na^+ concentration.

Figure 6. Extraction of Na^+ currents. **A** AIS of 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** Same for the bleach recording: the yellow trace is a recording from the same region without stimulation. The green dotted 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 the Basic Protocol 3 step 4. The three arrows point 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 5 trials.

TABLES:

Table 1. Troubleshooting

Protocol	Problem	Possible cause	Evaluation	Solution
1	Brain hemisphere unglued while slicing	Glue dried	Ensure the other hemisphere is well glued	Set the unglued hemisphere aside, out of reach from the steel blade but inside the slicing solution. Continue slicing the other hemisphere and when finished glue again the unglued hemisphere and slice.
2	Spilled dye	Too slow of an approach tocell before patching, insufficient front-filling of the pipette with intracellular solution or to strong pressure during the fast approach phase.	Check whether a large fluorescence background is observed after establishing the whole-cell configuration.	Patch another cell.
2	Axon moved out of focus in between recordings	Mechanical instability.	First, make sure table is pumped and mechanically uncoupled to external environment. You can check under transmitted light any potential drift of the pipette.	During recordings, reposition manually the AIS in its original position using low light. After termination of the experiment, improve the stability of your system.
3	The 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 neighbouring regions have similar signals	Fit the bleach with a different function such as the bi-exponential or the power2.

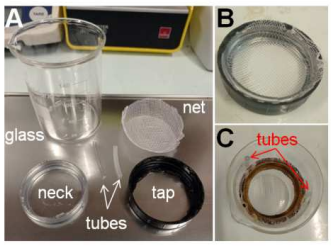

Fig1

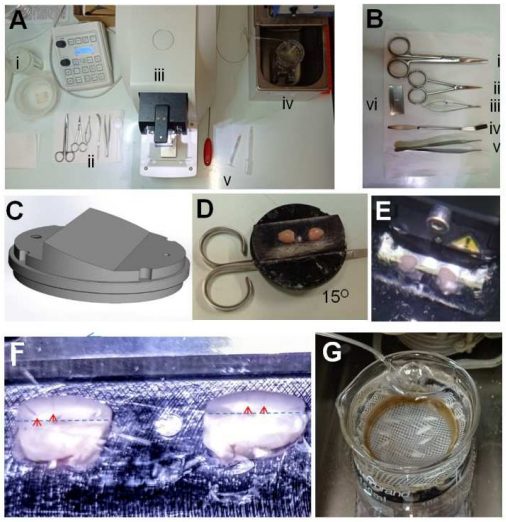

Fig2

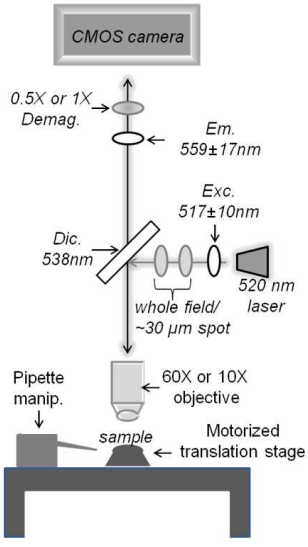

Fig3

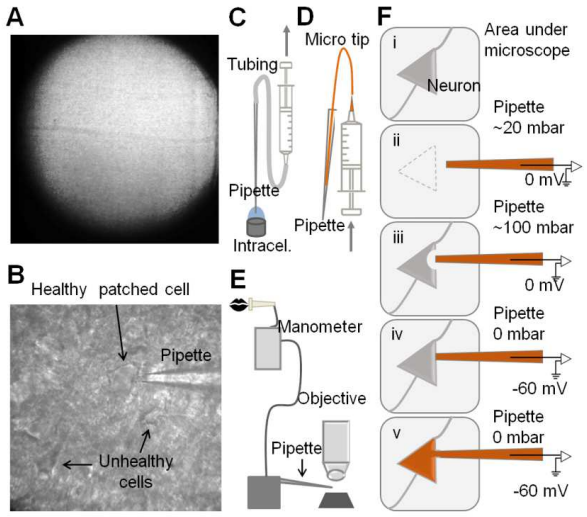

Fig4

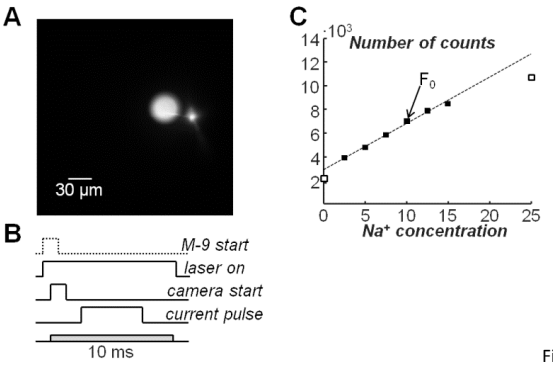

Fig5

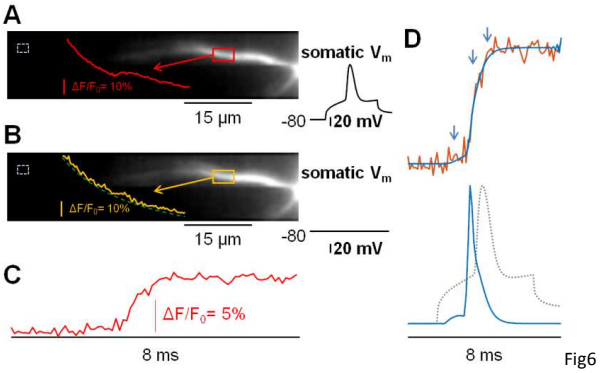

Fig6