Targeted axon-attached recording with fluorescent patch-clamp pipettes in brain slices

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Understanding the physiology of axons in the central nervous system requires experimental access to intact axons. This protocol describes how to perform cell-attached recordings from narrow axon fibers (φ < 1 μm) in acute and cultured brain slice preparations (with a success rate of ~50%). By using fluorophore-coated glass pipettes and Nipkow disk confocal microscopy, fluorescently labeled axons can be visually targeted under online optical control. In the cell-attached configuration, axonal action potentials are extracellularly recorded as unit-like, sharp negative currents. The axon morphology labeling and cell-attached recordings of axons can be completed within 1–2 h. The recordings are stable for at least 30 min.

INTRODUCTION

Axons in the mammalian cortex are generally considered to be cables through which action potentials (APs) conduct signals. Contrary to this prevailing notion, recent experimental evidence has suggested that the AP waveform can be modulated depending on the geometry of the axons and the activation state of voltage-gated ion channels[1,2]. Under physiological conditions, somatic depolarization induces the inactivation of low-threshold potassium channels, broadening axonal APs[3–10]. Furthermore, we have recently demonstrated that previously generated APs can be broadened in the middle of the axon when the membrane potentials of the axonal segment are depolarized[11]. Broadened APs more efficiently activate calcium channels at presynaptic terminals and facilitate synaptic transmission to postsynaptic neurons[9–14]. Therefore, an AP is capable of conveying an analog-like graded signal, a finding that is in contrast to the classic concept of a digital AP.

Several pioneering studies have performed direct whole-cell recordings from giant axonal structures (3–5 μm), such as the mossy fiber boutons of dentate gyrus granule cells[15,16] and the giant synaptic expansions of the Calyx of Held[13,17]. In the neocortex, Shu *et al.*[4] introduced a method to record from axonal blebs, which represent resealed swellings of the cut ends of axons in brain slices. Axonal bleb recording techniques have been subsequently used to study presynaptic and axonal electrical signaling in the neocortex[5,6] and hippocampus[11]; however, it is still difficult to access intact axonal fibers because of their small diameters (~1 μm)[18–21].

To overcome the inaccessibility of intact axonal fibers, we developed a novel protocol for direct patch-clamp recordings from intact unmyelinated axons in cortical brain slices with an ~50% success rate. Coating a glass pipette with commercially available Alexa Fluor–conjugated albumin allows visual targeting of a pipette to a fluorescently labeled axon under online confocal manipulation[11,22]. This technique can be achieved using a combination of Nipkow-type spinning-disk confocal microscopy and a back-illuminated electron-multiplying charge-coupled device (EM-CCD) camera, which enables rapid image acquisition with low phototoxicity and photobleaching rates[23,24]. The principal advantage of this optical setup is that one can couple images of axon tracts and patch-clamp pipettes without switching back and forth between fluorescence and transmitted images. The limitation of this method is that it is only applicable to cell-attached recordings of extracellular AP waveforms, which are likely to be a mixture of the inverse of intracellular membrane potential and its derivative[10]. We believe that this technique could potentially be a unique tool to directly investigate the density, properties and distribution of receptors and ion channels on axonal membranes in different cell types, which could elucidate the mechanisms underlying the generation and propagation of APs.

MATERIALS

REAGENTS

- Experimental animals: Wistar rats or ICR mice (Japan SLC) aged 12 to 17 d are suitable for acute slice preparations. Wistar rats aged 7 d are suitable for cultured slice preparations. The strain of the animal is not important for this protocol **! CAUTION** All animal experiments should be performed in accordance with the relevant animal experiment ethics committee and according to the institutional and national guidelines for the care and use of laboratory animals.
- Artificial cerebrospinal fluid (aCSF; see REAGENT SETUP)
- Hanks' balanced salt solution (HBSS; see REAGENT SETUP)
- Gey's balanced salt solution (GBSS; see REAGENT SETUP)
- Phosphate-buffered saline (PBS; see REAGENT SETUP)
- Horse serum (Cell Culture Technologies, cat. no. CC 3015-502)
- Minimal essential medium (MEM; Sigma-Aldrich, cat. no. M4655)
- Penicillin/streptomycin (Sigma-Aldrich, cat. no. A-4333)
- Carbogen (a mixture of 95% oxygen and 5% carbon dioxide)
- Chemicals for aCSF and patch pipette solution (all from Sigma-Aldrich): NaCl, KCl, $NaHCO_3$, NaH_2PO_4, KH_2PO_4, $CaCl_2$, $MgCl_2$, $MgSO_4$, D-glucose, sucrose, ascorbic acid, KOH, K-gluconate, HEPES, Mg-ATP, Na_2-GTP and Na-phosphocreatine
- NaN_3 (Sigma-Aldrich, cat. no. S-2002)
- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A-4161)
- BSA Alexa Fluor 488 conjugate (BSA-Alexa; Invitrogen, cat. no. A-13100)
- Alexa Fluor 488 hydrazide (Invitrogen, cat. no. A-10436)
- Diethyl ether

EQUIPMENT

- Dissection tools for removing the brain (scissors, forceps and spatula)
- Vibrating slicer (Leica Microsystems, cat. no. VT1200S)
- Incubation chamber for maintaining the slices (interface or submerge)
- Membrane filter (Omnipore JHWP02500, diameter: 25 mm, 0.45-μm pore; Millipore)

- O-shaped plastic plate, termed a 'doughnut' plate (custom made; Hazai-Ya; for more detail, please see Koyama *et al.*[25])
- Upright microscope (Nikon, cat. no. FN-1)
- Water-immersion objective lens (magnification: ×40; numerical aperture: 0.8; working distance: 3.5 mm; Nikon, CFI Apo 40XW)
- Optical magnifier turret (×1, ×1.5, ×2; Nikon)
- Microscope isolation system with an *XY* mover (Narishige, cat. no. ITS-FN1)
- Nipkow-type spinning-disk confocal unit (Yokogawa, cat. no. CSU-X1)
- EM-CCD camera (Andor, cat. no. iXon EM+ DU897)
- Laser diode (488-nm; Fitel, HPU50101PFS)
- Temperature-controlled imaging chamber (Warner Instruments, cat. no. RC-25)
- Inline solution heater (Warner Instruments, cat. no. SH-27B)
- Temperature controller (Warner Instruments, cat. no. TC-344B)
- U-shaped platinum grid to weigh down a slice in a recording chamber
- Two electromotive micromanipulators (Narishige, cat. no. EMM-3NV)
- Two patch-clamp amplifiers (Molecular Devices, cat. no. MultiClamp 700B)
- Horizontal electrode puller (P-97 horizontal puller, Sutter Instruments)
- Thick-walled borosilicate glass tubing (outer diameter: 1.5 mm, inner diameter: 0.84 mm; World Precision Instruments, cat. no. 1B150F-4)
- Patch pipette fillers with solution filter (diameter: 4 mm, 0.45-µm pore; Millex-LH, Millipore)
- Syringe pressurizer (10-ml; Terumo)
- C-mount connecting adaptor
- Solis software (Andor)

REAGENT SETUP

Sucrose-based slicing medium Sucrose-based slicing medium is prepared by mixing 27 mM $NaHCO_3$, 1.4 mM NaH_2PO_4, 2.5 mM KCl, 0.5 mM ascorbic acid, 7.0 mM $MgSO_4$, 1.0 mM $CaCl_2$ and 222 mM sucrose. The solution can be stored for several days at −20 °C.

aCSF Combine 125 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 and 10 mM D-glucose. ▲ **CRITICAL** aCSF should be prepared immediately before use and saturated with carbogen for at least 10 min.

GBSS Combine 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.57 mM $MgSO_4$, 27 mM $NaHCO_3$, 0.8 mM NaH_2PO_4, 0.22 mM KH_2PO_4 and 36 mM D-glucose. The solution must be filter-sterilized before use. ▲ **CRITICAL** Freshly prepare the solution just before each experiment.

HBSS HBSS is prepared by mixing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4 and 5.6 mM D-glucose. The medium must be filter-sterilized before use. ▲ **CRITICAL** Freshly prepare the solution just before each experiment.

PBS Combine 132 mM NaCl, 7 mM Na_2HPO_4 and 2 mM NaH_2PO_4 (pH 7.4). The solution can be stored for several days at 4 °C.

Standard intracellular solution Combine 135 mM K-gluconate, 4 mM KCl, 10 mM HEPES, 10 mM Na_2-phosphocreatine, 0.3 mM Na_2-GTP, 4 mM Mg-ATP and 0.2 mM Alexa Fluor 488 hydrazide. The solution can be stored for several days at −20 °C. ▲ **CRITICAL** Keep the solution on ice throughout the experiment.

Culture medium (for 100 ml) Culture medium is prepared by mixing 50 ml of MEM, 25 ml of HBSS and 25 ml of heat-inactivated horse serum supplemented with 50 µl of antibiotics (50 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin). ▲ **CRITICAL** Freshly prepare the solution just before each experiment.

BSA-Alexa solution To prevent nonspecific adsorption of BSA-Alexa, rinse the plastic tips and tubes used for preparation and stock for 60 s with 0.1% (wt/vol) BSA in advance. Dissolve BSA-Alexa and NaN_3 in PBS to yield final concentrations of 0.02% (wt/vol) and 3 mM, respectively. The solution can be stored at 4 °C for more than 3 months in a plastic tube. For short-term storage, the addition of NaN_3 is not necessary.

EQUIPMENT SETUP

Spinning-disk confocal microscope Mount the CSU-X1 confocal unit on a Nikon NK1 upright microscope through a C-mount connecting adaptor. Then, connect an Andor EM-CCD camera through the adaptor. Other compatible EM-CCD cameras with wide imaging fields and high spatial resolutions (more than 500 × 500 pixels) can also be used. Intercalate an optical magnifier turret between the microscope and the confocal unit. Split a laser line sent to the confocal unit into multiple beams through a confocal Nipkow disk and ensure it reaches the preparation. The microlens array on the Nipkow disk is designed to minimize light loss by focusing each laser beam on the corresponding pinhole. Excite fluorophores with a 488-nm laser beam, and capture fluorescent signals through a 507-nm long-pass emission filter. Acquire images at 5–20 frames per second (f.p.s.) using Solis software. ▲ **CRITICAL** The advantage of Nipkow-type spinning-disk confocal microscopy is that it enables online experimental manipulations owing to the fast scanning rates (up to 2,000 f.p.s.). Another important advantage is its slow photobleaching (and thus low phototoxicity). Conventional epifluorescence microscopy is inapplicable to our protocols because of its fast photobleaching. ▲ **CRITICAL** Use an objective with a higher numerical aperture (more than 0.7). An objective with long working distance is desirable in order to prevent interference with patch pipettes; note that a pipette for axonal recording approaches at an angle of ~30°. The Yokogawa CSU-X1 confocal unit can obtain images that are approximately twice as bright as the older CSU10 or CSU22 version. In our experience with various versions of Andor EM-CCD cameras (i.e., DU860, DU887, DU888 and DU897), the DU897 was the best for the detection of axonal branches. ▲ **CRITICAL** The spatial resolution of images is crucial. The thickness of an axonal fiber should be imaged at a size of at least 4 pixels.

Image-acquisition software Solis is our first choice. Another option is iQ (Andor), a higher-grade software designed for various experimental purposes.

Pipette puller We recommend a dual-carriage (horizontal) puller. In general, the puller has several parameters, including the number of pulling cycles, pulling strength, heating duration and temperature, and cooling gas flow. The number of pulling cycles should be between three and five. The overall shape of the pipette for axonal recording is similar to those used for somatic recordings. There is no need to prepare pipettes having unique shapes for axonal recordings. The shaping of the pipette tips for axonal patching can be determined by the pulling conditions only at the final cycle.

Patch-clamp setup Useful tricks for the assembly of a functional patch-clamp setup can be found in the Molecular Devices Axon Guide. No special setups for axonal patch-clamp recording are required.

PROCEDURE

Preparation of brain slices ● TIMING 90 min

1| Prepare brain slices using either an acute brain slice preparation (option A) or organotypically cultured brain slices (option B). We usually use 10- to 17-d-old mice for acute neocortical and hippocampal slices and 6- to 7-d-old rats for cultured hippocampal slices. Further details can be found in refs. 16,26.

(A) Preparation of acute neocortical slices

(i) Anesthetize the mice with diethyl ether before decapitation. After decapitation, quickly remove the brain and gently drop it into ice-cold sucrose-based slicing medium bubbled with 95% O_2 and 5% CO_2.
▲ **CRITICAL STEP** The brain must be removed with care, but the removal should be completed in <1 min. Low quality of slice preparations is often attributable to technical failure at this step.

(ii) Mount the hemisphere in the tray of a slicer and cut 400-µm-thick slices with a vibratome in sucrose-based slicing medium.

(iii) Dissect the targeted region with forceps and microknives.
(iv) Transfer the slices to a maintenance chamber filled with carbonated aCSF using a wide-lumen pipette. The slices should initially be kept at 32 °C for 30 min and then maintained at room temperature (22–24 °C).

(B) Preparation of cultured hippocampal slices

(i) Anesthetize postnatal 7-d-old rats with hypothermia.
(ii) Prepare 300-μm-thick hippocampal slices with a vibratome in GBSS.
(iii) Place two to three slices on an Omnipore membrane filter in a 35-mm dish with 1 ml of culture medium[26]. Put the dishes in a cell culture incubator with 5% CO_2 at 37 °C. The slices should be cultured for 7–10 d *in vitro*. Change the culturing medium every 3–4 d.

Somatic recording and visualization of axon morphology ● TIMING 30–60 min

2| Transfer a slice into a recording chamber that is perfused with aCSF at room temperature or near physiological temperature (32–34 °C). Place a platinum ring with nylon grids on the slice surface to keep the slice stable against the solution perfusion (**Fig. 1**).

▲ **CRITICAL STEP** For good recordings, use slices within 4 h of transferring the slices into the chamber.

▲ **CRITICAL STEP** The orientation of the slice is important. Targeted soma and axons should be positioned so that the patch electrodes can access them without any interference (**Fig. 1**).

3| Set an optical magnifier turret to ×1. Search and focus on a healthy neuron with an ×40 objective lens. If necessary, use a differential interference contrast video microscope.

▲ **CRITICAL STEP** In acute slices, the cells that are close to the surface of the slice may be damaged or dead. The cells that are located at depths of more than 10 and 50 μm should be selected in cultured and acute brain slices, respectively.

4| For visualization of the axon with fluorophores, perform whole-cell recording from the soma with the intracellular solution containing 200 μM Alexa Fluor 488 hydrazide dye. Use a glass pipette with a resistance of 4–6 MΩ to obtain the access resistance of 10–25 MΩ. Full details of this procedure are described in ref. 27. Briefly, carefully approach the selected soma with a positive pressure of 50–120 hPa. Once the pipette tip has been positioned, release the pipette pressure and apply gentle suction (~20 hPa). Patch configuration can be assessed by applying repetitive 1–10-mV test pulses using a patch-clamp amplifier.

▲ **CRITICAL STEP** The data should be discarded if the access resistance changes by more than 20% during the experiment.

5| After constructing the whole-cell configuration, pause until the axonal fibers are sufficiently labeled with the fluorescent dye. In the case of pyramidal cells in the neocortex or hippocampus, recording should be maintained for at least 30 min to anterogradely trace axonal branches more than 300 μm away from the soma.

▲ **CRITICAL STEP** This period is a trade-off. Longer staining time allows clearer visualization of the axon morphology, but cell health may be compromised by phototoxicity.

▲ **CRITICAL STEP** In cortical neurons, the axons are distinguishable from the dendrites by the typical morphology: the axons are thinner and longer than the dendrites, the axons lack spine-like structures, and they often branch at obtuse angles (**Fig. 1a**).

6| Adjust the parameters of image acquisition for optimal results. We find the following settings work best: frame rates: 10–20 f.p.s.; spinning-disk rotation speeds: 5,000–10,000 r.p.m.; laser intensities: 0.5–2 mW. Image cropping and binning are not necessary.

▲ **CRITICAL STEP** The remainder of the procedure should be accomplished as rapidly as possible to avoid phototoxicity and photobleaching. If the total duration of the laser exposure exceeds 10 min, undesirable side effects may occur in the neurons, such as pathological depolarization of membrane potentials, AP broadenings and anomalous activation of ion channels. Damage may be observed as a beading of axonal varicosities.

? TROUBLESHOOTING

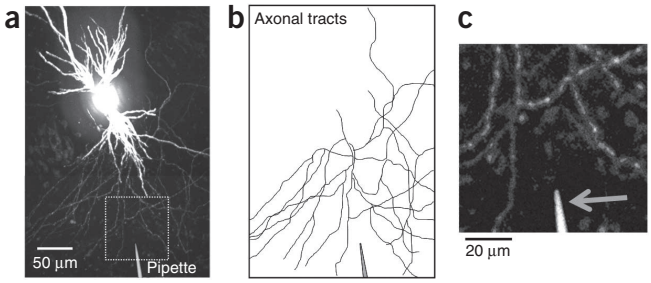

Figure 1 | Confocal images of axonal fibers in a hippocampal CA3 pyramidal cell. (**a**) An overview showing fluorescently labeled axonal fibers and a fluorophore-coated pipette that is positioned 20 μm away from the fibers. Under these imaging conditions, the somatic fluorescence intensity is completely saturated. (**b**) Line-traced axon tracts for the axonal fibers shown in **a**. (**c**) A high-magnification image of the boxed region in **a**. The arrow indicates the fluorophore-coated pipette.

7| Start image acquisition. The amplifier gain of the EM-CCD camera should be carefully optimized to reliably recognize the tracts of the axonal fibers.
▲ **CRITICAL STEP** Visualization of the axonal fibers requires higher gain and stronger digital contrast compared with the soma. Although the somatic fluorescence intensity will be completely saturated, this does not matter for axon patching (**Fig. 1a**).
▲ **CRITICAL STEP** In some cases, the images may contain wave-like periodic noise (i.e., interference fringes due to differential interaction between the Nipkow disk rotation speed and the camera scanning rate[28]). To reduce the interference, adjust the rotational speed of the disk or the video rates.

8| Select an axonal fiber under confocal monitoring. The accessible fiber should have highly contrasted outlines and should be located at a depth of 20–50 μm from the surface of the slice. Lower contrast structures arise from thinner fibers, more deeply located fibers or unhealthy axons. They should not be selected for patching.
▲ **CRITICAL STEP** Select the unmyelinated part of an axon for patching. Myelinated regions are barely visible under confocal monitoring.

Axon patch-clamp recording ● TIMING 15–30 min
9| Check that the borosilicate glass pipette you will be using for axon-attached recording has an open-tip resistance of 5–12 MΩ when filled with aCSF.
▲ **CRITICAL STEP** In the case of the extracellular solution containing pharmacological reagents, the same solution should be used for the internal solution.
▲ **CRITICAL STEP** Pipettes with resistances of 5–12 MΩ are recommended. Lower resistance gives a higher success rate of patching, but lower resistance may decrease the stability of the recording.
▲ **CRITICAL STEP** If the tip of the pipette has jagged edges, subtle heat-polishing might be useful to make the edge round and smooth. This optional process is similar to those widely used for somatic whole-cell recordings. Under eye inspection using a ×40 objective, polishing results in little change in the pipette tip shape. Therefore, care must be taken so as not to melt the tip too much.

10| To coat a pipette with fluorophores, dip the pipette tip into BSA-Alexa solution in a 1.5-ml tube for 5–30 s (please also see Ishikawa *et al.*[22]). Dip vertically and keep the pipette from touching the side of the tube.
▲ **CRITICAL STEP** The duration of dipping is a compromise; longer coating periods increase the brightness of the pipette and reduce the success rate of axon patching. In an ideal scenario, the fluorescence intensity of the patch pipettes is similar to or slightly higher than the fluorescence intensity of the targeted fibers (**Fig. 1c**). In each experiment, the brightness of the pipette tips should be checked by confocal monitoring.
▲ **CRITICAL STEP** Prevent the pipette tip from touching the edge of the tube.
▲ **CRITICAL STEP** Inspection of the pipette tips is necessary. In the coating procedure, dust may attach to the pipette tip. If the dust problem persists, apply a positive intrapipette pressure of 50–60 hPa during dipping to prevent the pipette tips from clogging.
? TROUBLESHOOTING

11| Fix the fluorophore-coated pipette with a head stage at an angle of ~30° (**Fig. 2**).

12| Place the fluorophore-coated pipette into a recording chamber with a positive pressure of 20–30 hPa.

13| Monitor the pipette resistance by measuring the currents in response to 1- to 5-mV test pulses using the internal stimulus generator of the patch-clamp amplifier.

14| Move the pipette tip to a point that is ~20 μm from the targeted fiber (**Figs. 1** and **2**). This procedure is similar to those used for somatic recordings.

15| Switch the optical magnifier turret from ×1 to ×2. To further enhance the visibility, an imaged field including the targeted fiber and the fluorescent pipette can be magnified with an ×4–8 digital zoom on the computer monitor (**Fig. 2c**). Images of size greater than 100 × 100 pixels are desirable.

16| Approach the selected axon under confocal monitoring with caution (**Fig. 2b**,**c** and **Supplementary Video 1**). To guide the pipette tip to the perfect spot, a fine manipulator that can move the pipette at steps of <0.3 μm should be used. **Figure 2** shows an example pipette trajectory for axon-attached recording. First, set the pipette tip 5–10 μm above the targeted

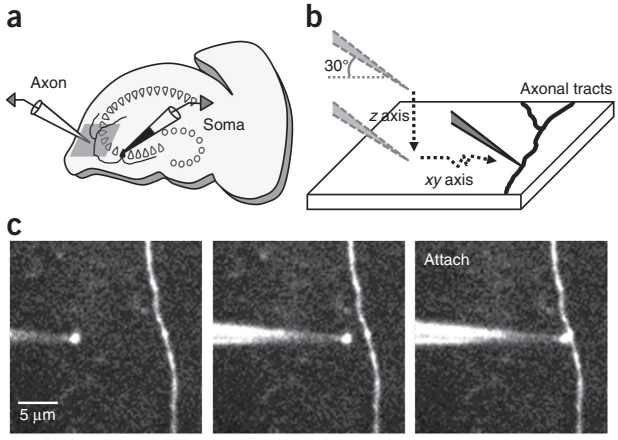

Figure 2 | Online targeting of an axon fiber under confocal monitoring. (**a**) A schematic illustration showing the axon tracts of a pyramidal cell and the positions of two pipettes in a hippocampal slice. The shaded area is magnified in **b**. (**b**) The pipette trajectory for axon-attached recording (the black lines indicate axons). First, lower the pipette to the level of the targeted axon vertically by manipulation of the *z* axis. Second, approach the axon horizontally by *xy*-axis manipulation. All of the procedures should be performed under online confocal monitoring. (**c**) Representative sequential images (from left to right) showing a pipette tip that is gradually approaching an axonal fiber by *xy*-axis manipulation. The right picture shows a cell-attached recording. The images are magnified with an ×2 intermediate lens and an ×5 digital zoom (100 × 100 pixels).

fiber by *xyz*-axis manipulation. Second, lower the pipette tip vertically to the level of the axon by *z*-axis manipulation. Third, approach the axon horizontally ~1 µm apart from the targeted fiber by *xy*-axis manipulation. Finally, approach and touch the fiber. At this final stage, approaching from a direction that is more perpendicular to the axonal tract leads to a higher success rate of patching. In some cases, subtle *z*-axis manipulation may be required to modify the position of the pipette tip during approach.

▲ **CRITICAL STEP** If the fiber moves away from the pipette tip along the tip movement, reduce the pipette pressure slightly.

17| When the pipette tip is positioned on the axon fiber (**Fig. 2c**, right), release the pipette pressure and apply gentle suction with a negative pressure of <20 hPa to achieve the axon-attached configuration.

▲ **CRITICAL STEP** The total duration of laser exposure should be <10 min to prevent phototoxicity and photobleaching. The resting potentials and AP waveforms of the whole-cell patched neuron should be continuously checked during the recording period.

▲ **CRITICAL STEP** If the pipette tip is positioned on the accurate spot, just a single application of the negative pressure will be needed to obtain AP signals (see Step 19).

18| Depending on the conditions, the seal resistance should range from 20 MΩ to 10 GΩ. Check that this is the case before proceeding. Even a low seal resistance allows stable recording of axonal APs with the so-called 'loose axon-attached' patch and allows the quantification of the AP width. Signals should be low-pass filtered at 2–10 kHz and sampled at 20–100 kHz. Fast and slow pipette capacitive transients are minimized in the axon-attached configuration.

? TROUBLESHOOTING

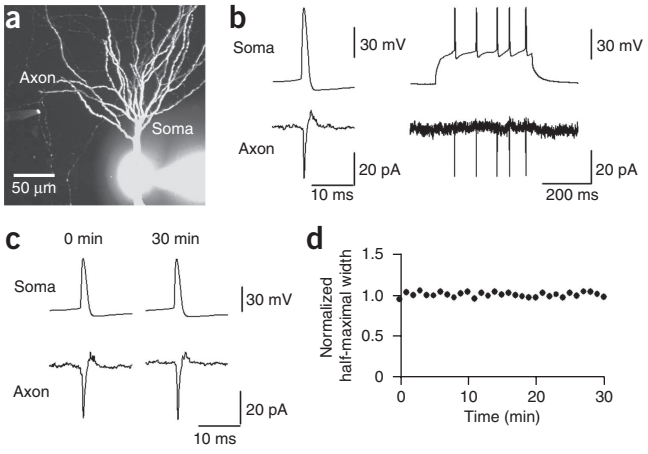

Figure 3 | Dual patch-clamp recordings from the soma and the axon of a hippocampal CA3 pyramidal cell. (**a**) A representative image of simultaneous recordings from the soma and the axon of a single pyramidal cell. (**b**) AP trains evoked at the soma (top) by the injection of rectangular current elicit sharp negative currents at the axon (bottom). (**c**) AP waveforms obtained 0 and 30 min after axon patching. (**d**) The half-maximal width of the axonal APs is stable during a recording period of 30 min.

19| Inject a rectangular current (2–3 ms duration, 1–2 nA) into the somatic pipette to evoke an AP. If axon recording is successfully achieved, a sharp negative current will occur in the axon pipette in the voltage-clamp configuration[29] (**Fig. 3a**,**b**). Burst firings can be evoked by long pulse stimulation (**Fig. 3c**). The recording should be stable for at least 30 min (**Fig. 3d**). See also **Figure 4**.

▲ **CRITICAL STEP** If no signal is recorded from the axon pipettes, the pipette might have been placed incorrectly. Restart the procedure from Step 9. If there is a weak signal with a low signal-to-noise ratio, further application of a slight suction with a negative pressure of up to 10 hPa may be effective in enhancing the signal amplitude.

▲ **CRITICAL STEP** In the cortex, the half-maximum width of the sharp negative currents should range from 0.1 to 0.25 ms. The peak amplitudes of the currents can vary depending on the seal resistances.

▲ **CRITICAL STEP** The latencies of axonal APs relative to the somatic APs increase with the distance between the recording site and the axon hillock (**Fig. 4**).

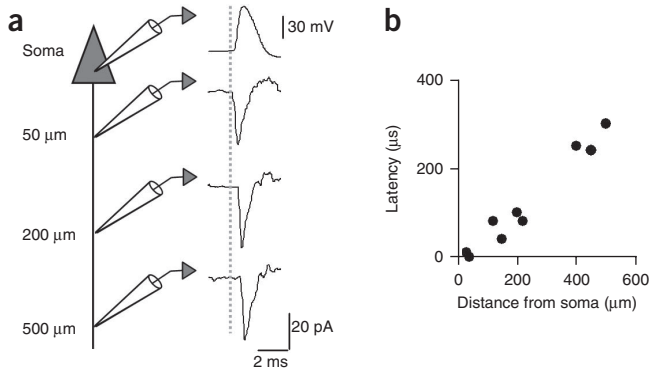

Figure 4 | Latency of axonal APs. (**a**) Axonal APs were recorded at 60-, 200- and 500-µm distances from the soma of the identical CA3 pyramidal cell. (**b**) A plot of axonal AP latency relative to the somatic AP as a function of the distance from the soma (n = 9 recordings). Individual recordings were performed independently from the same neuron.

? TROUBLESHOOTING

Table 1 summarizes the problems, potential sources of error and solutions. Basic troubleshooting information about slice preparations and somatic patch-clamp recordings can be found in other protocols[16,25–27].

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
6 (visualization of axon morphology)	Membrane potentials depolarize with time	Bad preparation or phototoxicity	Cell health and recording stability depend on the preparation and the laser exposure. Improve the slice quality or reduce the laser exposure time. Unless it is necessary, the laser shutter should be closed
	Axonal branches are barely detectable	The amount of fluorescent dye injected into the soma may be insufficient	Wait for a longer time for the somatic whole-cell recording or increase the concentration of the dye. In our experience, sufficient dye diffusion takes more than 30 min when 200 µM Alexa Fluor 488 hydrazide is used as the internal solution of a whole-cell pipette. A low detection rate may be caused by photobleaching. The laser exposure time should be as short as possible. If the problem persists, use an optical apparatus with a higher sensitivity
10 (axon patch-clamp recording)	Glass pipettes are not stained with fluorophores	Coating duration is too short	Increase the coating duration or use higher concentrations of fluorophores. Longer coating times result in higher fluorescence intensities. If the problem persists, make a new BSA-Alexa solution or increase the photosensitivity of the optical setup
18 (axon patch-clamp recording)	No signal is obtained from the axonal braches	The resistance of the pipette is not optimal	The resistance of the patch pipette is compromised: lower resistances (larger pipette tips) increase the success rate of patching and reduce the stability of recording. We recommend that beginners first try to use pipettes with lower resistances (5–7 MΩ). In this case, however, application of negative pressure to attach axons should be gentle in order to avoid destroying the axonal structures
	No signal is obtained from the axonal braches	The positioning of the patch pipette is not appropriate	Record from thicker fibers. Reduce the pipette pressure while accessing a fiber. Approach the axonal fiber at an angle that is as perpendicular to the axon tract as possible (**Fig. 2**) and reduce the overlap of the pipette and axon during the approach. Optimize the positioning of the pipette tip by rotating the recording chamber or by moving the electrode manipulator. Proficiency in one's own setup is necessary for this procedure

● TIMING

Step 1, preparation of brain slices: 90 min
Steps 2–8, somatic recording and visualization of axon morphology: 30–60 min
Steps 9–19, axon patch-clamp recording: 15–30 min

ANTICIPATED RESULTS

The major advantages of this protocol include the visibility of the glass pipettes during the fluorophore-coating strategy and the low phototoxicity and low photobleaching rates because of the use of spinning-disk confocal microscopy. When following our protocol, those who are trained in electrophysiological experiments should establish the axon-attached configuration with a 30% success rate in acute slices and a 50% success rate in cultured slices. Over the past 2 years, experienced experimenters have been able to obtain approximately four axon recordings per day. Typical AP currents recorded from axons

are shown in **Figures 3** and **4**. Under the most optimized conditions, recordings remain stable for up to 30 min. Beginners sometimes have trouble identifying the location of the pipette tip relative to the targeted axon. Our procedure for pipette visualization is designed to solve this problem. **Figure 2** and **Supplementary Video 1** will help you find the appropriate positions of the pipette tips. Approximately 2 to 3 months of daily training will be required to obtain stable recordings.

On the basis of electrophysiological principles, cell-attached recordings of APs from axonal fibers are similar to extracellular recordings of unit signals. From our previous experiments, however, the recorded currents seem to reflect a combination of the inverse of intracellular membrane potentials and their derivatives[10]. Therefore, care must be taken in interpreting the AP waveforms. Our experimental protocol will be applicable to unmyelinated axons in a variety of regions, including the neocortex, hippocampus, striatum, cerebellum, spinal cord and even peripheral nerves in both rats and mice. In addition, we have used our protocols for whole-cell recordings from fluorescent dendrites in the cortex[22,30], which will be useful to study the physiological properties of back-propagating APs and dendritic spikes. In conclusion, we expect that our protocol will advance the understanding of the fundamental characteristics of axonal AP conduction and provide information about how and where axonal APs are modulated by neurotransmitters and ion channels.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS T.S. collected experimental data and carried out the data analysis. T.S. and Y.I. wrote the manuscript. N.M. supervised the project and provided feedback on the manuscript.

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1. Debanne, D. Information processing in the axon. *Nat. Rev. Neurosci.* **5**, 304–316 (2004).
2. Astman, N., Gutnick, M.J. & Fleidervish, I.A. Persistent sodium current in layer 5 neocortical neurons is primarily generated in the proximal axon. *J. Neurosci.* **26**, 3465–3473 (2006).
3. de Polavieja, G.G., Harsch, A., Kleppe, I., Robinson, H.P. & Juusola, M. Stimulus history reliably shapes action potential waveforms of cortical neurons. *J. Neurosci.* **25**, 5657–5665 (2005).
4. Shu, Y., Hasenstaub, A., Duque, A., Yu, Y. & McCormick, D.A. Modulation of intracortical synaptic potentials by presynaptic somatic membrane potential. *Nature* **441**, 761–765 (2006).
5. Kole, M.H., Letzkus, J.J. & Stuart, G.J. Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. *Neuron* **55**, 633–647 (2007).
6. Shu, Y., Yu, Y., Yang, J. & McCormick, D.A. Selective control of cortical axonal spikes by a slowly inactivating K^+ current. *Proc. Natl. Acad. Sci. USA* **104**, 11453–11458 (2007).
7. Alle, H. & Geiger, J.R. Combined analog and action potential coding in hippocampal mossy fibers. *Science* **311**, 1290–1293 (2006).
8. Scott, R., Ruiz, A., Henneberger, C., Kullmann, D.M. & Rusakov, D.A. Analog modulation of mossy fiber transmission is uncoupled from changes in presynaptic Ca^{2+}. *J. Neurosci.* **28**, 7765–7773 (2008).
9. Christie, J.M., Chiu, D.N. & Jahr, C.E. Ca^{2+}-dependent enhancement of release by subthreshold somatic depolarization. *Nat. Neurosci.* **14**, 62–68 (2011).
10. Sasaki, T., Matsuki, N. & Ikegaya, Y. Effects of axonal topology on the somatic modulation of synaptic outputs. *J. Neurosci.* **32**, 2868–2876 (2012).
11. Sasaki, T., Matsuki, N. & Ikegaya, Y. Action-potential modulation during axonal conduction. *Science* **331**, 599–601 (2011).
12. Awatramani, G.B., Price, G.D. & Trussell, L.O. Modulation of transmitter release by presynaptic resting potential and background calcium levels. *Neuron* **48**, 109–121 (2005).
13. Hori, T. & Takahashi, T. Mechanisms underlying short-term modulation of transmitter release by presynaptic depolarization. *J. Physiol.* **587**, 2987–3000 (2009).
14. Alle, H. & Geiger, J.R. Analog signalling in mammalian cortical axons. *Curr. Opin. Neurobiol.* **18**, 314–320 (2008).
15. Geiger, J.R. & Jonas, P. Dynamic control of presynaptic Ca^{2+} inflow by fast-inactivating K^+ channels in hippocampal mossy fiber boutons. *Neuron* **28**, 927–939 (2000).
16. Bischofberger, J., Engel, D., Li, L., Geiger, J.R. & Jonas, P. Patch-clamp recording from mossy fiber terminals in hippocampal slices. *Nat. Protoc.* **1**, 2075–2081 (2006).
17. Forsythe, I.D. Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, *in vitro*. *J. Physiol.* **479** (Part 3), 381–387 (1994).
18. Palmer, L.M. *et al.* Initiation of simple and complex spikes in cerebellar Purkinje cells. *J. Physiol.* **588**, 1709–1717 (2010).
19. Rudolph, S., Overstreet-Wadiche, L. & Wadiche, J.I. Desynchronization of multivesicular release enhances Purkinje cell output. *Neuron* **70**, 991–1004 (2011).
20. Atherton, J.F., Wokosin, D.L., Ramanathan, S. & Bevan, M.D. Autonomous initiation and propagation of action potentials in neurons of the subthalamic nucleus. *J. Physiol.* **586**, 5679–5700 (2008).
21. Monsivais, P., Clark, B.A., Roth, A. & Hausser, M. Determinants of action potential propagation in cerebellar Purkinje cell axons. *J. Neurosci.* **25**, 464–472 (2005).
22. Ishikawa, D. *et al.* Fluorescent pipettes for optically targeted patch-clamp recordings. *Neural Netw.* **23**, 669–672 (2010).
23. Sasaki, T., Takahashi, N., Matsuki, N. & Ikegaya, Y. Fast and accurate detection of action potentials from somatic calcium fluctuations. *J. Neurophysiol.* **100**, 1668–1676 (2008).
24. Takahashi, N., Sasaki, T., Matsumoto, W., Matsuki, N. & Ikegaya, Y. Circuit topology for synchronizing neurons in spontaneously active networks. *Proc. Natl. Acad. Sci. USA* **107**, 10244–10249 (2010).
25. Koyama, R. *et al.* A low-cost method for brain slice cultures. *J. Pharmacol. Sci.* **104**, 191–194 (2007).
26. De Simoni, A. & Yu, L.M. Preparation of organotypic hippocampal slice cultures: interface method. *Nat. Protoc.* **1**, 1439–1445 (2006).
27. Debanne, D. *et al.* Paired-recordings from synaptically coupled cortical and hippocampal neurons in acute and cultured brain slices. *Nat. Protoc.* **3**, 1559–1568 (2008).
28. Takahashi, N. *et al.* High-speed multineuron calcium imaging using Nipkow-type confocal microscopy. *Curr. Protoc. Neurosci.* **57**, 2.14.1–2.14.10 (2011).
29. Perkins, K.L. Cell-attached voltage-clamp and current-clamp recording and stimulation techniques in brain slices. *J. Neurosci. Methods* **154**, 1–18 (2006).
30. Takahashi, N. *et al.* Locally synchronized synaptic inputs. *Science* **335**, 353–356 (2012).