Please cite this article as: Carola *et. al.*, (2017). Extracellular Axon Stimulation, *Bio-protocol* 7 (5): e2151. DOI: 10.21769/BioProtoc.2151.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

Extracellular Axon Stimulation

Carola Städele*, Margaret Louise DeMaegd and Wolfgang Stein*

School of Biological Sciences, Illinois State University, Normal, IL, USA
*For correspondence: carola@neurobiologie.de; wstein@neurobiologie.de

[Abstract] This is a detailed protocol explaining how to perform extracellular axon stimulations as described in Städele and Stein, 2016. The ability to stimulate and record action potentials is essential to electrophysiological examinations of neuronal function. Extracellular stimulation of axons traveling in fiber bundles (nerves) is a classical technique in brain research and a fundamental tool in neurophysiology (Abbas and Miller, 2004; Barry, 2015; Basser and Roth, 2000; Cogan, 2008). It allows for activating action potentials in individual or multiple axons, controlling their firing frequency, provides information about the speed of neuronal communication, and neuron health and function.
Keywords: Action potentials, Electrophysiology, Neuron, Threshold, Artifact, Anode, Cathode

[Background] Extracellular axon stimulation elicits action potentials (APs) without the need of introducing electrodes into neurons. This protocol describes cathodal stimulation, which uses the fact that the membrane potential of a neuron at rest is negative while the extracellular surrounding is positive in comparison. Two electrodes are needed: (1) a stimulation electrode (cathode) placed in close proximity to the axon, and (2) a reference electrode (anode) placed in the bath. When activated, the stimulation electrode adds electrons and thus negative charge to the outside of the axon. This makes the outside of the axon less positive and, as a consequence, decreases the potential difference between inside and outside of the neuron. The result is a local depolarization inside the axon. If sufficient in magnitude, this elicits an AP. The elicited AP originates close to the stimulation electrode and propagates bi-directionally along the axon.

The threshold current needed to elicit APs depends on several parameters, including (1) axon diameter (thicker axons are depolarized first), (2) the distance between stimulation electrode and axon, and (3) stimulation amplitude and duration. The duration must be limited to less than the duration of an AP to prevent the neuronal membrane from becoming refractory. Thus, short current pulses at threshold amplitude are typically used to elicit individual APs. Since thicker axons are recruited at lower stimulus amplitudes, extracellular stimulation works best if the axon of interest is the one with the largest diameter in the nerve. If smaller axons are targeted, larger stimulus amplitudes are required, which may activate larger axons first, in addition to the smaller axons of interest.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

Materials and Reagents

Note: The materials and equipment listed refer to the equipment used in Städele and Stein (2016). The principles of retrograde extracellular axon stimulations are universal and the procedures can be easily adapted to other preparations. To reduce costs, comparable materials, equipment and software may be used that serve the same functions. For the general public or a teaching classroom, we suggest utilizing equipment from Backyard Brains (https://backyardbrains.com).

1. Syringe, filled with petroleum jelly for preparing extracellular recording and stimulation wells
   For preparing syringes, the following materials will be needed:
   a. Petroleum jelly (100% pure, pharmacy)
   b. 100 ml glass beaker (for melting petroleum jelly)
   c. 5 ml Luer lock syringe (*e.g.,* BD, catalog number: 309603)
   d. Injection needle (20 G x 1.5”, *e.g.,* Santa Cruz Biotechnology, catalog number: sc-359535)
   e. Sand paper (80 to 100 grit, hardware store)
2. Recording/stimulation electrodes
   For preparing electrodes, the following materials will be needed:
   a. Stainless steel wire, uncoated (A-M Systems, catalog number: 794800)
      Low-cost alternative: Minutien pins (see below) or sewing pins
   b. Electrical wire, red and black PVC insulated (Southwire, model: 22 gauge stranded, catalog number: 57572444, hardware store)
   c. Wire stripper (hardware store)
   d. Needle-nose pliers (hardware store)
   e. Heat shrink tubing (hardware store)
   f. Tin solder, 3/32 in. (Forney, catalog number: 38109)
3. Petri dish lined with silicon elastomer (*e.g.,* Sylgard 184, Sigma-Aldrich, catalog number: 761036; or Elastosil RT 601, Wacker Chemie, catalog number: 60063613)
4. Minutien pins (Fine Science Tools, catalog number: 26002-10)
5. Modeling clay (craft store)
6. Dissected nervous system
   Note: We are using adult Jonah crabs (Cancer borealis), purchased from The Fresh Lobster Company (Gloucester, MA).
7. Physiological saline (see Recipes)
   The recipe for *C. borealis* saline can be found in Table 1
   a. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9625)
   b. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Sigma-Aldrich, catalog number: M9272)
   c. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: C7902)
   d. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
   e. Trizma base (Sigma-Aldrich, catalog number: T1503)

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

f. Maleic acid (Sigma-Aldrich, catalog number: M0375)

Equipment

1. Heating plate (Thermo Fisher Scientific, Thermo Scientific™, model: Nuova™ Stirring HotPlates, catalog number: SP18425Q)
2. Stereomicroscope (*e.g.,* Leica Microsystems, model: MS5)
3. Stimulator (A.M.P.I, model: Master8 Pulse Stimulator)
   Low cost alternative: Pulse Pal V2 (Sanworks, catalog number: 1102)
4. Amplifier (A-M Systems, model: Differential AC Amplifier 1700, catalog number: 690000)
   Low cost alternative: Spikerbox (Backyard Brains, model: Neuron SpikerBox)
5. Data acquisition board (CED, model: Power 1401-3A)
   Low-cost alternative: by using the BYB Spike Recorder, data can be digitized by using the microphone jack and soundcard on a computer/laptop. A second low-cost alternative is Spikehound (http://spikehound.sourceforge.net), which also allows recording through the computer soundcard
6. Soldering station (Apex Tool, Weller, model: Station 50/60W 120 V WES51, catalog number: WES51)
7. Voltmeter (FLIR Systems, Extech, model: EX330, catalog number: 203489911)
8. Forceps (*e.g.*, Fine Science Tools, model: Dumont #5, catalog number: 11251-10)

Software

1. Recording software (Spike2 version 7.12, Cambridge Electronic Design Limited)
   Low cost alternative: BYB Spike Recorder (freeware, available on https://backyardbrains.com/products/spikerecorder) or Spikehound (http://spikehound.sourceforge.net)

Procedure

Figure 1 illustrates the experimental setup and electrode placement. To stimulate an axon, a section of the nerve of interest will be electrically isolated from the rest of the nervous system by using a petroleum jelly well. The success of the stimulation will be monitored by extracellularly recording the elicited AP on a spatially distant part of the axon.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

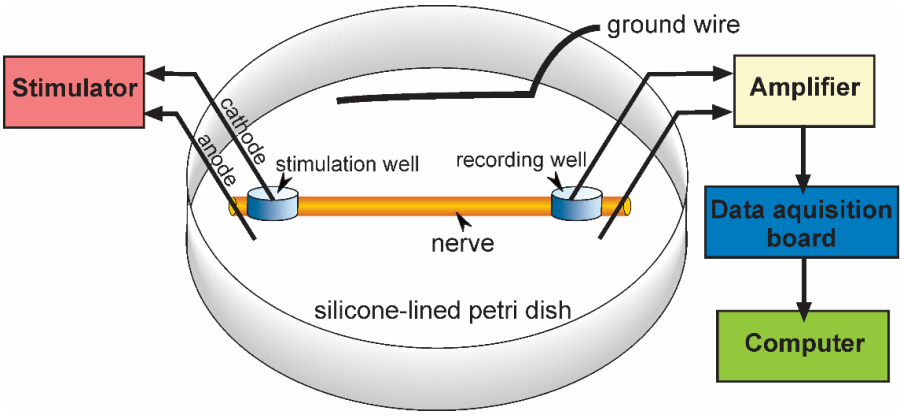


Figure 1. Schematic of the experimental setup and electrode placement. Illustrated is a Petri dish that contains the nerve. The blue cylinders represent petroleum jelly wells, the black lines illustrate electrode placement and their connection to stimulation and recording equipment.

1. Preparing petroleum jelly syringes
   a. Transfer petroleum jelly to a glass beaker.
   b. Place glass beaker on heating plate and warm petroleum jelly up until it is fully melted.
      Note: The melting point of petroleum jelly is between 45 °C and 60 °C. Avoid boiling! Boiling will create air bubbles.
   c. Take the 5 ml Luer lock syringe and fill it with the melted petroleum jelly by pulling the jelly up through the tip.
      Note: Wear gloves to protect yourself from burns.
   d. Let the filled syringe sit at room temperature until petroleum jelly is solidified.
      Note: To prevent air bubbles, place the filled syringe upwards (with the tip towards the ceiling) until the petroleum jelly solidifies.
   e. Cut off the tip of a 20-gauge injection needle using the wire-cutting pliers.
      Note: Use sand paper to smoothen the sharp edges.
   f. Bend the front part of the injection needle by an angle of ~70° (Figure 2).
   g. Add the needle to the syringe.

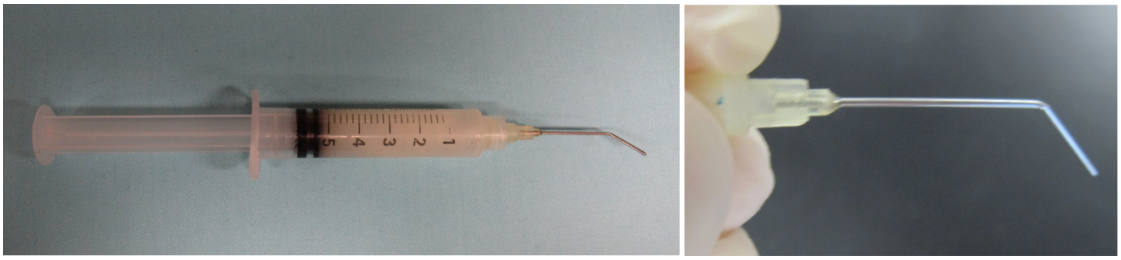


Figure 2. Petroleum jelly filled syringe used for preparing the extracellular stimulation/recording wells

2. Preparing stimulation and recording electrodes

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

Note: Repeat the following steps to create all necessary electrodes including the stimulating, recording, and grounding electrodes. The stimulating and recording electrodes require both a red and black wire, while the ground only needs a single wire.

a. Cut the electrical wires to the desired length.
b. Use the wire stripper to remove 5 to 10 mm of the insulation on each side of each red/black wire.
c. Take the pre-heated soldering iron and coat the stripped part of the cable with tin-solder (Figure 3A).
d. Take needle-nose pliers and bend the tin-solder covered wire part into a loop (Figure 3B).
e. Pass the stainless steel wire through the loop.
   Note: Instead of stainless steel wire, minutien or sewing pins can be used as well.
f. Fill the loop with tin-solder to attach the stainless steel wire to the black/red wire (Figure 3C).
g. Use a voltmeter to check if the stainless steel wire has the proper electrical contact with the wire.
h. Solder a suitable plug onto the other side of the cable to attach it to the stimulator/amplifier.
i. Use heat shrink tubing to insulate the blank solder joints.
j. In addition to stimulation and recording electrodes, a single ground electrode is necessary to reduce recording noise and stimulation artifact. Repeat the above steps using a black wire to build a single electrode.

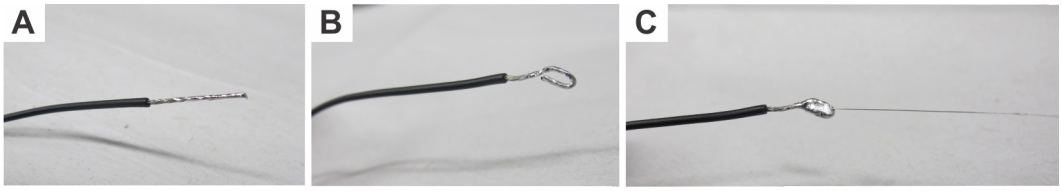


Figure 3. Preparation of extracellular stimulation/recording electrode. A. Electrical wire with insulated and tin-solder coated front part; B. Same wire, bent into a loop; C. Stainless steel wire attached to loop with tin-solder.

3. Dissect the nerve of interest
   Note: For a detailed protocol how to dissect the stomatogastric nervous system of C. borealis please review (Gutierrez and Grashow, 2009).
4. Transfer the nervous system to a silicone lined Petri dish.
   Note: The silicon elastomer should coat the bottom ~5 mm of the Petri dish.
   Place the nervous system in the middle of the dish.
   a. Fill the Petri dish with physiological saline (see Table 1 for *C. borealis* saline).
   b. Use minutien pins to mount the nervous system by either directly pinning through the end of nerves or by pinning through connective tissue still attached to the nervous system.
      Note: Do not pin through the section of nerve being recorded as this will damage axons.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

5. Secure the dish from displacement by attaching it to the work surface with modeling clay (Figure 4).
6. Place the ground electrode into the bath. Use the modelling clay to prevent the ground electrode from displacement. Connect the ground wire to the amplifier ground.

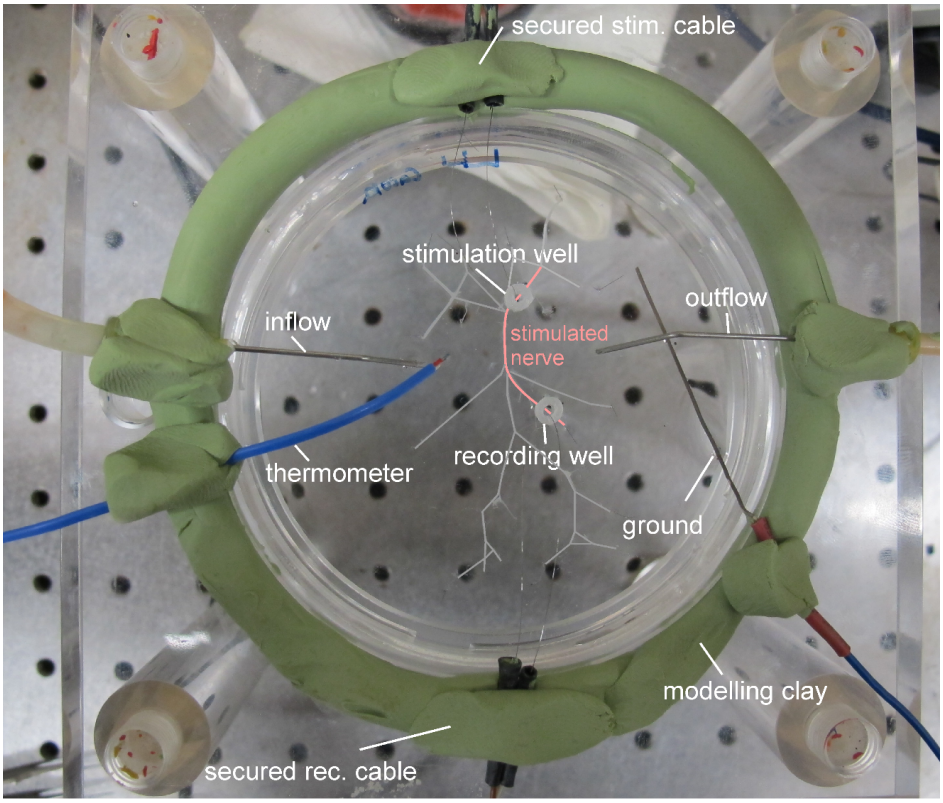


Figure 4. Petri dish with isolated stomatogastric nervous system. The photo shows the placement of the stimulation and recording wells along a stomatogastric nerve, saline inflow and outflow, and ground wire. The Petri dish is attached to the work surface with modelling clay. For extracellular stimulation and recording, no vibration isolation laboratory tables are needed. For better visualization, nerves have been retraced (gray and pink lines).

7. Prepare the extracellular stimulation/recording wells.
   a. With the use of a stereomicroscope make two concentric circles at two locations on the nerve of interest with the petroleum jelly filled syringe as shown in Figure 5. One well will serve as stimulation well, the other will serve as recording well. Add layers of petroleum jelly until the wells extend above the saline level (Figures 5A and 5B).
      Note: The well isolates a small section of the nerve from the rest of the bath as petroleum jelly is nonconductive.
   b. Ensure that the wells are tight and not leaky.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

Note: Add one or two drops of saline to the inside of the well. If the level of saline does not decrease, the well is sufficiently tight and isolates the nerve section from the rest of the dish (Figure 5C).

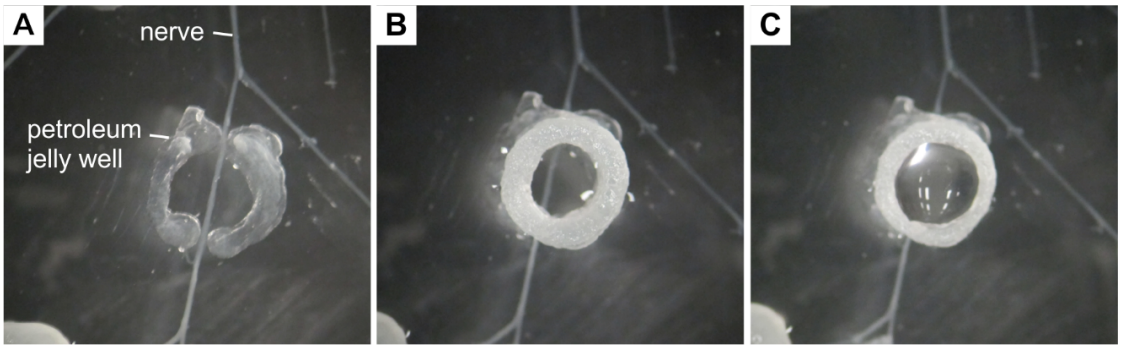


Figure 5. Petroleum jelly well preparation. A. An initial concentric circle was formed around a small section of the nerve of interest. B. Finished well. Petroleum jelly layers were added until the top most layer extended out of the saline. C. Leak test. Saline was added inside the well to test if it is sufficiently isolating the nerve section from the rest of the bath.

8. Place stimulation electrodes.
   a. Connect the stimulation cable with the stimulator (red wire = cathode, black wire = anode).
   b. Use forceps to place the steel wire (cathode) inside the stimulation well (Figure 6). Firmly press the wire into the silicone.
      Note: Avoid puncturing nerve or well. Place the end of the electrode deep enough into the silicone so the wire will not become dislodged at any point during the remainder of the experiment.
   c. Use forceps to place the anode outside of the well, but still close to it.
      Note: Wires must not touch each other since this will create a short circuit and axons will not be stimulated.
   d. Secure the wires using modelling clay.

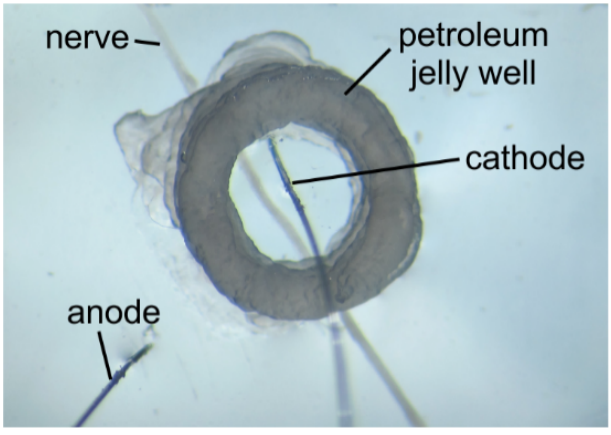


Figure 6. Arrangement of stimulation wires in the petroleum jelly well

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017 DOI:10.21769/BioProtoc.2151

9. Repeat step 8 for the recording wires.
10. Turn the extracellular amplifier on and check if spontaneous APs are visible on the extracellular recording.

    Notes:

    a. *For C. borealis, the amplifier filter settings should: amplification: 10,000; high pass filter: low cut-off 100 Hz; low pass filter: high cut-off 500 Hz.*

    b. *Detected AP waveforms do not directly reflect membrane potential changes. APs are detected using a differential recording between the inside and the outside of the recording well.*
11. Stimulate the nerve extracellularly.

    Initially, set the stimulation parameters on the Master8 to 1 ms stimulation pulse duration, 1 Hz stimulation frequency, and 0 nA/Volt. After the stimulation threshold has been determined, more physiological parameters may be used.

    a. Use the extracellular recording to first identify the stimulus artifact (Figures 7A and 7B).

    Note: Stimulation artifacts can be easily distinguished from APs because the size of the artifact increases linearly with stimulus amplitude. If the stimulation artifact is too large, the polarity of the cables should be reversed. The stimulation artifact will be smallest when the cathode (and not the anode) is placed inside the stimulation well.

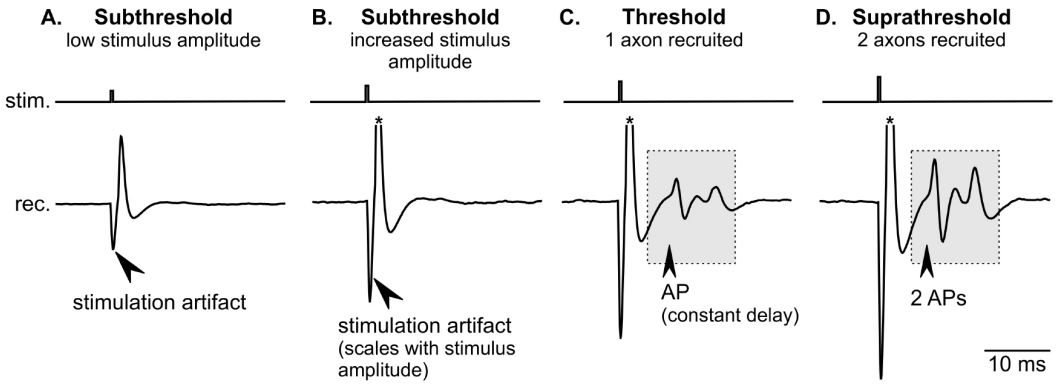


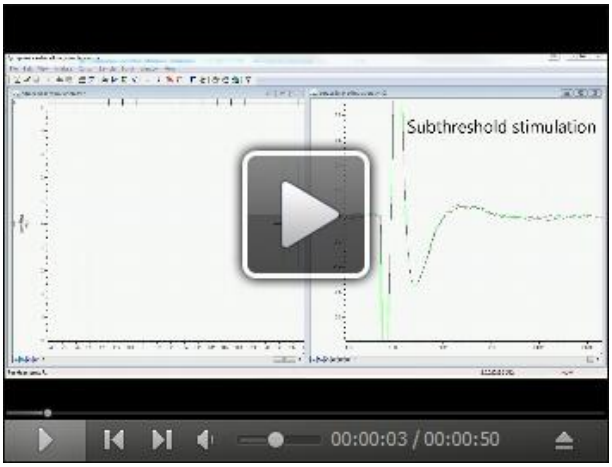
Figure 7. Adjustment of the extracellular stimulation threshold. Shown are extracellular recordings of a nerve in the stomatogastric nervous system during subthreshold (A, B), threshold (C) and suprathreshold stimulation (D). A. During subthreshold stimulation with low stimulus amplitude only the stimulation artifact can be observed, but no AP. The artifact occurs simultaneously with the stimulus pulse. B. Increasing stimulation amplitude below threshold will not elicit APs, but increase the stimulation artifact amplitude. C. Increasing stimulus amplitude to threshold elicits an AP. Its waveform appears with a fixed time delay after the stimulation artifact (gray box). The axon with the largest diameter in a nerve will be recruited first. Note that the AP waveform does not represent the different phases of an AP, but rather the potential difference between the inside and outside of the petroleum jelly well as the AP enters and leaves the well. D. Increasing the stimulation amplitude above threshold recruits additional, smaller diameter axons. Note that the shape and amplitude of the detected waveform changes when

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

other axons are recruited (compound AP). For better visualization, the artifact amplitude was cropped (indicated by *).

b. Determine the stimulation threshold by slowly increasing the stimulation amplitude until an AP becomes visible (Figure 7C).
Note: The AP should appear with a time delay after the stimulation artifact. APs can be distinguished from the artifact because they occur only after stimulus threshold is reached, and their time of occurrence and amplitude does not increase linearly with the stimulus amplitude. Note that if stimulus amplitude is further increased, APs from other axons may be recruited, which will change the size and shape of the detected signal (compound APs during suprathreshold stimulation, see Figure 7D and Video 1).
c. If the recording software has a trigger function, use it to control the sweeps of the extracellular recording to permit a fast time base, and a comparison of stimulus artifact and AP shapes.

Video 1. Video clip illustrating subthreshold, threshold and suprathreshold stimulation



Data analysis

Data analysis will differ depending on the experiment, but the following steps should be kept in mind while designing an experiment using extracellular axon stimulation.

1. To ensure that APs are elicited, it is essential to differentiate between stimulus artifact and arriving APs at the recording site. Elicited APs arrive with a constant delay after the stimulus pulse, and are independent of stimulus amplitude (Figure 7C). In contrast, the stimulation artifacts scale with stimulus amplitude and arrive almost instantaneously (Figures 7A and 7B).
2. At subthreshold stimulation amplitudes, only the stimulation artifact is visible (Figure 7A). To test whether the recorded signal is indeed the artifact, the time between stimulus onset and signal arrival at the recording site can be measured, using vertical cursors. For signal arrival, the first obvious peak of the signal can be used. Further, the amplitude of the arriving signal should be

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

measured between signal minimum and maximum, using horizontal cursors. At least 10 consecutive stimuli should be averaged to determine mean arrival time and amplitude.

3. Increase and decrease stimulus amplitude slightly, and measure arrival time and amplitude of the recorded signal again. If the arrival time occurs at the same time as the stimulus pulse and amplitude scales with stimulus amplitude (Figure 7B), the recorded signal is the stimulation artifact. Measure at least 10 consecutive signals at each stimulus amplitude.
   Note: A t-test can be used to compare measurements at different stimulus amplitudes (significance cut off value of $P < 0.05$).
4. Increase stimulus amplitude further until a second, delayed signal arrives at the recording site (threshold stimulation, Figure 7C). If this is the AP, it should occur suddenly when stimulus amplitude is raised (no slow build-up). Measure arrival times and amplitudes for at least 10 consecutive APs.
   Note: APs are unaffected by changes of stimulus amplitude. Confirm that the recorded signal is indeed the AP by slightly increasing the stimulus amplitude before measuring arrival times and amplitudes again. Arrival times of APs depend on the distance between stimulation and recording sites.
5. Increasing the stimulus amplitude further (suprathreshold, Figure 7D) may recruit additional APs, whose delay and amplitudes can be measured using the same analysis.

Notes

1. Prior to attempting axon stimulation, record spontaneous AP activity of at least 10 consecutive APs. There may be need to further reduce noise by grounding the stimulator to the amplifier and by utilizing a faraday cage.
2. Instead of steel, silver chloride may be used for the ground wire. This is particularly important if additional intracellular recordings are planned and/or a drift of the recorded membrane potential must be avoided.
3. If the stimulation artifact is large and has a long duration, the anode and cathode of the stimulator may be inverted. In this case, the polarity of the stimulation electrodes needs to be reversed, because the anode causes the extracellular space to become more positive and, as a consequence, the neuron hyperpolarizes locally. Since this hyperpolarization is surrounded by areas which, relatively, are depolarized, APs can still be elicited (anodic stimulation). However, APs stimulated by anodic stimulation have a higher threshold, causing the stimulus artifact to be larger.
4. If no extracellular recording of the stimulated nerve is feasible, the success of the stimulation can be assessed by monitoring changes in activity of postsynaptic neurons, circuits, muscles, or behavior. In this case, higher stimulus frequencies are recommended to achieve clear postsynaptic responses.

bio-protocol www.bio-protocol.org/e2151 Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2151

Recipes

1. *Cancer borealis* physiological saline recipe (see Table 1)

Table 1. *Cancer borealis* physiological saline recipe

Salt (Molecular Weight)	Concentration [mM]
NaCl (58.44)	440
$MgCl_2 \cdot 6H_2O$ (203.31)	26
$CaCl_2 \cdot 2H_2O$ (147.01)	13
KCl (74.56)	11
Trizma base (121.14)	11.2
Maleic acid (283.3)	5.1

Note: Adjust pH to 7.4-7.6 with Trizma base and maleic acid.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG STE 937/9-1), National Science Foundation (NSF IOS 1354932), Illinois State University, and the German Academic Exchange Service.

References

1. Abbas, P. J. and Miller, C. A. (2004). Biophysics and physiology. In: F.-G. Zeng and R. R. Fay. (Eds). Cochlear Implants: Auditory prostheses and electric hearing. *Springer*, 20: 149-212.
2. Barry, J. M. (2015). Axonal activity *in vivo*: technical considerations and implications for the exploration of neural circuits in freely moving animals. *Front Neurosci* 9: 153.
3. Basser, P. J. and Roth, B. J. (2000). New currents in electrical stimulation of excitable tissues. *Annu Rev Biomed Eng* 2: 377-397.
4. Cogan, S. F. (2008). Neural stimulation and recording electrodes. *Annu Rev Biomed Eng* 10: 275-309.
5. Gutierrez, G. J. and Grashow, R. G. (2009). *Cancer borealis* stomatogastric nervous system dissection. *J Vis Exp* (25)
6. Städele, C. and Stein, W. (2016). The site of spontaneous ectopic spike initiation facilitates signal integration in a sensory neuron. *J Neurosci* 36(25): 6718-6731.