Earthworm Lab.

The goal of this laboratory is to learn the basics of electrophysiology. There are three main components to this area of research: instrumentation, software and biological preparations. We need to learn the necessary skills in each category to be able to perform electrophysiology experiments.

**I thank Biology Machine Shop for constructing worm chambers.

Instrumentation:

Fig.1

Instrumentation in electrophysiology has two aspects: mechanical and electronic. The mechanical aspect involves the use of fine and stable manipulators, typically with micrometer resolution, to move sharp or patch electrodes in order to approach and record from neurons without tearing them apart. These manipulators can be manual, motor or piezoelectric driven. The use of these manipulators typically takes a dedicated training session because they are delicate and expensive and often don't survive a class of 120 students! This project is designed in such a way that we don't need to use manipulators.

The second aspect of instrumentation is electronic. Traditionally, this includes amplifiers to measure the electric activity of neurons, and oscilloscopes to display the recordings. These instruments are full of knobs, with 10-15 on each, and have frustrated biology students for decades. After all, who can remember which knob does what in the span of 1 to 2 lab sessions? (Engineering and physical sciences majors have an advantage here because they have learnt about similar instruments systematically elsewhere.) Fortunately, advances in modern electronics have collapsed the amplifier and oscilloscope into something more familiar. There is only one amplifier you will have to use. It is a differential amplifier, M3000 (Fig.1). We choose M3000 here because it has a very good signal to noise ratio. In order not to

make this lab too long, we only ask you to turn the power on. All the knobs and switches are preset for you. Don't change them. Do check that all the knobs and switches are in the same positions as those in Fig.1.

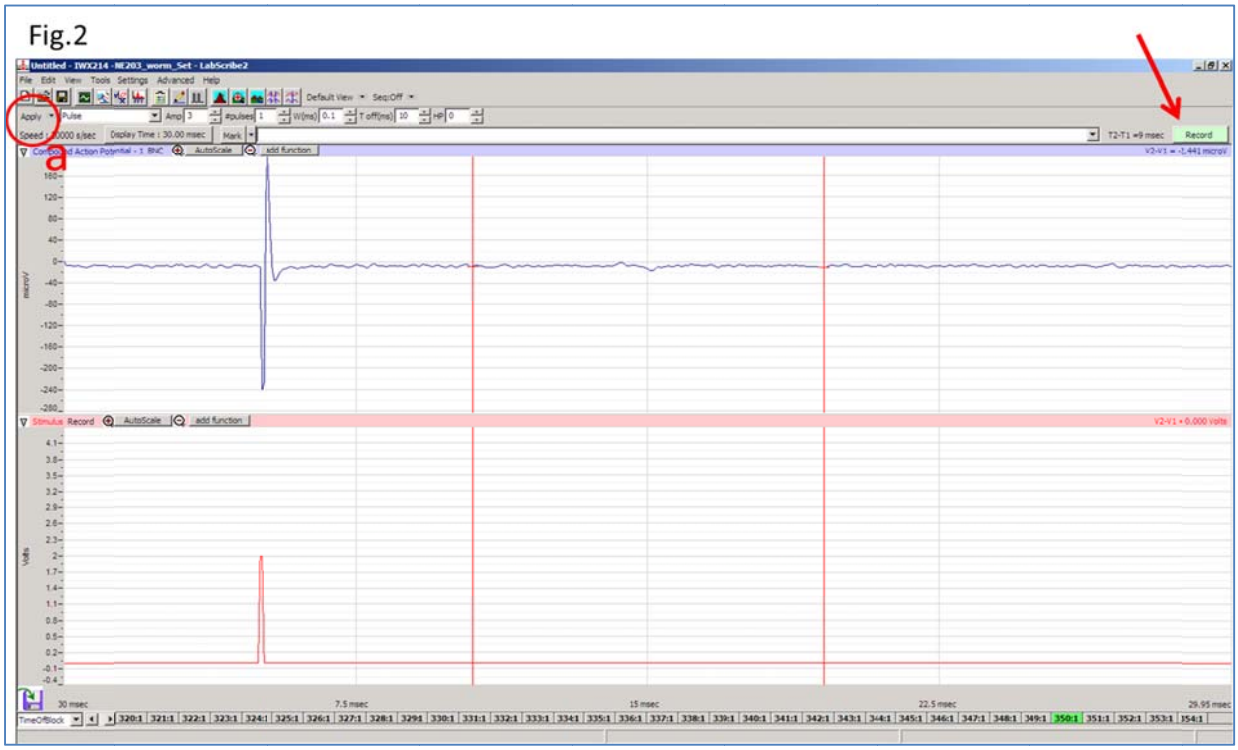
Software:

The data acquisition software we use is called LabScribe. It looks pretty but is fairly complicated, and can get us all lost if we are not careful or if we're too quick to click on buttons. After all, the software has to do all the functions previously performed by the many knobs on both amplifier and oscilloscope.

Rather than spend hours making you learn the software systematically, we will just launch LabScribe by clicking on "NE203_worm_Set.iwxset" in the earthworm folder. Meanwhile, you must resist the urge to click on buttons out of curiosity. For now, we would like to avoid getting into any part of the software we don't need to spend time on.

There are two main panels on your Mac (Fig.2). The top panel (blue) shows the recording from the earthworm, the bottom one (pink) shows the stimulation. We need the bottom panel to visualize the parameters used to stimulate the earthworm axons. These parameters include: the time when the stimulus is delivered, the duration of the stimulus and the amplitude of the stimulus.

Fig.2



How is this stimulation delivered? The black box on your bench, officially called IX214 (Fig.3), manages this process. The red section of IX214 (stimulator), is where the stimulation will be sent out from in order to shock the worm. The parameters of our stimulation defined

in LabScribe will be sent via the USB cable from the Mac to the back of IX214. These numerical parameters will in turn be translated into amplitude, duration and timing of stimulation.

Fig.3

How is recorded data collected? We will use a recording technique called differential recording. As the name suggests, the recording will actually be taken from two electrodes, i.e. two wires, placed near each other under the earthworm. The circuit in M3000 takes the difference between the signals recorded by the two electrodes. Signals from the two wires come into IX214 via the Ch1, in the middle (Non Isolated Inputs) section.

Optional, dense reading!

Why go to the trouble of taking the difference between the two channels? Because action potential signals picked up from the metal wires under the worm are very small. AP's you see in textbook figures or simulations are recorded from **inside** of a neuron or axon (intracellular recording) and have an amplitude of ~100mV. The amplitude of a 100mV AP will drop down to tens of **microvolts** if an electrode is placed near the axon but in the extracellular space (extracellular recording). The actual AP amplitude of an extracellular recording depends on the distance between the axon and recording electrode, the size of the axon, and the conductivity of tissue between the axon and electrode. In our case, the recording wires are separated from the axons by a gap of ~1 millimeter, including muscles and skin of the worm.

Typical instruments noise on a bench, without special protection and with cables attached, is in the range of tens to hundreds of microvolts. An extracellular AP, also tens to hundreds of microvolts in amplitude, will be difficult to detect in the presence of the instrument noise. Another main source of the noise in the recording comes from powerlines, at 60Hz, picked up by the cables coming out of M3000. Since the two channels pick up the same powerline noise, taking the difference between the two channels easily cancels out this main source of noise. A similar approach is used in EEG, ECG and EMG recordings. To further reduce the noise level, the trace you see on the computer monitor will have been filtered and cleaned up.
End of dense reading.

One last thing you need to know for now is how to start your recording. All you need is to click on the button "Record" above and to the right of the upper panel (Fig.2 red arrow). You can click it now, but it will only give you a flat line because you don't have a worm set up yet. We will discuss how to save and analyze your data later.

Biological Preparation.

The earthworm has been one of the mainstays in the undergraduate laboratory. Although it is not as glamorous as squid or frog, there is a great deal to be learnt from this creature. In fact, the measurements we will do today are similar to the basic tests a neurologist performs on patients. Simple parameters such as action potential conduction velocity, stimulation threshold, refectory period and EMG are useful variables from which to infer the source of a patient's neurological problems. The following procedure has been adapted from a laboratory instruction manual used at Smith College[1] and a paper[2] published in 2010.

We are going to use the whole earthworm for our experiment. We take this approach because we don’t want to spend a disproportional amount of time dissecting animals. As an alternative, some lab manuals advise students to use pins to pierce and immobilize the animals, and then use the same pins as stimulating and recording electrodes. Pinning the worms down has the advantage of keeping the electrodes in the same place throughout, and recordings are especially consistent and have good resolution. However, it is difficult to keep an animal anesthetized for more than 20 minutes. The sight of a pinned worm waking up and starting to squirm would be sure to distract students and make many of you cringe. Instead, we will adopt an approach suggested in a recent paper where the earthworm is kept in a confined space, with recording wires placed under the animal[2]. This way, the animal, though it may complain, is not seriously harmed. (While preparing this manual, I ran through more than 20 experiments, with as many animals, and they all survived as long as I finished the tests within 4 hours.) There is, however, a price to pay when using this humane approach, namely that stimulation and recordings will not be strictly consistent between trials. This is because although the

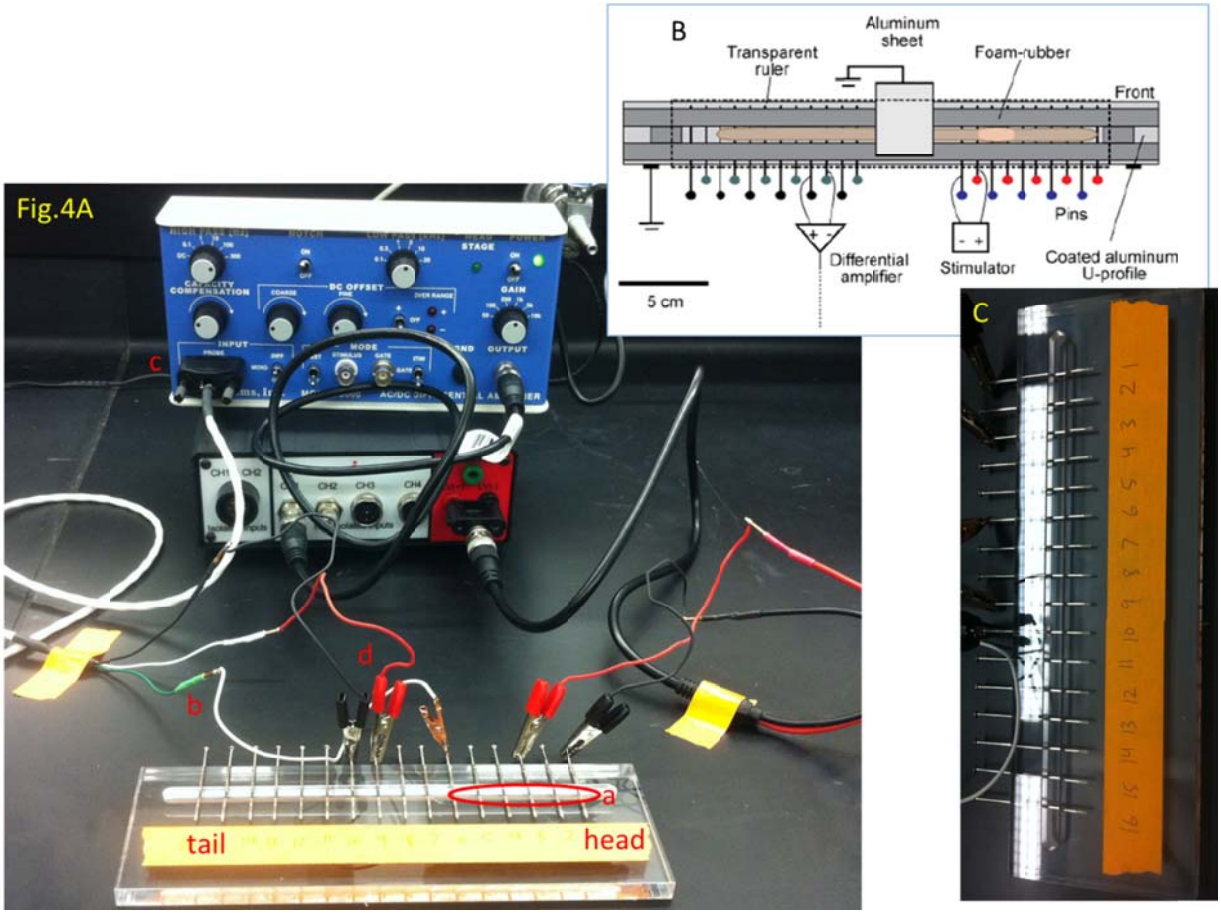
earthworm is confined, it can still move. As a result, the distance between our stimulating and recording wires and the axons will have varied slightly from time to time. Nevertheless, useful information can be obtained, and experts like us will be able to extract the data we are after.

1. Anesthetize the animal. Handle the animal gently. You may want to wear gloves for the first step. Pick up the animal by hand or use the spatula to hold it by its mid-section. Put your animal in the beaker, rinse it with tap water, and drain the tap water without pouring the earthworm down the sink. Pour in 10% ethanol, enough to cover the animal. (Make sure that you drain the water properly first, so that the alcohol is not diluted.) We need to wait until the worm stops moving, about 5-10 minutes. (Finish reading the next two paragraphs while you are waiting. You don't want to swing the worm around while reading and figuring out how to put it in the recording chamber.) You should keep an eye on the worm after 5 minutes, moving the beaker a little to see if the worm still moves. Take it out of alcohol when it has stopped moving. Alcohol should immobilize its muscles, and block the function of sensory receptors on the skin, so that the worm doesn't move too vigorously when we record from it.

We will next put the worm on a paper towel so the animal is not dripping wet and the slime it secretes is wiped off, but the worm should remain moist. Too much water on the skin will short out the current and degrade our recordings. Then again, a dry worm is a dead worm. Just let the paper towel soak up excess water, then transfer the animal to the worm cage we have built. (You can tilt the paper towel so the worm rolls on it, to remove excess fluid.) The spatula is useful for tugging the worm into the recording chamber (Fig.4)

This chamber is designed to hold the animal in place, by not leaving too much wiggling room. The pins attached to the side of the cage will make contact with the ventral side of the animal. (We will discuss the pins in detail in a minute.) The most pressing issue right now is to place the worm in the chamber in the correct orientation. First, the anterior should be at the end of the chamber marked by smaller numbers. (The numbers indicate the distance from the end of chamber in centimeters.) Which end of the worm is the anterior? Check Fig.5. In addition, the paler side of the worm should face down. (There is a dark streak on the dorsal side of the animal.) This orientation is important because the nerve cord where axons are located is on the ventral side, and the skin on that side is thinner and should provide better electric signals (Fig.5). Lay the animal down and cover the top of the chamber with the clear ruler on your bench. Use the spatula to tug the worm in if necessary. Hold the ruler down with the aluminum bar on the ruler. The aluminum bar holds the ruler down and the opening on the bar allows you to keep an eye on the worm. You should check the location of the worm regularly, to make sure that it rests on the pins you have connected for stimulation or recording. The entire contraption is designed to minimize movement and to prevent the

loss of moisture, so that the animal stays in a good physiological state for as long as possible.

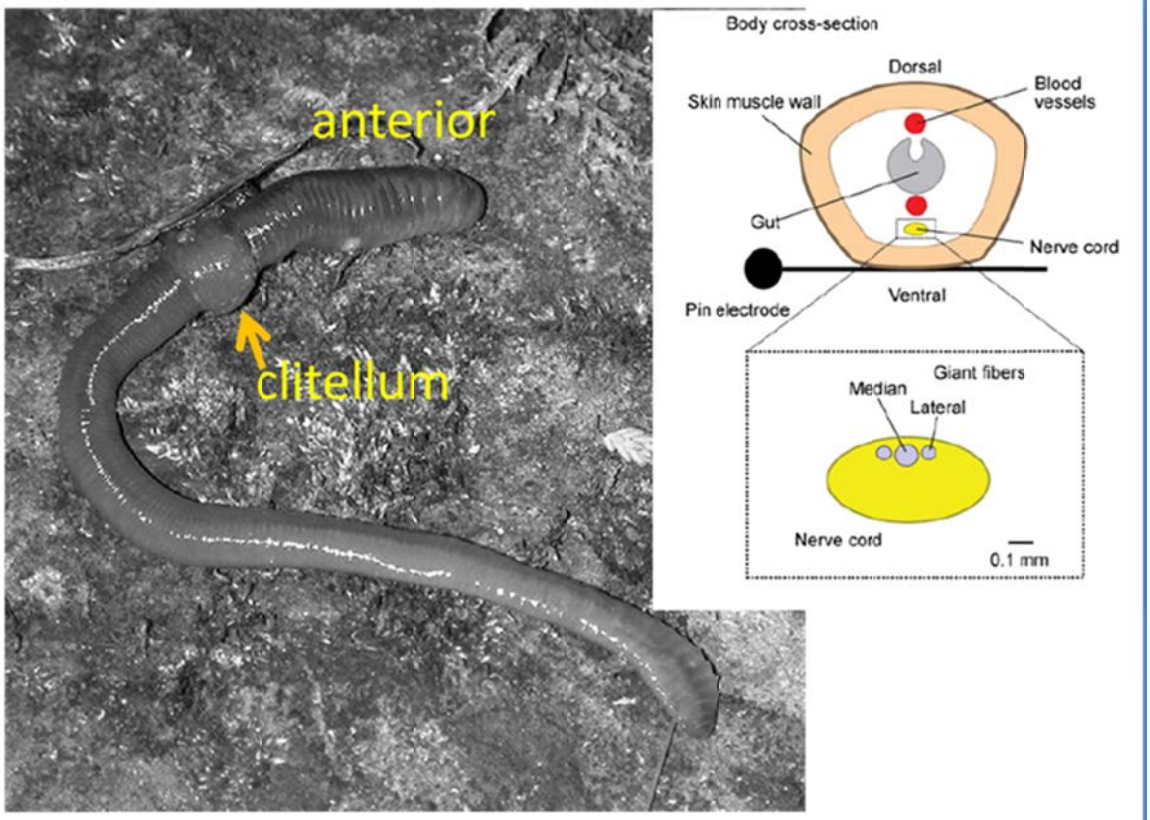


Pins: If you place the animals down correctly, you should have pins 1-7 near the anterior of the animal (Fig.4Aa). (To confirm the orientation, check the inset at Fig.4C.) These pins are for stimulation. The stimulation parameters are displayed in the bottom panel in LabScribe. When a voltage pulse is applied between two pins, current flows between them, and, if large enough, the current should depolarize the axon and initiate action potentials. This kind of extracellular stimulation is quite inefficient and there is a lot excess current flowing everywhere. This current will be picked up by our recording electrode and obscure the signals generated by AP. To "mop up" the excess current overflow, we place a ground electrode between the pair of stimulation electrodes and the pair of recording electrodes. This way, the excess current delivered by the stimulation electrodes will mostly "sink" into the ground wire and not reach the recording electrodes. Recording electrodes will be connected to 2 of the 8 pins (9-16), while the ground electrode should be **between** the stimulation and recording pairs.

Connections: Cables are already connected to the stimulator and INPUT of M3000. At the ends of these cables, there should be thin wires with alligator clips. You will clip these wires to the pins on the chamber

to stimulate and record from the worm. You should clip stimulating leads near the anterior by using any of the four pins, 1-5 (Fig.4 Aa). It doesn't matter whether the red wire is ahead of or behind the black wire. Find the white wire connected to the green lead (Fig.4Ab) bundled in a white cable coming out of the "INPUT" section of M3000 (Fig.4 Ac). Clip it to a pin immediately posterior to the posterior stimulation pins. This is your ground lead, for mopping up the excess current due to stimulation. Finally, clip the remaining red and black wires to a pair of neighboring pins in the middle of the tail end. As a start, clip the red wire near the head and the black wire posterior to it (Fig.4 Ad).

Fig.5



Source: Photo from Wikipedia, drawing from Ref.#2.

We can now start the experiment:

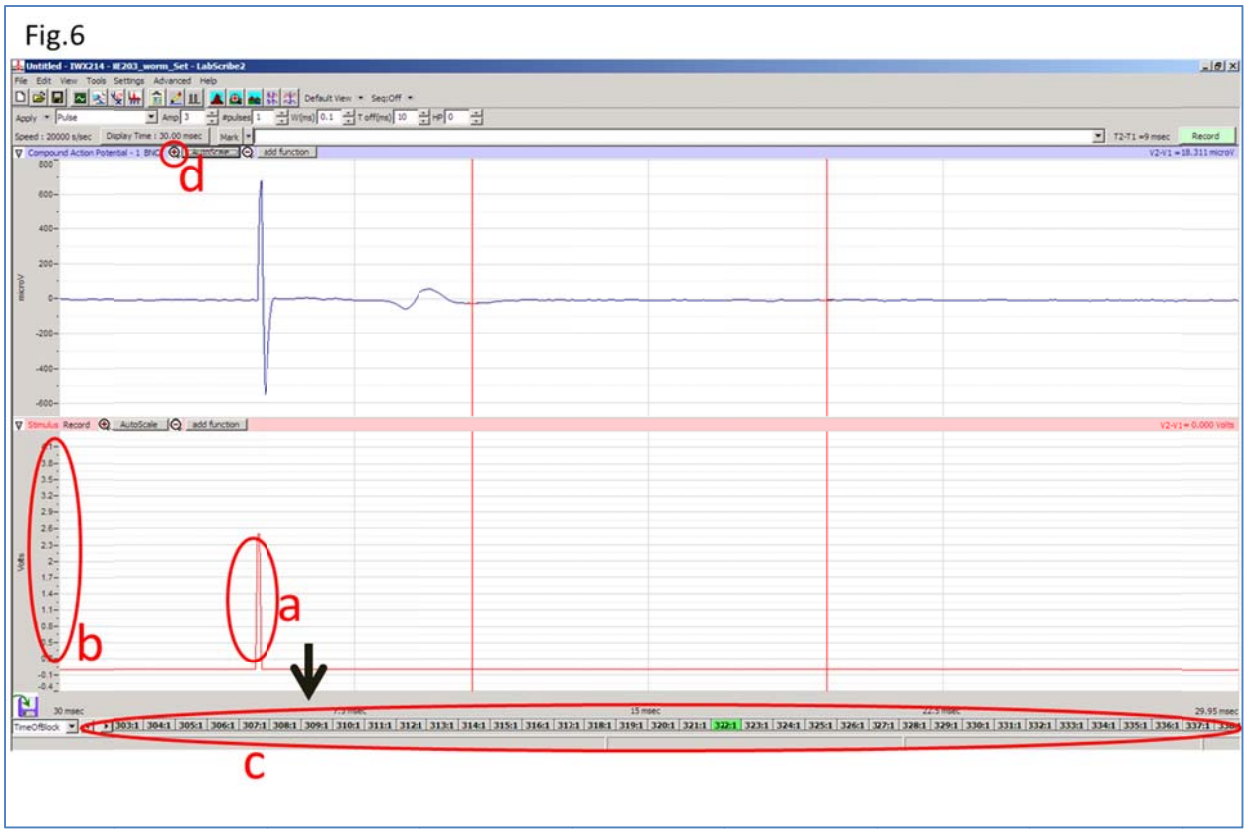
Go to the stimulation strip on LabScribe. It is the horizontal strip marked Apply on the left and below all the colorful buttons (Fig.2 circle a). The information on this strip is important. The left most box is marked "Pulse", meaning we will use a brief voltage pulse for stimulation. The amplitude of this voltage pulse is defined in the box next to Amp. The pulse width (0.1 ms) is defined in the box next to W(ms). The main parameter we will adjust is the amplitude. Set it to 1 volt now. We will deal with all other boxes later, so don't change them for now. **You must click on the "Apply" button every time you**

change the stimulus amplitude, otherwise the changes you make will not take effect.

Set the amp to 1 V. Click the "Apply" and then the "Record" button. You should see something that looks like the Figure 6. There are two panels. The bottom panel displays your stimulation, delivered at 5ms (Fig.6 circle a). You can read the amplitude of stimulation by referring to the left axis (circle b). The height of the stimulation pulse at 5ms should match with the number in the amp box in the "Apply" strip. The top trace is our differential recording. Both panels use the same time scale, marked at the bottom, from 0 to 29.95 ms. The recorded trace at the top is the complicated part. Let's examine the recording in several steps:

If you don't see a trace at all, click on the "AutoScale" button. A blue trace should show up. You may have to click on the magnifying glass (Fig.6d) in order to observe details on your trace that are similar to those in Fig.6. You can also drag the trace up or down so that it remains visible after magnification.

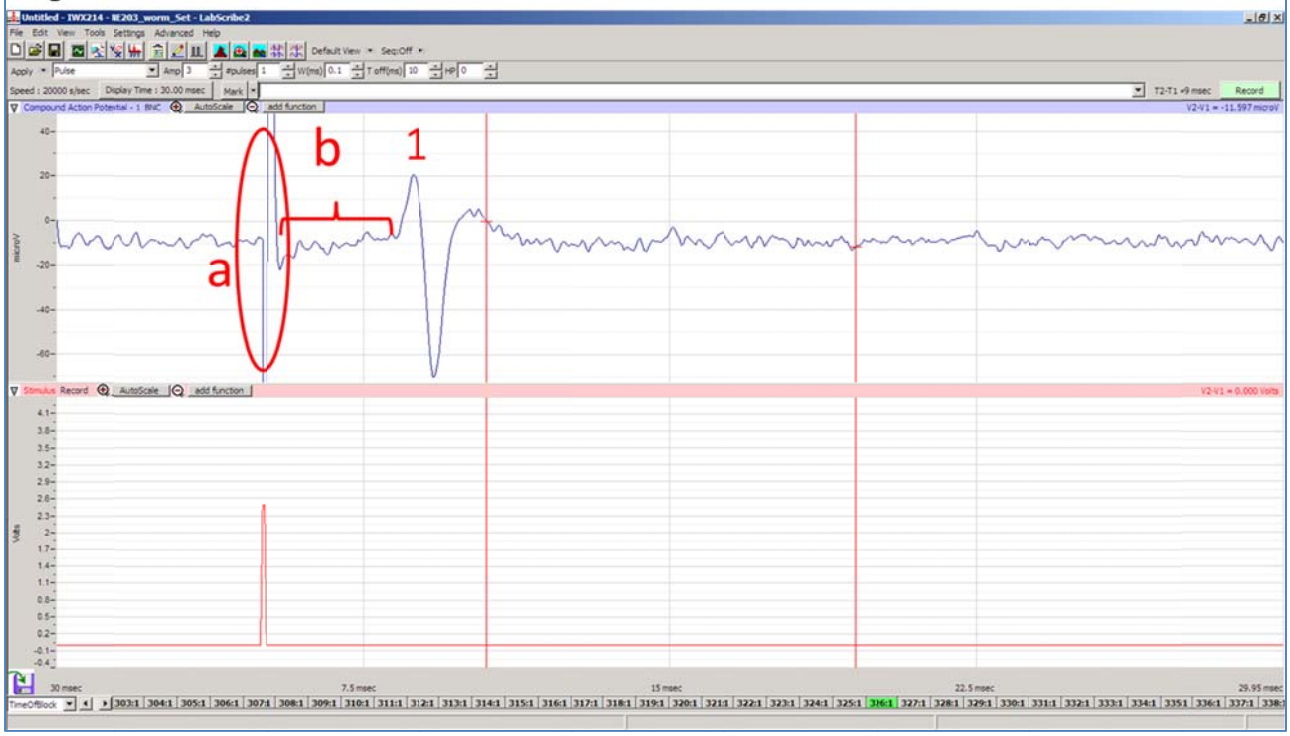
Fig.6



**You should still read on, even if your trace doesn't look like the example in Fig.6. There are some features to which you should pay attention, and other features you can ignore. We will mainly pay attention to the part of the trace after the stimulus artifact (Fig.7a). The signals following the stimulus pulse are small and we don't see much detail. We can enlarge them by clinking on the

magnifying glass, left to Autoscale. You can drag the panel up or down if the trace goes off scale after you magnify it. After magnification, the trace should look like this (Fig.7).

Fig.7



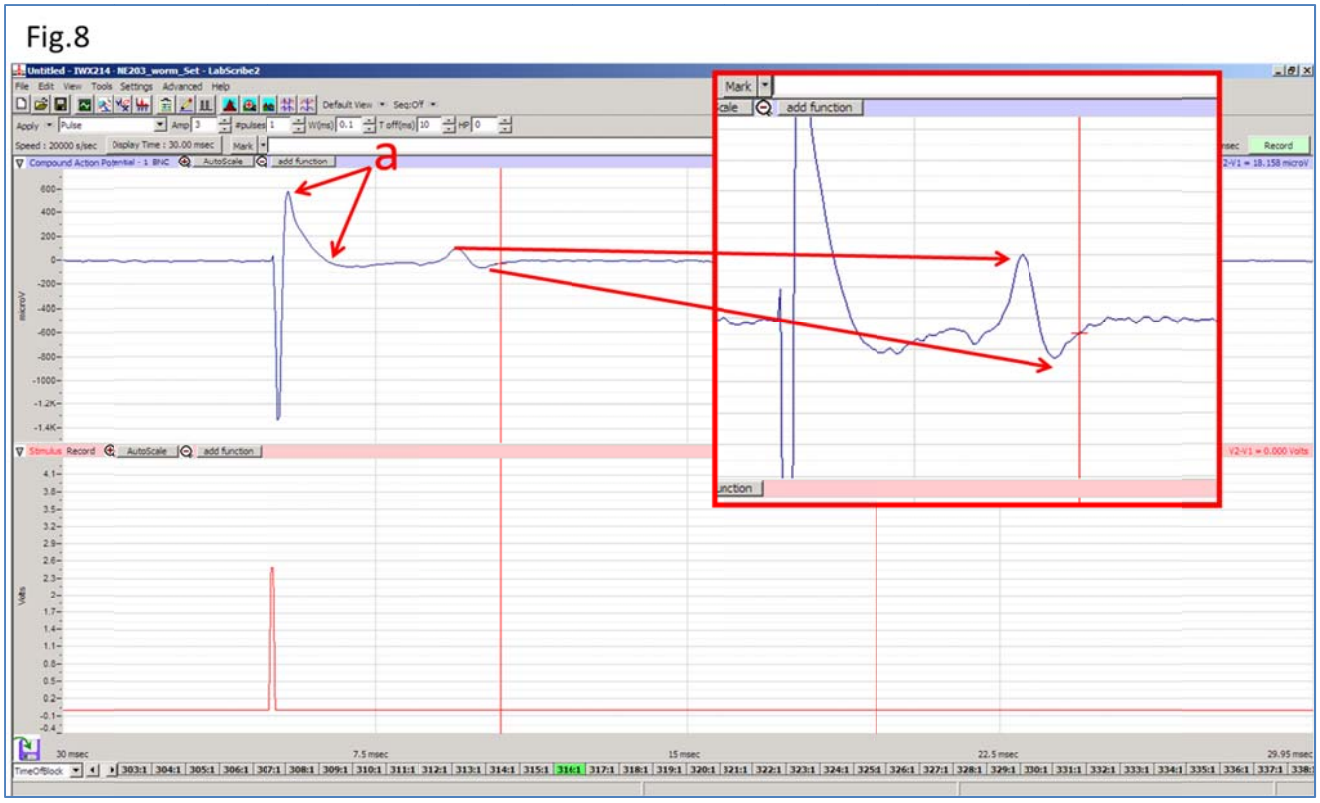
The stimulus artifact has an up and down spiky shape (Fig.7 circle a). The shape of the artifact is not important; the amplitude is in the range of 0.2 mV or larger. Note that we delivered a 5 volt shock near the head. Most of the shock current has been picked up by the ground electrode. We record only a fraction of one mV with our recording electrode. Enough about the artifact!

There is a main a peak following the stimulus artifact (1), this is the action potential generated by the median giant fiber. We are going to make some measurements from this signal.

However, not everyone has beginner's luck. Your trace may not look like the example in Fig.6&7.

Yours may look like that in Fig.8

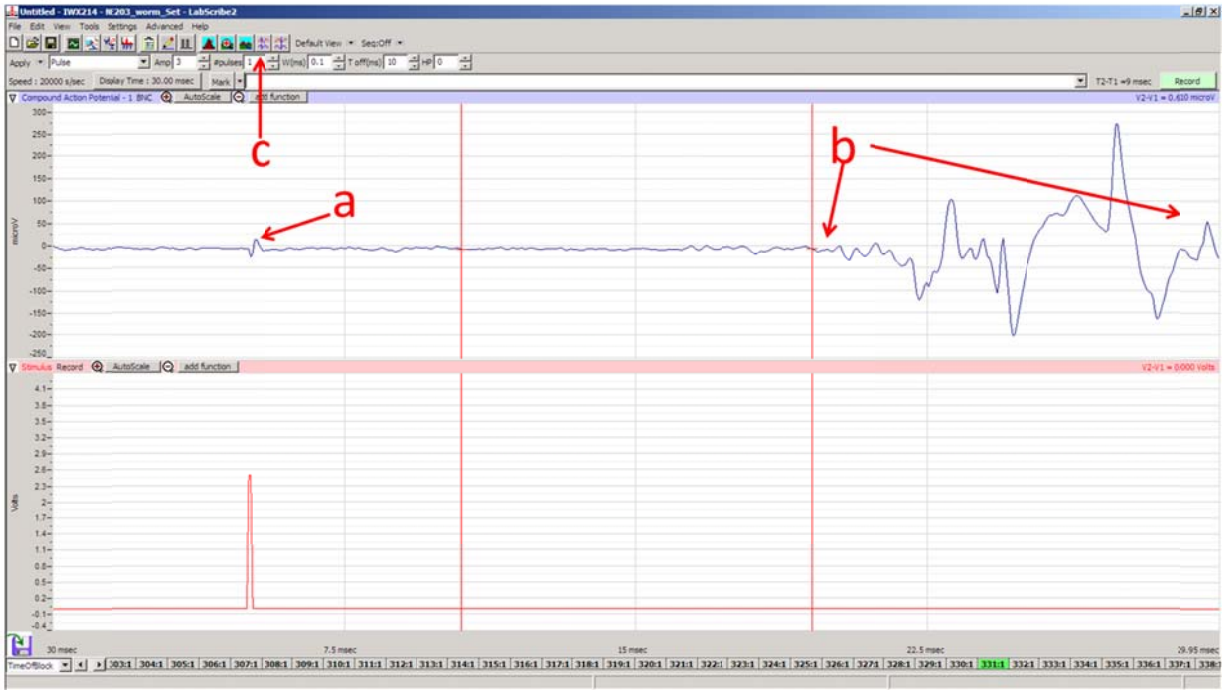
Fig.8



Not as beautiful as that in Fig.6&7, since the artifact is very wide (a). It happens in some traces. If the artifact remains wide in all your trials, check your ground clip. Make sure that it is not in contact with other pins or wires. In any case, not a pretty trace, but it is your baby. It has the essential information you need. (See inset for the enlarged AP, red arrows.)

What about a trace that looks like that in Fig.9 after you have clicked "AutoScale" and magnified it?

Fig.9

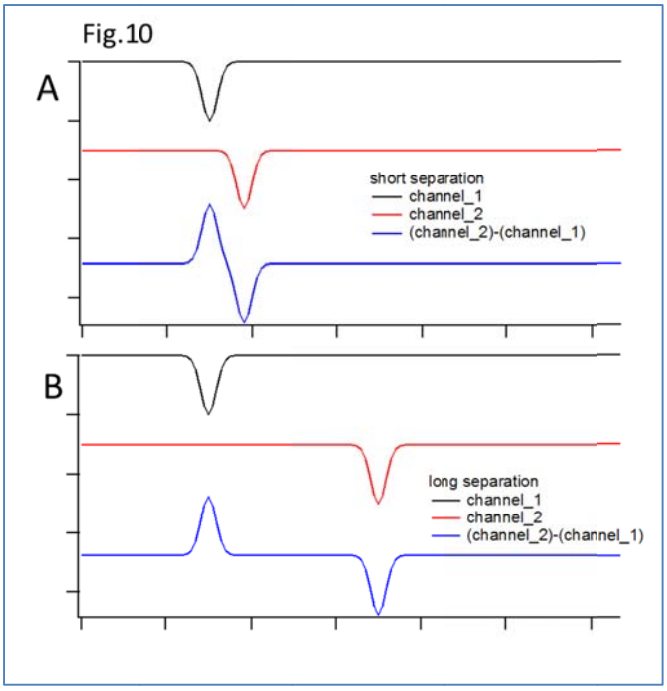


This trace is not completely flat, with funny spikes at wrong places. This is not good. First, the stimulus artifact is not the right size. A normal recording should give us stimulus artifact of ~0.2mV or larger. It is less than 0.05mV in this case (a). It is not your fault. The instrument we use is excellent in terms of amplifying small signals but, in the process of trying to amplify a very small action potentials, a capacitor in M3000 can become saturated. Don't try to click it right away, wait for 10-20 second and try again. The right signal, namely traces look like those in Fig.6-8 will come. In addition, there is "junk" in this trace (b). We know it is junk because it occurs so late in the traces that it cannot be related to AP conduction. In addition, the entire event lasts for about 10ms and we know that earthworms don't generate such spiky and complex response by a single shock.

The AP traces in Fig.6-8 look very good but may not be as pretty as what you will see on internet or books. In those published recordings, people show their best recordings after hundreds or thousands of trials. It is unlikely that you will get something as good looking in the first trial, although one never knows. Nevertheless, there is information in your trace.

First, you need to identify the stimulus artifact (Fig.7 circle a). This is the signal picked up by your recording electrode when the stimulating electrode is delivering the shock. The timing of the stimulus artifact coincides with the pulse shown in the bottom panel. The trace goes flat for 3-5 ms (Fig.7 b) then you should see a sharp spike, often going positive first then negative (biphasic) (Fig.7 1). The polarity of the spikes, namely whether they point up or down,

depends on how your electrode is connected. If you reverse the order of the pin connections of the recording electrodes, you will get a spike going down first and then swinging upward. (You can try to reverse the order now. Also, the amplifier often becomes saturated when you move the clips. One trick that helps, sometimes, to get the amplifier out of saturation is to use the metal spatula to short the electrode leads with the ground pin. Do this carefully and don't end up pulling the wires and moving the pins.)



The reason for the biphasic AP is simple. The trace is the result of a subtraction by the amplifier, [(channel_red)-(channel_black)]. With a recording from a single wire, extracellular AP should be negative. If AP travels from head to tail and is recorded by channel_black (Fig.10 A black) first and channel_red (Fig.10A red) second-as AP propagate from black clip to red clip—then the difference trace should deflect in a positive direction first, then swing to negative (Fig.10 A blue). If the two recording electrodes are separated by several centimeters, i.e. very far and AP traveling between the clips takes longer, then the positive peak returns to baseline for a while before it swings downward (Fig.10B). The gap between the two peaks represents the time AP takes to travel from electrode black to electrode red.

After the AP, the trace continues. Sometimes, there are additional bumps. Many papers and websites on internet suggest that there is a second, slightly delayed sharp peak mediated by the lateral giant axons. During my preparation and testing for this manual in the Fall semester of 2013, I found it difficult to unambiguously identify APs generated by the lateral giant. Not wanting to sweep the lateral giant under the rug, the first version of this manual offer extra credit to students who could clearly demonstrate the AP from the lateral giant. In 2014, one group succeeded and eleven groups succeeded in 2015. This is an example of advances in science and technology, we, as both students and instructors, do get better and achieve more over time if we are willing to pay attention and make efforts. The identification of lateral giant is part of this paboratory (Project 4) from 2016 onward.

Before we start our project, we need to keep track of what we have done. You should open a powerpoint file, in order to take notes in the notes window of slide 1. (Labscribe has a sophisticated lab note taking feature, but I want to get on with the experiment and not

spending more time teaching software you will use only once in your life.) For the traces you have generated, each one is identified by a specific numerical code (x:1) where "x" increases sequentially. The trace ID's are displayed at the bottom of your window (Fig.6 circle c). If you click on a particular box, the corresponding trace will appear and the little box marked green (Fig.6 black arrow). You should make notes of the voltage you use for each or a range of traces, from trace x to trace y for example, in your notebook. Also make a note of particularly good traces. Keep your notes simple and informative, such that anyone reading it can quickly reconstruct your experiment. (It may not be convenient to type in the notes while LabScribe is running, write the info down on a piece of paper and type in later. Your notebook counts as part of your lab report.)

Finally, use the "File" menu in LabScribe and use "Save As" to save your data and your notes, with a filename identifying your group. For the rest of this lab session, just save your results from time to time so you have all data under one filename.

Project 1: What is the threshold of the median giant axon?

If you remember Project 1 of your first lab, you should have a rough idea about how to find threshold. You need to adjust the stimulation amplitude up and down until you find the AP threshold where a change of 0.1V will make the difference between failing/succeeding to evoke AP.

In the note section of your powerpoint slide 1, you should write down the trace IDs for which AP stimulation failed and succeeded in triggering AP. (Select one good looking example for each condition.) The difference in stimulating voltage used for the two IDed traces should be 0.1V. In this experiment, it is reasonable to repeat the stimulation evoked by a given voltage several times, especially when you are testing voltage around the threshold, to convince yourselves of your accuracy and to increase your chance of obtaining a trace with a flat baseline. However, don’t overdo it. You will regret later if you have to find a particular trace and have to sort through too many traces. Of course, you need to stimulate as many times as you need to get your data. Another important reason for not overdoing your stimulation is that your worm may get too agitated and move around too much. That will definitely affect the consistency of your results.

In addition to the note taking details I have already mentioned, you should also make a note of the position of your worm, namely head at x cm, tail at x cm. A division of labor may be useful here—one person working LabScribe, one taking notes and one noting worm and pin position etc.

In order to prepare for your presentation next week, you need to choose your best traces now, both below and above threshold, and incorporate them into slide 2 of your file. (Slide 1 is reserved for note taking.) You may have to size the two figures to fit into one

slide. You can write the figure legend after you go home. In the legend, you should include:

(1) The time when the peak of AP occur. A precise measurement can be made by moving the red vertical cursor to the peak and reading where the cursor is on the bottom—time—axis.

(2) The amplitude of the AP. Do this by placing one cursor on the positive peak and the other cursor on the negative peak. The numerical readout of this difference is displayed to the right of the colored strip, as V2-V1=xx. (You may have to click on the "two cursor" button (Fig.9c) for the two cursors and difference measurement.)

(3) Location of your worm, and position of electrodes, both stimulation and recording.

Do the measurements now, LabScribe is not available to make measurement from your personal PC. If you are not sure about identifying AP, or of the appropriate criteria for baseline/peak choice, check with your LI or LA.

***If you have been watching the worm, you must have noticed that the worm twitches slightly after each stimulation. (It is not good if there is absolutely no movement when you shock the worm. Ask your LI for advise, don’t just crank up stimulation. Something is wrong if you have to use a stimulation voltage above 4V!) In fact, it is important to keep an eye on your worm because they do try to escape, and to contract or lengthen itself. As a result, your worm may move away from your stimulation or recording electrode over time. Move your clips to where the worm is. No worm, no threshold!

Project 2. AP threshold is dependent on stimulation duration.

This project builds on the threshold you have established for 0.1ms stimulation in Project 1. We now would like to know if the threshold stays the same if we increase the stimulation duration to 0.2ms and then 0.5ms.

First, repeat the supra-threshold stimulation you have established in Project 1, in order to make sure that it still works. If not, the worm has probably moved, so adjust the amplitude and find the new threshold. (Check your note on worm position from Project 1, as the threshold may change significantly if the worm has crawled or shortened itself.)

Decease the stimulation amp to half that of your previous voltage, and increase W(ms) to 0.2ms. (It is important to start with a low voltage as you increase the duration, because your previous stimulation amplitude will be way too high for a duration 2x longer. We don’t want the worm to get all jumpy.) Don’t forget to click "Apply" before you run your test. After you click record, check the stimulation trace first to make sure that the correct shock was delivered. (Also, check the worm as well. If you have been watching the animal, you have some

idea about how much the worm should twitch when your shocks are just above threshold. If you see that the worm is twitching more vigorously with the new shock parameter, you should reduce the stimulus strength further.) Depending whether you get AP or not, adjust the voltage amplitude until you find the threshold for a stimulation duration of 0.2ms.

Go through the same procedure, namely lowering the amplitude and increasing the duration, to find the threshold for 0.5ms.

In your notes under slide 1, you should identify trace IDs where APs failed and succeeded for 0.1, 0.2 and 0.5ms trials. Paste traces containing APs activated by the three stimulus durations onto slide 3. (You will have to shrink them so they fit onto one slide.) Write a figure legend that includes: (1) the time of AP occurrence, (2) AP amplitude and (3) the pin locations. In a separate paragraph, discuss whether the amplitudes stay the same for different stimulation durations? Explain your observation.

Project 3: Conduction velocity of the median giant.

This project is for you to figure out. You have information on the distance between different recording pins from marks on the chamber, and the timing of AP from your recording. From the two pieces of information, you should be able to estimate AP conduction velocity.

Here are a few hints: the simplest approach will be to place the two recording clips on pins next to each other. You will need to have the following information:

	pin location as marked on cage	Time at artifact and AP	Distance btwn pins (1) & (2)	Difference btwn times (1) & (2)
stimulus pin (posterior) (1)				
Recording pin (anterior) (2)				
			Conduction velocity (m/sec)	

In your report, you should also show the trace from which you get the numbers from. (By using the inset➔screenshot routine.)write a figure legend for the traces and, in a separate paragraph, explain how you calculate the conduction velocity.

Optional: If you have time, you should try to repeat the estimate by using a different pair of pins for recording, to see if your estimate is consistent.

Alternatively, you can place the two recording clips as far apart as possible, but still underneath the worm. In this case, you should get

recording similar the blue trace in Fig.10B. Show the recording, write a legend and explain your calculation. (You get extra credit for doing this one properly.)

	Location (pin # as marked on cage)	Time of AP (include trace ID)	Distance between pins	Difference in AP time
Recording pin (red)				
Recording pin (black)				
			Conduction velocity (m/sec)	

Project 4: Identification of APs generated by the lateral giant axons.

It has been about two hours since we first anesthetized the earthworm, it is likely to be restless and moving in the recording chamber. An animal in such state is not fit for our goal of recording lateral giant. It would be necessary to put the earthworm back to the alcohol and wait until the worm stop writhing. The animal should be completely relaxed in that when you lay it in the recording chamber, it should be stretched out over the entire length of the recording chamber and make no effort to shorten itself or squirm.

If you think you have found the lateral giants, show the traces, write a figure legend. To really prove your point, you need to show that the threshold for the lateral giant is higher than the medial giant. At a minimum, you will have to show a pair of traces, one with sub- and the other supra-threshold stimuli for the lateral giant. Of course, the medial giant will have to be there in both traces. Talk to LI, LA if the requirement—that the medial giant will have to be there while the laterals go sub- and supra-threshold—doesn't make sense.

Extra credit: There are two lateral giants, what kind of experimental evidence do you need to demonstrate the existence of two lateral giants? Discuss this question among yourselves and with instructors. Show your evidence in a separate Powerpoint slide if you think you have found evidence for two lateral giants.

References:

1.http://www.science.smith.edu/departments/NeuroSci/courses/bio330/labs/L4giants.html

2. Teaching basic neurophysiology using intact earthworms. Kladt N, Hanslik U, Heinzel HG. J Undergrad Neurosci Educ. 2010 Fall;9(1):A20-35