Experiment AN-4: Action Potentials in Earthworms

Equipment Required

PC or Mac Computer

IXTA, USB cable, IXTA power supply

iWire-B3G input cable

NBC-401 or 501 Nerve Bath Chamber

C-BNC-P2 Stimulator Cable - BNC to Dual pin jack stimulating cable

C-ISO-FP5 Recording Cable - 5 Lead Pin jack recording cable

Room-Temp & Chilled Ringer's solution

Bottles containing 10% ethanol solution

Note – the iWire-B3G should be connected to the IXTA prior to turning it on.

Earthworm Recording Setup

1. Plug the BNC adapter of the C-BNC-P2 stimulator cable into the Stimulator 1 input of the IXTA stimulator.
2. Locate the iWire-B3G recording cable and plug it into the iWire 1input on the front of the IXTA.

Figure AN-4-S1: The iWire-B3G recording cable and stimulator cables attached to the IXTA.

3. **Preparation of the Earthworm**
    - Obtain an earthworm and rinse it off with tap or distilled water.
    - In a well ventilated area, place 10-25 ml of the 10% ethanol solution into a beaker.
    - Anesthetize the worm by placing it into the beaker. Remove the worm as soon as it stops moving. This takes approximately 5 minutes, but may take a little longer. Note that too much ethanol can kill the worm and too little will cause the worm to wriggle while attached to the electrodes.
    - Dispose of the ethanol solution properly into a waste container. DO NOT REUSE the ethanol for another worm.
    - Place the worm with its ventral side down on the NBC-401 Nerve Bath Chamber so that the worm lays the length of the electrodes.
    - The anterior portion of the worm is marked by the clitellum (the thickened area). This end of the worm will be at the stimulating end of the NBC-401 with the 3 pins.
    - Gently stretch the worm the length of the nerve bath chamber.

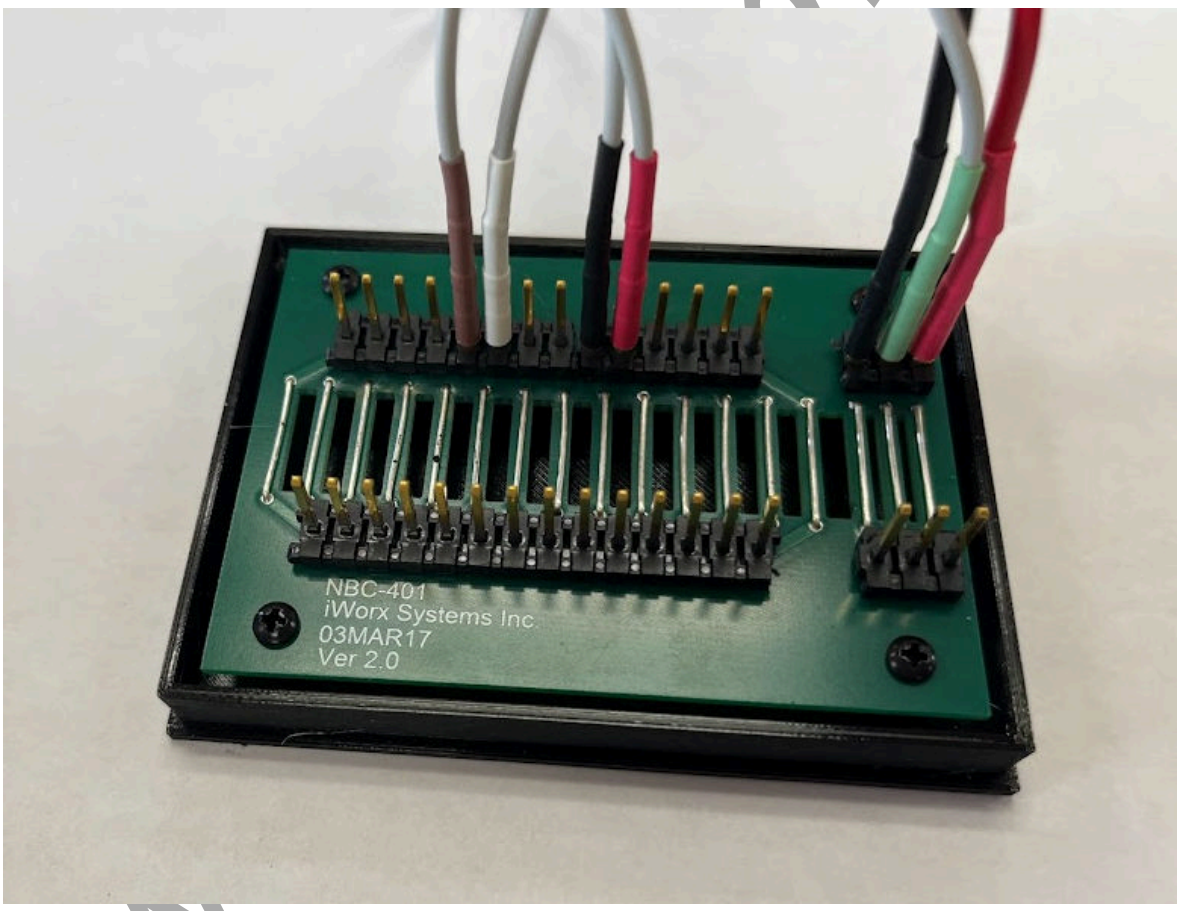

Figure AN-4-S2: NBC-401 Nerve bath chamber with the C-BNC-P2 and C-ISO-FP5 cables for recording worm action potentials.

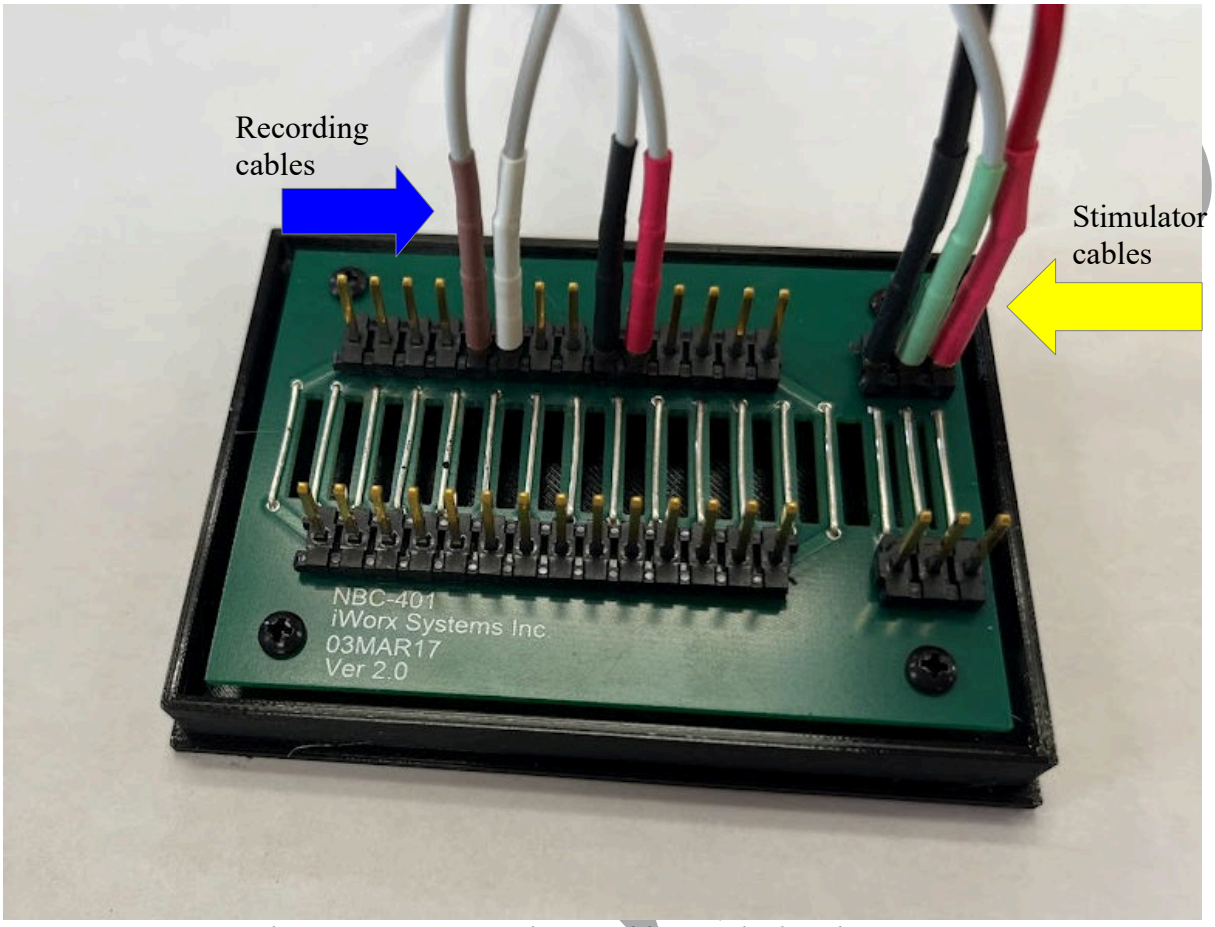

Figure AN-4-S3: The C-BNC-P2 Stimulator cable attached to the NBC-401.

Setting up the Nerve Bath Chamber

1. Firmly push the leads onto the pins of the chamber so that:
    - The red lead is on the outer most pin of the stimulating section of the chamber.
    - The green lead is on the pin between the red and black lead.
    - The black lead is on the third pin.
2. Locate the C-ISO-FP5 recording lead wires with pin jacks in the collection of equipment needed for this experiment.
3. Attach the red, black, and green C-ISO-FP5 recording lead wires to the corresponding sockets on the lead pedestal of the iWire-B3G biopotential cable.

4. Attach the leads to the pins on the nerve bath chamber as shown in figure above. Firmly push the leads onto the pins so that:
    - Place the red (+1) lead on the 5th pin.
    - Place the black (-1) lead on the 6th pin.
    - Leave one or two pins empty, then place the white (+2) lead on the next pin.
    - Place the brown (-2) lead right next to the white one.

Note: *The photo shows the white and brown leads set up for short path recording - this setup will be changed during the Nerve Conduction Velocity exercise.*

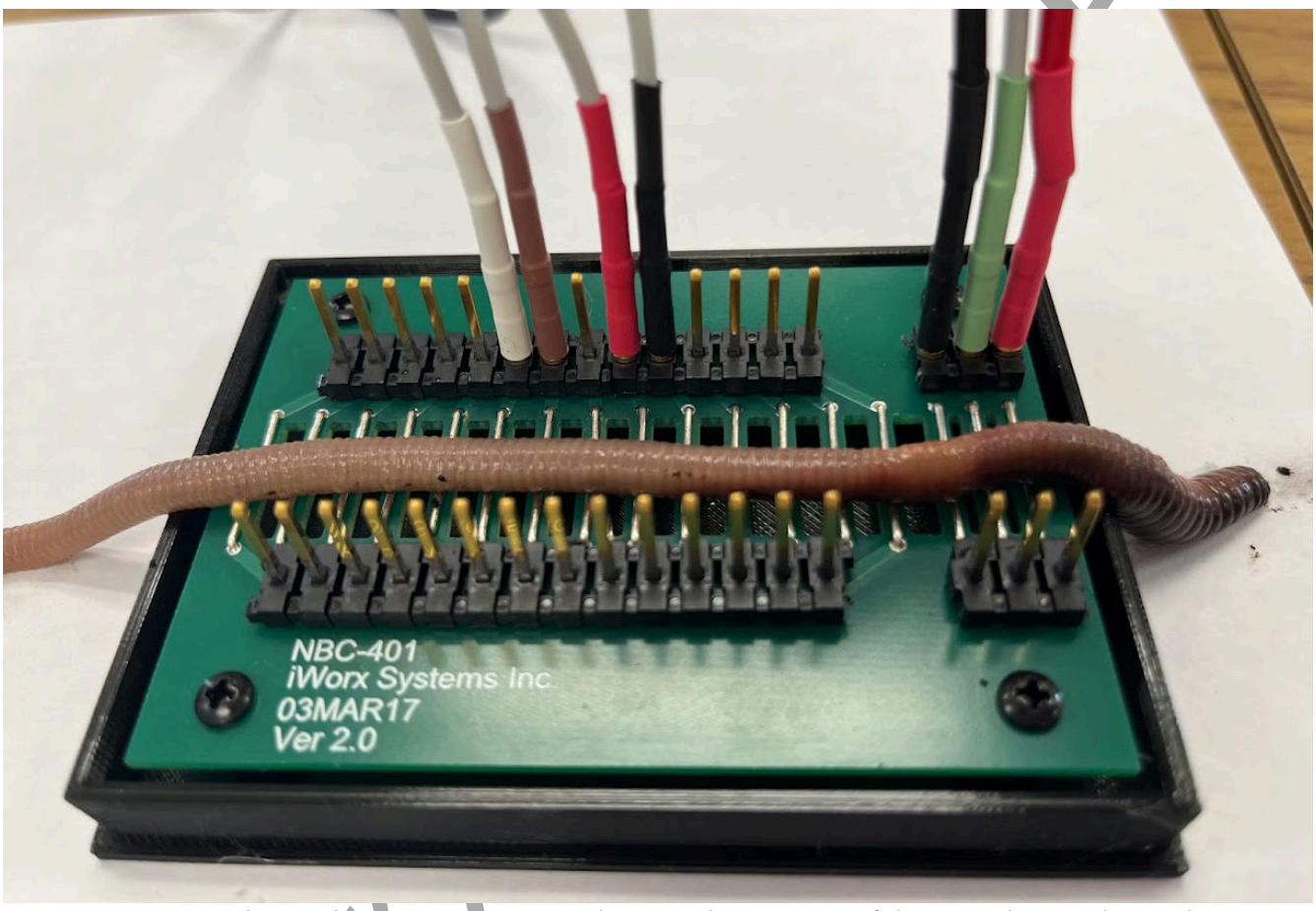

Figure AN-4-S4: The earthworm preparation showing the position of the recording and stimulating electrodes

Warning: The earthworm preparation used in this experiment is functional for a limited period of time. To conserve time, complete all the exercises in the experiment before analyzing the data. Keep an eye on the earthworm during the experiments. If the worm begins to move, remove the electrodes and re-anesthetize it, or carefully drip the ethanol solution on it and quickly wick away the excess. Dispose of all waste in properly labeled containers.

Warning: You WILL need to blot any liquid from between the electrodes of the nerve bath chamber to prevent bridging. If bridging occurs, there will be no conduction of current and no reaction from the specimen.

NOTE – If there is a problem with electrical noise in the room, please see the Appendix.

Experiment AN-4: Action Potentials in Earthworms

Exercise 1: Viability of the Neurons

Aim: To test the viability of the nerve cord by stimulating the nerve cord with a stimulus amplitude that should exceed the threshold of the neurons.

Approximate Time: 30 minutes

Procedure

1. Click the Stimulator Preferences icon on the LabScribe toolbar to open the stimulator control panel if it does not open automatically.

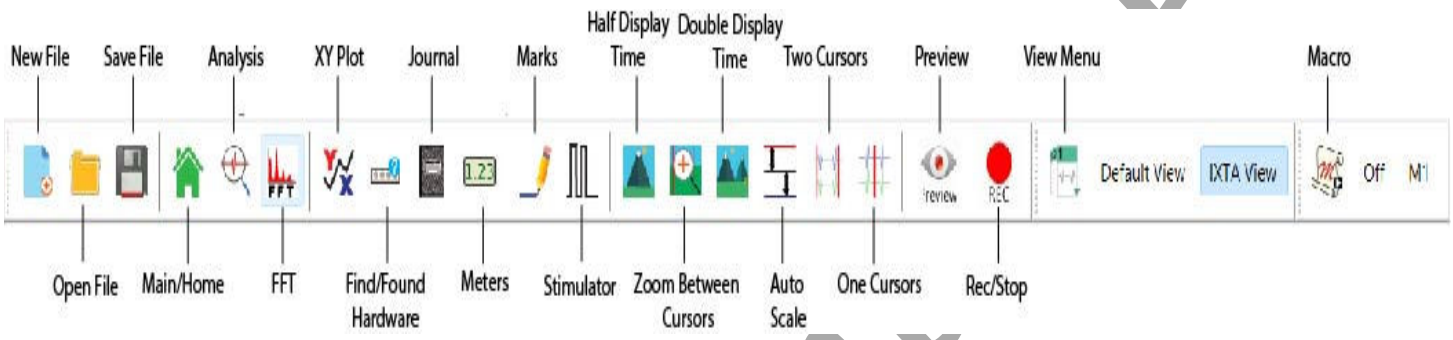

Figure AN-4-L1: The LabScribe toolbar.

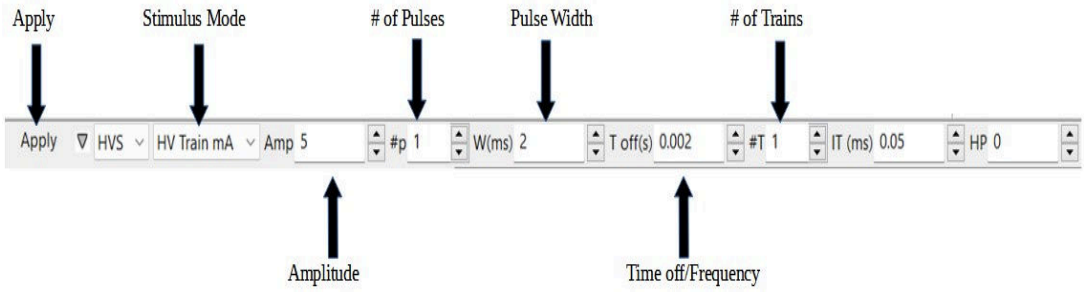

Figure AN-4-L2: The stimulator control panel

2. Check the values for the stimulus parameters that are listed in the stimulator control panel on the Main window. The pulse amplitude (Amp) should be set to 3.0 V, the number of pulses (#pulses) to 1, the pulse width (W) should be set to 0.1 msec, and TimeOff to 10 msec. Click on the Apply button to insure that the values set for the stimulus parameters are the ones that are used in the exercise.
3. Click the Record button to stimulate and record from the nerve cord. The recording stops automatically after one sweep. On the FilteredCAP-1 channel, use the **+ button** to the left of the AutoScale button to zoom in on the action potential.
4. Click the AutoScale button on the upper margin of the Stimulus channel. The recording should be similar to the one shown below.

Note: *The stimulus amplitude and width set for this exercise should be strong enough to cause an AP in a healthy neuron. If there is a single AP, the medial neuron with its lower threshold is the one being stimulated. If two adjacent APs are seen, both the medial and lateral neurons are being stimulated.*

Figure AN-4-L3: The monophasic action potential shown on both channel 1 and channel 2 of the earthworm action potential recording.

5. If two adjacent action potentials are seen in the recording and in the above figure, lower the stimulus amplitude until only one AP is seen. Use the down arrow next to the pulse amplitude (Amp) window to lower the stimulus amplitude. Click on the Apply button, before clicking on the Record button, to finalize the change in the stimulus amplitude.
6. On extracellular recordings of action potentials, a stimulus artifact may appear: The size of the artifact depends on the width and amplitude of the stimulus pulse and the effectiveness of the ground electrode separating the stimulating electrodes from the recording electrodes. If the end of the stimulus artifact merges with the beginning of the action potential, move the proximal (black) recording electrode away from the ground electrode and closer to the distal (red) recording electrode. Record another action potential.

Note: *You may find that these giant neurons "fatigue" with continued stimulation. If this happens, stop stimulating for a few minutes to give the neurons time to recover, then try again.*

7. If an action potential did not appear on the screen, check:
    - The wiring between the stimulator of the iWorx unit and the stimulating electrodes
    - The wiring between the isolated inputs of the iWorx unit and the recording electrodes.
    - The values for the stimulus parameters are shown on the stimulator control panel. These values should match the ones listed in the table in the Setup document. If they do not match, make the necessary adjustments. Click the Apply button on the stimulator control panel to finalize the stimulus settings.
8. Click the Record button to stimulate and record from the neuron. If the neuron does not respond after the connections and the settings have been verified, increase the stimulus amplitude (Amp) by 0.5 V. Remember to click the Apply button to finalize the change.
9. Click the Record button to stimulate and record from the neuron again. If no action potential is generated, continue to increase the stimulus amplitude (Amp) in increments of 0.5V and record from the nerve until the AP appears.

Warning: Do not exceed a stimulus amplitude of 4.0V without consulting your instructor.

10. Select Save As in the File menu, type a name for the file. Click on the Save button to save the data file.

Note: *Remember to reanesthetize the earthworm as needed by dripping a small amount of 10% EtOH on it. Quickly wick away the excess. If the earthworm is clearly dead, replace it with a new anesthetized worm. Be careful not to over anesthetize your worm.*

Exercise 2: Conduction Velocity

Aim: To measure the conduction velocity of the action potential in the medial neuron.

Approximate Time: 20 minutes

Procedure

1. Change the stimulus amplitude (Amp) to the lowest voltage that creates an action potential in the medial neuron. Click the Apply button to finalize the change in the stimulus amplitude.
2. Make sure the recording electrodes are positioned as they were for Exercise 1.
3. Type **Short Path** in the Mark box. The mark will be made automatically when you click Record. Click Record to stimulate the neuron.
4. Move the white and brown recording electrodes to pins 8 and 9 as shown in the figure below.
5. Type **Long Path** in the Mark box. The mark will be made automatically when you click Record. Click Record to stimulate the neuron.
6. Measure the distance between the two positions of the proximal (black and white) recording electrodes that were used.

7. Select Save in the File menu.

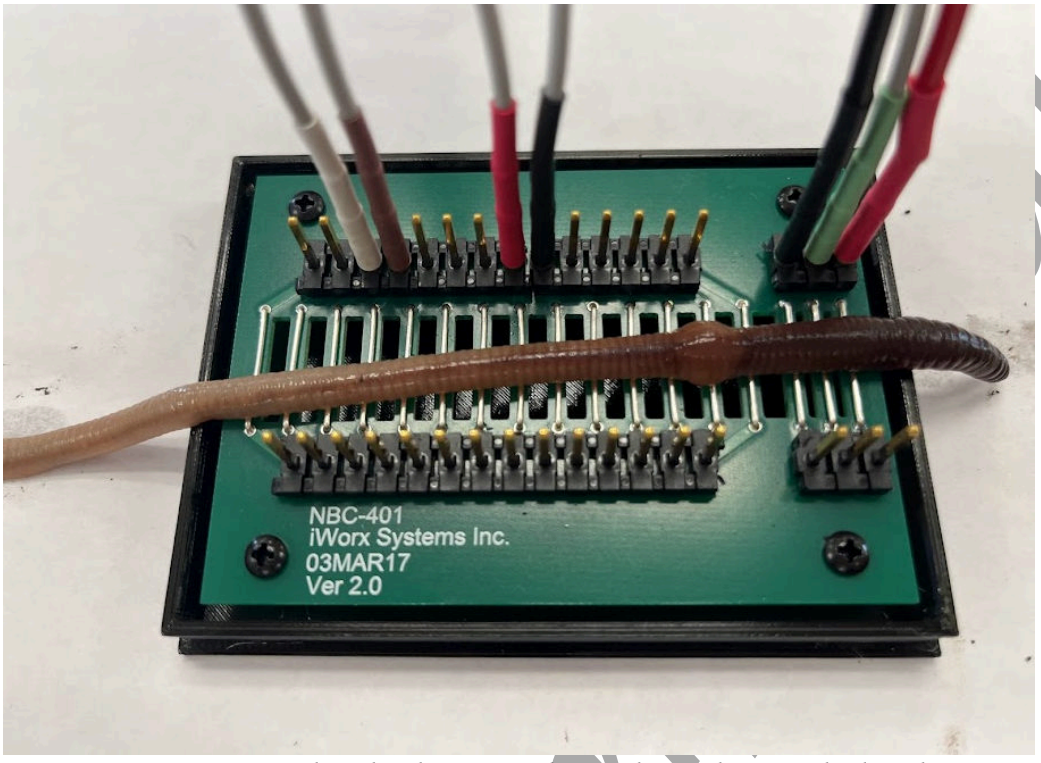

Figure AN-4-L4: C-ISO-FP5 recording lead wires with pin electrodes attached to the NBC-402 for long path recording.

8. This exercise can be repeated, moving the white and brown recording electrodes to pins farther away from the red and black electrodes. Remember to measure the distance between the proximal electrodes as stated in step 6.
9. Select Save in the File menu.

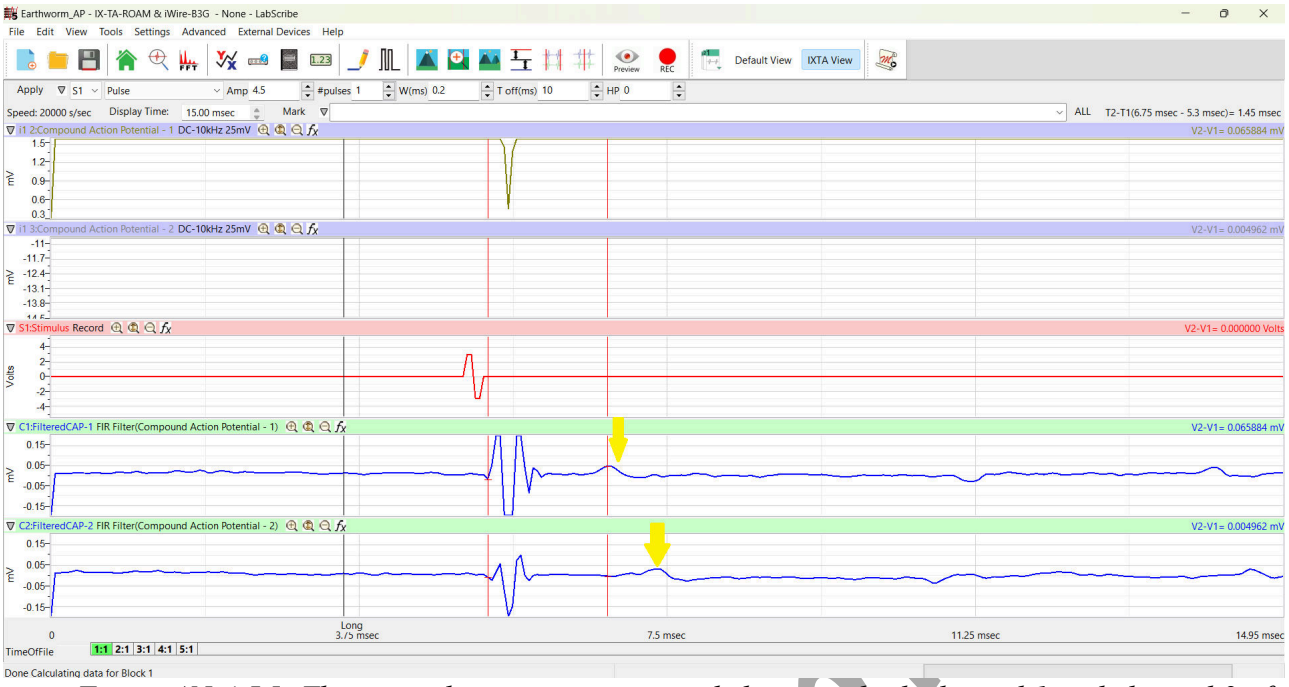

Figure AN-4-L5: The monophasic action potential shown on both channel 1 and channel 2 of the earthworm action potential recording for the long path setup.

Exercise 3: Conduction Velocity and Temperature

Aim: To examine the effects of cooling the worm on the conduction velocity of the action potential in the medial neuron.

Approximate Time: 20 minutes

Note: *This part of the experiment must be done quickly since the earthworm will begin to warm as soon as the cold paper towels are removed.*

Procedure

1. Carefully cover the anesthetized worm with a few KimWipes or paper towels soaked in very cold worm Ringer's solution. Keep the earthworm covered for 2 minutes to get chilled.
2. Remove the paper towels and quickly measure the conduction velocity of the neuron using the same procedures used in Exercise 3.
3. Remember to start with the electrodes in the Short Path position and move them to the Long Path position.
4. Repeat Exercise 3 after the earthworm has warmed back up to room temperature.

Exercise 4: Refractory Period

Aim: To measure the effect of stimulus frequency on the occurrence of multiple action potentials in a neuron.

Approximate Time: 30 minutes

After a neuron generates an action potential, it is unable to generate a second action potential until the membrane recovers or the strength of the stimulus is increased. This time is known as the refractory period. The refractory period can be divided into two sections: the absolute refractory period, which occurs directly after the first action potential. During the absolute refractory period, the neuron is unable to generate a second action potential even though the stimulus strength is set to a very high level. The other section of the refractory period is known as the relative refractory period, and it occurs after the neuron has been given additional time to recover. In the relative refractory period, the neuron is able to generate a second action potential, but the strength of the stimulus needed to do this is greater than the threshold stimulus of the neuron. As the neuron is given more time to recover, the strength of the stimulus required to trigger the second action potential decreases. The end of the relative refractory period is defined as the time when the stimulus required to trigger the second action potential is the same as the threshold stimulus that can trigger the first action potential in the pair.

Procedure

1. Adjust the stimulus parameters to the values listed in Table 1 using the same techniques as above. The stimulus amplitude must be set to the lowest voltage that will generate an action potential. Click the Apply button.
2. Type**10 msec** in the Mark box. The mark will be made automatically when you click Record. Click Record to stimulate the neuron.
3. Change the time interval between stimulus pulses (T Off) to 9 msec. Click the Apply button to finalize this change.
4. Type **9 msec** in the Mark box. The mark will be made automatically when you click Record. Click Record to stimulate the neuron.
5. Repeat Steps 3 and 4 to record the effects of decreasing the time interval between the two stimuli on the amplitude of the second action potential. Change the time interval between the stimulus pulses (T Off) to 8, 7, 6, 5, 4, 3, 2, and 1 msec. Put the appropriate mark in the Mark box and record the action potentials.
6. Select Save in the File menu.

Table AN-4-L1: Stimulus Parameters Required for Measuring the Refractoriness of the Neuron.

Stimulus Parameter	Value
Stimulus Amplitude (Amp)	Threshold Level
Number of Pulses (#pulses)	2
Pulse Width (W)	0.1 msec
Time Between Pulses (T Off)	10 msec
Holding Potential (HP)	0

Exercise 5: Stimulus Strength and Duration

Aim: To demonstrate the inverse relationship between the stimulus amplitude and the stimulus duration needed to generate action potentials.

Approximate Time: 30 minutes

Stimulus pulses change the flow (current) of ions through the neuronal membrane. In turn, changes in ion currents lead to changes in the membrane potential of the neuron. If the change in the membrane potential exceeds the critical level known as the threshold, the neuron generates an action potential. The changes in ion currents are dependent on the stimulus amplitude and the stimulus duration. For example, a stimulus pulse with a low amplitude and a long duration can stimulate a neuron just as easily as a stimulus pulse with a high amplitude and a short duration.

Procedure

1. Adjust the critical stimulus parameters to the values listed in Table 2 using the same techniques as before. Click the Apply button to finalize the changes to these stimulus parameters.
2. Type **0.100 msec,** and the stimulus amplitude in the Mark box. Click Record to stimulate the neuron, the mark will be made automatically.
3. Change the stimulus duration to 0.05 msec. Click the Apply button to finalize this change.
4. Type **0.05 msec** and the stimulus amplitude in the Mark box. Click Record to stimulate the neuron.
5. If an action potential is not detected when the stimulus duration is 0.05msec:
    - Increase the stimulus amplitude by an increment of 0.5 V, and click the Apply button.
    - Mark the recording with 0.05 msec and the current stimulus amplitude. Click Record to stimulate the neuron.
    - Continue to raise the stimulus amplitude by 0.5 V and record until an action potential appears. Mark the sweep with the stimulus duration, 0.05msec, and the value of the stimulus amplitude that first caused an action potential at that stimulus duration.

6. Enter the stimulus amplitude that first causes an action potential at the duration of 0.05 msec on Table 3.
7. Change the stimulus duration to 0.06 msec. Click the Apply button to finalize this change.
8. Repeat Steps 4 and 5 for the stimulus duration of 0.06 msec.
9. Repeat Steps 3, 4, and 5 for the following stimulus durations: 0.06, 0.08, 0.16, 0.32, 0.64, and 1.28msec.
10. Select Save in the File menu.

Table AN-4-L2: Stimulus Parameters Required for Determining the Stimulus Strength-Duration Relationship of the Neuron.

Stimulus Parameter	Value
Stimulus Amplitude (Amp)	Threshold Level
Number of Pulses (#pulses)	1
Pulse Width (W)	0.05 msec
Time Between Pulses (T Off)	0.9 msec
Holding Potential (HP)	0

Data Analysis

Exercise 1 - APs from Medial Neurons

1. Use the tabs in the Sweep Selection bar at the bottom of the Main window to find the sweep displaying the action potential from the medial neuron caused by the stimulus amplitude of 3 V.
2. Click on a tab in the selection bar and the sweep associated with the tab will appear on the Main window. On the FilteredCAP-1 channel, use the + button to the left of the AutoScale button to zoom in on the action potential as shown.
3. Click the AutoScale button on the Stimulus channel to maximize the height of the stimulus.
4. Once the sweep is displayed on the Main window, transfer the sweep to the Analysis window by clicking on the Analysis window icon in the toolbar or selecting Analysis from the Windows menu.
5. The values for V2-V1 and T2-T1 on each channel are seen in the table across the top margin of each channel.
6. Once the cursors are placed in the correct positions for determining the amplitude of the action potential, the amplitude (V2-V1) can be recorded in the on-line notebook of LabScribe by typing its name and value directly into the Journal, or on a separate data table.

Table AN-4-L3: Stimulus Amplitudes that First Cause Action Potentials in the Medial Neuron at Specified Stimulus Durations.

Stimulus Duration (ms)	Stimulus Amplitude (V)
0.100	
0.140	
0.160	
0.180	
0.200	
0.260	
0.320	
0.640	
1.280	

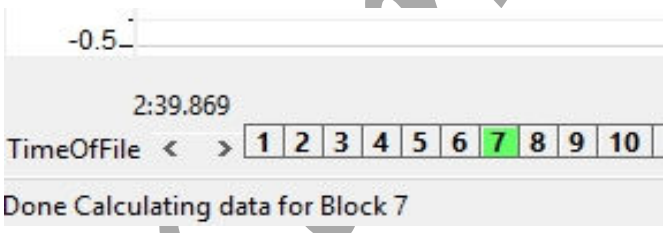

Figure AN-4-L5: The Sweep Selection bar showing the tab for Sweep 7 highlighted.

7. The functions in the channel pull-down menus of the Analysis window can also be used to enter the name and value of the parameter from the recording to the Journal. To use these functions:
    - Place the cursors at the locations used to measure the amplitude of the action potential.
    - Transfer the names of the mathematical functions used to determine the amplitude to the Journal using the Add Title to Journal function in the Action Potential Channel pull-down menu.
    - Transfer the values for the amplitude to the Journal using the Add Ch. Data to Journal function in the Action Potential Channel pull-down menu.
8. To measure the amplitude of the action potential, place one cursor on the baseline before the stimulus artifact displayed on the FilteredCAP-1 channel. Place the other cursor on the peak of the AP. The value for the V2-V1 function on the Filtered channel is the amplitude of the action potential.

9. Record the amplitude of the action potential generated by 3V in the Journal using the one of the techniques described in Steps 5 or 6, and on Table 4.

Figure AN-4-L6: Action potentials (AP) and stimulus pulse displayed in the Analysis window, with cursors positioned to measure the amplitude of the AP.

Table AN-4-L4: Amplitude of Action Potentials Generated by Stimulus Pulses of Different Amplitudes.

Stimulus	StimulusAmp (V)	Action Potential Amplitude (mV)	
		Medial Neuron	Lateral Neuron
Initial Trial Stimulus	3		
Threshold for Medial Neuron			
Maximum for Medial Neuron			

10. Use the tabs in the Sweep Selection bar at the bottom of the Analysis window to find the sweep with the action potential from the medial neuron that was caused by the threshold stimulus amplitude.

Note: *Threshold amplitude may be higher or lower than the initial setting of 3 V.*

11. To take measurements from a sweep displayed on the Analysis window, select its name from the Sweep menu in the upper left margin of the data display window.
12. Use the same techniques described earlier to measure and record the amplitude of the action potential from the medial neuron caused by its threshold stimulus amplitude.

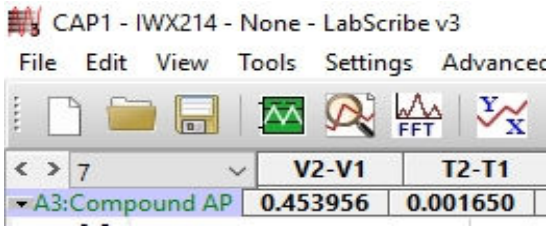

Figure AN-4-L7: The upper left corner of the Analysis window showing the Sweep menu used to select the sweep from which the data is measured.

13. Select Save in the File menu.
14. Click on the Main Window icon to return to that window.

Questions

1. When was threshold reached? Was it at 3 V, or higher, or lower?
2. Does the amplitude of the action potential in the medial neuron increase when the stimulus amplitude is increased?

Exercise 2 - Conduction Velocity

1. Use the Sweep Selection bar at the bottom of the Main window to display the first sweep needed to calculate the conduction velocity of the neuron. Click on the tab of that sweep and it will appear on the Main window.

Note: *Use the + button to maximize the action potential.*

2. Transfer the sweep to the Analysis window using the Analysis window icon in the toolbar or the Analysis listing on the Windows menu.
3. This sweep contains the APs from Filtered channels 1 and 2. Channel 1 represents the black and red recording electrodes; channel 2 represents the white and brown recording electrodes.
4. Place one cursor on the peak of the AP shown in FilteredCAP-1 channel and second cursor on the peak of the AP in FilteredCAP-2 channel and measure T2-T1:
    - The value for T2-T1 is the time it takes the AP to move the distance between the two positions of the recording electrodes used in this exercise.

- Record the value for T2-T1 (in msec) in the Journal, and on Table 5, for temperature and direction labeled as Room-Normal.

5. Measure the conduction distance (in mm) between the two positions used for the proximal (black and white) recording electrodes. Record this distance (in mm) in the Journal, and on Table 5, as the conduction distance for temperature and direction labeled as Room-Normal.
6. Calculate the conduction velocity (in meters per second). Divide the distance (in mm) between the two positions of these electrodes by T2-T1, which is the time (in msec) between the peaks of the two action potentials.

   For example: 10 mm/0.2 msec = 50 mm/msec = 50 m/sec
7. Record the conduction velocity in the Journal, and on the data table for the temperature and direction labeled as Room Temp.

Figure AN-4-L8: Two sweeps used to determine the conduction velocity of the neuron.

8. Go back to the main window to select another sweep at Room Temp when the electrodes were moved farther apart. Follow the same directions in Steps 3-6 to calculate Conduction Velocity.
9. Select Save in the File menu.

Exercise 3 - Conduction Velocity at Low Temperature

1. Use the same techniques used in the analysis of Exercise 2 to make and record the measurements needed to determine the conduction velocities of the neuron. Record the values for these measurements in the Journal and on Table 5.

2. Calculate and record the conduction velocity of the neuron at the cold temperature (Cold Temp).
3. Calculate and record the conduction velocity of the neuron at room temperature (Room-Post) after the neuron cooled and warmed.
4. Select Save in the File menu.

Questions

1. Does the conduction velocity change when the earthworm is cooled?
2. What properties of the ion channels may change with temperature?
3. After warming back up to room temperature, was the conduction velocity the same as it was prior to cooling? Why or why not?

Table AN-4-L5: Conduction Velocities of a Neuron at Two Different Temperatures.

Temperature	Distance (mm)	Time (msec)	Conduction Velocity (m/sec)
Room Temp			
Cold Temp			
Room - Post			

Exercise 4 - Refractory Period

1. Use the Sweep Selection bar at the bottom of the Main window to display the sweep with a pair of stimulus pulses that are 10msec apart. Click on its tab to make it appear on the Main window.
2. Transfer the sweep to the Analysis window using the Analysis window icon in the toolbar or the Analysis listing on the Windows menu.
3. Measure and record the amplitudes of the two action potentials using the techniques described in the analysis of Exercise 2. Record the amplitudes of the first and second action potentials on Table 6.
4. To display the other sweeps where the times between the stimulus pulses are getting shorter, click on the tabs for those sweeps in the Sweep Selection bar at the bottom of the Analysis window. The sweeps that are selected will appear together on the Analysis window.
5. To take measurements from another sweep displayed on the Analysis window, select its name from the Sweep menu in the upper left margin of the data display window. Measure and record the amplitudes of these action potentials as described in Step 3.
6. Select Save in the File menu.

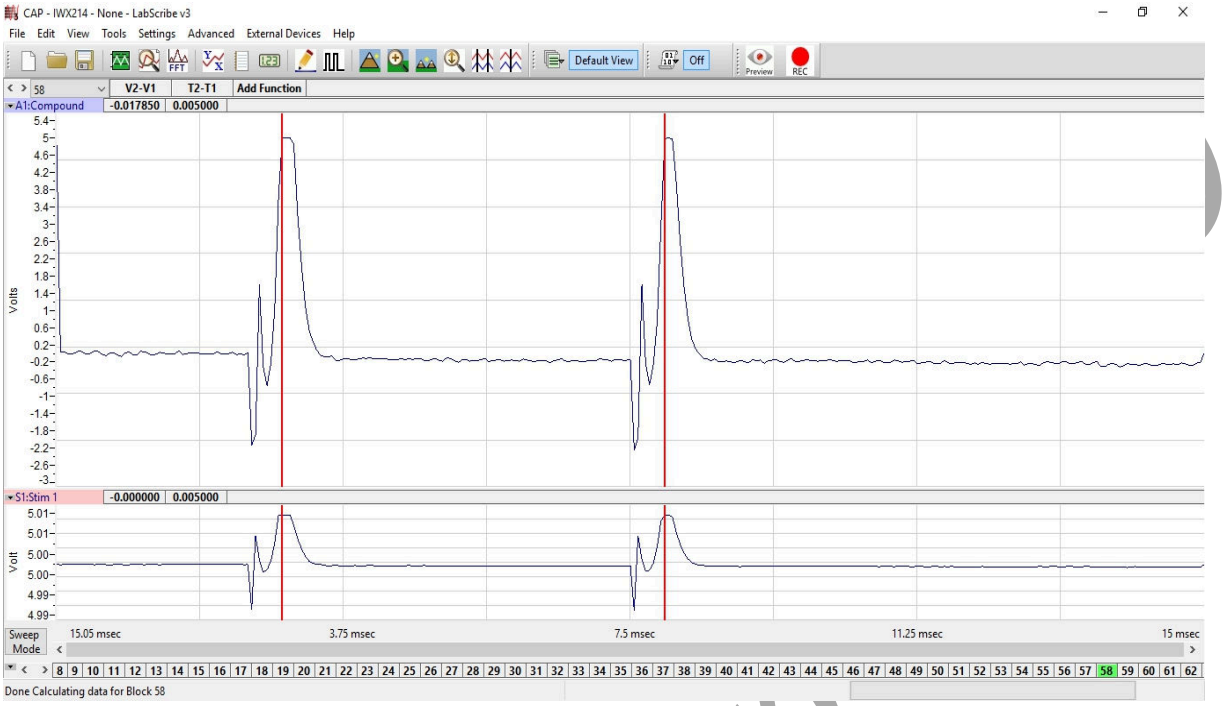

Figure AN-4-L9: Pair of action potentials stimulated by a pair of pulses that are 5 msec apart. Note - channel 2 is not displayed in this image.

Questions

1. Does the first AP in the pair have the same amplitude at all of the frequencies?
2. Does the second AP in the pair have the same amplitude at all of the frequencies?
3. At which frequency, or interpulse interval, does the second AP in the pair decline or disappear?
4. Is a neuron that generates a full-sized action potential with a stimulus of increased amplitude in the absolute or relative portion of its refractory period?

Table AN-4-L6: Amplitudes of Action Potentials Occurring at Different Frequencies.

Interpulse Interval (ms)	Effective Frequency (Hz)	Amplitudes (mV)	
		First AP	Second AP
10	100		
9	111		
8	125		
7	143		
6	167		
5	200		
4	250		
3	333		
2	500		
1	1000		

Exercise 5 - Stimulus Strength - Duration

1. Graph the stimulus amplitude needed to create an action potential as a function of the stimulus duration. Data is in Table 3. Place the values for the stimulus amplitudes on the Y-axis, and the values for the stimulus durations on the X-axis.
2. The minimum stimulus amplitude which will elicit action potentials at an infinitely long stimulus duration is a value known as the rheobase. Another value, chronaxie, is the stimulus duration where the stimulus amplitude is twice the value of the rheobase. Chronaxie values are used as measures of the excitability of neurons. The most excitable neurons have the smallest chronaxies. Using the graph created in Step 1, determine rheobase and chronaxie for the medial neuron.

Questions

1. How does the excitability of your medial neuron compare to the medial neurons used by other groups in your lab section?
2. Collect conduction velocity and chronaxie data from the other groups in your lab section. Is there a relationship between conduction velocity and neuron excitability? If so, what is it?