

Experiment AN-12: Crustacean Neuromuscular Junction

Equipment Required

PC or Macintosh computer

IXTA, USB cable, IXTA power supply

C-BNC-P2 stimulator cable (used for AN-12-B)

iWire B3G recording unit

Suction electrode assembly and glass tips (See Appendix II for construction instructions and a commercial alternative)

IC-200 Intracellular probe

Glass microelectrodes

Micromanipulator with base (2 each)

Faraday cage (if necessary)

Steel base plate floating on a bicycle inner tube.

Headstage tester (18-megohm resistor with clip)

Preparation dish, crayfish and crayfish saline (See Appendix I)

Dissection microscope and light source

Bath ground electrode with cable

Disposable plastic transfer pipettes

Assorted banana cables and alligator clips

3M KCl electrode filling solution

Equipment Setup

Connect the iWire-B3G prior to turning on the IXTA.

1. Locate the iWire-B3G recording cable.
2. Insert the connector on the end of the recording cable into the isolated inputs of the iWire 1 channel on the front of the IXTA.



Figure AN-12-S1: The iWire-B3G recording cable.

This experiment uses a suction electrode.

1. Attach the three connectors of the suction electrode assembly to the B3G so that:
 - the recording electrode, which is the wire inside the lumen of the suction tubing, is connected to the red (+) connector.
 - the indifferent (reference) electrode, which is the wire wrapped around the suction tubing down to the glass microelectrode tip, is connected to the black (-) connector.
 - the ground electrode, that is in the solution in the bath chamber, is connected to the green connector.
2. Place the barrel of the suction electrode on a micromanipulator placed close to the crayfish preparation dish.

Note: The default filter settings listed are suggested for use in ideal recording conditions. If noise is present in the recording environment, the high and low pass filters can be set at different levels to create a recording with less noise. If noise is caused by AC line voltage used to power the equipment in the lab, a digital notch filter (as described in the Electrical Noise section) can be used.

IC-200 Setup

1. Locate the IC-200 intracellular probe and the microelectrode holder in the iWorx kit.
2. Mount the intracellular probe in the micromanipulator and place it near the dissection microscope.
3. Position the preparation dish on the microscope stage so that the center of the dish is visible through the microscope. Orient the light so that it shines on the center of the dish.

4. Plug the DIN connector of the IC-200 intracellular probe cable into Channel A5.



Figure AN-12-S3: The IC-200 intracellular probe.

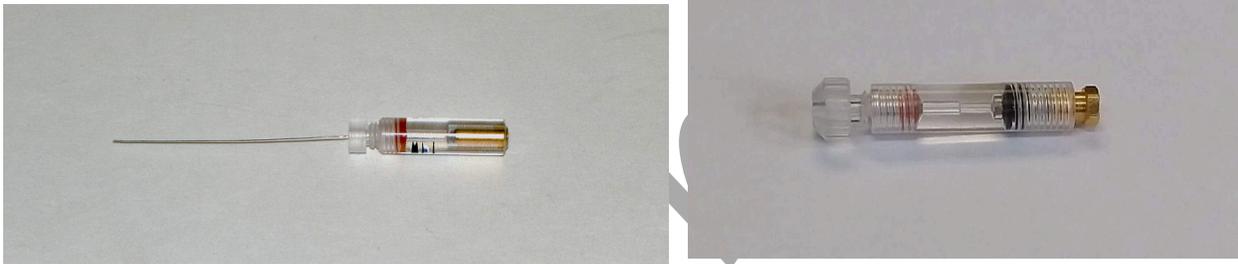


Figure AN-12-S4: Microelectrode holder options for the IC-200.



Figure AN-12-S5: The IC-200 intracellular probe with a microelectrode holder connected to an IXTA.

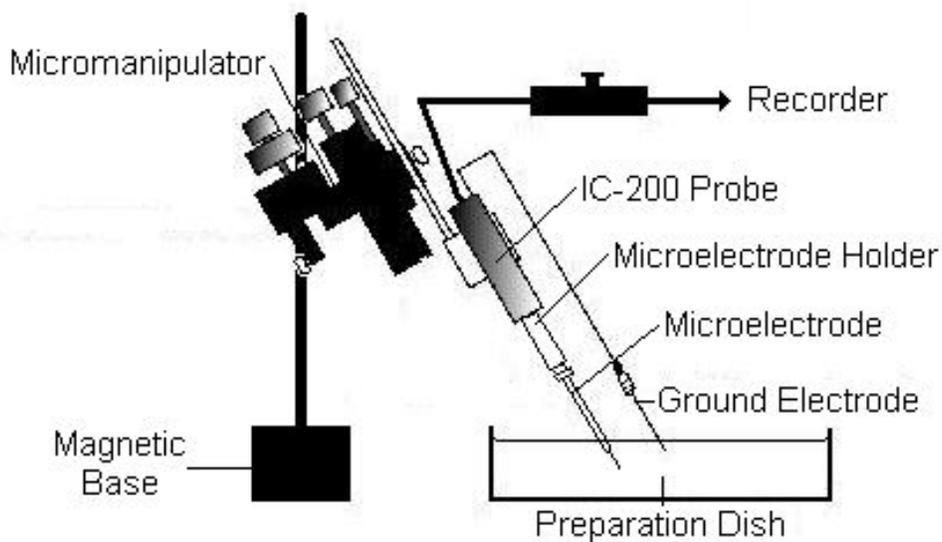


Figure AN-12-S6: Diagram showing the arrangement of equipment used to record resting membrane potentials from fast abdominal extensor muscles.

Intracellular Probe Test

1. Obtain an 18-megohm resistor (1/4 watt), and attach a small alligator clip to one of its ends.
2. Attach the end of the resistor with the alligator clip to the pin on the intracellular probe. Fasten the alligator clip on the black wire of the indifferent input to the other end of the resistor.

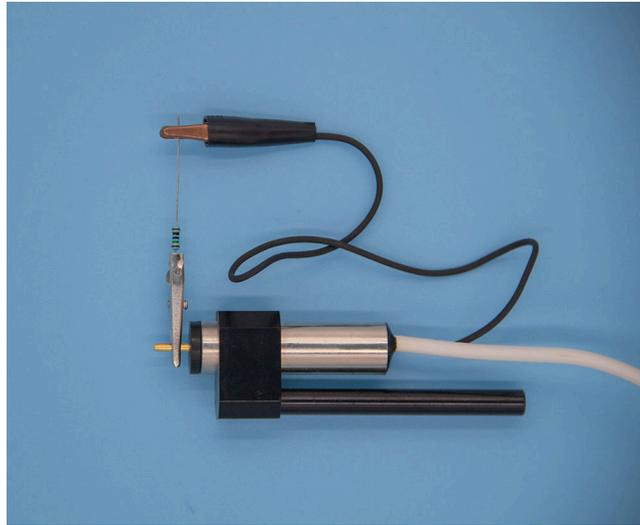


Figure AN-12-S7: Circuit for testing the output of the IC-200 probe. The pin of the probe input is connected to the ground electrode with an 18-megohm resistor that simulates the cell membrane.

3. Type Probe Test in the Mark box.
4. Click Record and click the Mark button. The trace should appear near the center of the screen.
5. Locate the test module on the cable of the intracellular probe. Depress and hold the electrode test button for a few seconds. The trace should deflect to a higher amplitude as the button is depressed, and return to the baseline when the button is released. Click Stop to halt recording.
6. Select Save As in the File menu and type a name for the file. Choose a destination on the computer in which to save the file, like your lab group folder. Designate the file type as *.iwxdata. Click on the Save button to save the data file.
7. Click the Double Cursor icon so that two vertical lines appear over the recording window.
8. Click and drag one cursor to the baseline prior to the electrode test button being depressed. Place the second cursor on the plateau recorded while the test button was depressed.

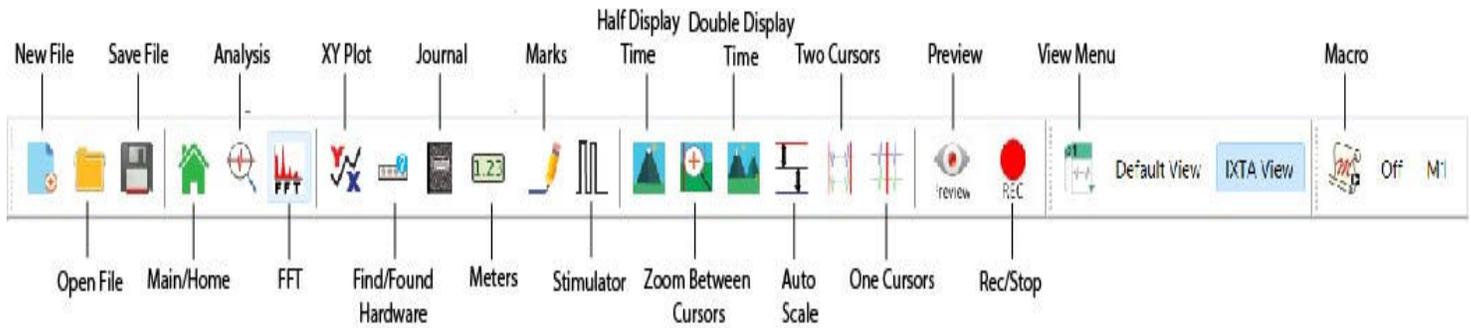


Figure AN-12-S8: The LabScribe toolbar.

- On the upper right margin on the Membrane Potential channel, the voltage difference between the positions of the two cursors, V2-V1, is the output of the IC-200 intracellular probe during the electrode test. The output of the IC-200 is 10 mV for every megohm of load applied to the circuit by the resistor used to connect the two poles of the IC-200. If an 18-megohm resistor is used on an IC-200 with a 1X gain, the output of the IC-200 during the test is 180 millivolts (mV):

$$18 \text{ megohm} \times 10 \text{ mV/megohm} = 180 \text{ mV}$$

If an 18-megohm resistor is used on an IC-200 with a 10X gain, the output of the IC-200 during the test is 1800 millivolts (mV):

$$(18 \text{ megohm}) (10 \text{ mV/megohm}) (10\text{X gain}) = 1800 \text{ mV}$$

- If the IC-200 intracellular probe tests to the level that is appropriate for the gain of the unit, the IC-200 probe and the iWorx unit are working properly.

The Dissection

- To anesthetize it, bury a crayfish in ice for 10 minutes.
- Separate the abdomen (the "tail") from the thorax.
- Securely pin the abdomen dorsal side down in the dish's wax or Sylgard. Cover the preparation with cold crayfish saline. Cut off the swimmerets which are the paired appendages on the ventral surface of each segment. The movements of the swimmerets interfere with the recording process if they are not removed.
- Choose one of the middle segments to dissect. Use a scalpel to make a shallow incision along the anterior sternite (the ribs that separate the segments). Cut all the way through the membrane but avoid cutting too deep and damaging the underlying nerve cord and muscles.

5. Use fine forceps to lift up the membrane and make a midline incision extending for the anterior half of the segment. Lift the corner on one side and use small scissors to cut a small piece of the membrane off. This will expose the ventral nerve cord, including the ganglion and the three nerves (I, II, and III) coming out of each side. Identify the superficial branch of the third nerve (SBIII) which extends to the superficial flexor.
5. The superficial flexor muscle should be visible through the membrane on the posterior part of the segment. The anterior ends of the muscle fibers are attached to a cuticle which extends across the segment about a third of the way back from the anterior sternite. The posterior ends of the muscle fibers attach to the posterior sternite.
6. Carefully remove enough of the membrane over the superficial flexor to create a window that exposes the superficial flexor muscle fibers.
7. In this experiment, you will be attaching a suction electrode to record from the superficial branch of Nerve 3 while simultaneously recording intracellularly from the superficial flexor fibers.

Microelectrode Preparation

1. Fill a glass microelectrode with 3M KCl using a fine syringe needle (Hamilton type) on a 3cc syringe.
 - The microelectrode is filled from the back. Gently lower the needle into the lumen of the microelectrode until the tip of the needle is close to the taper in the microelectrode.
 - As you slowly inject the 3M KCl into the microelectrode, pull the needle up the microelectrode to allow space for the fluid. Keep the tip of the needle under the meniscus of the fluid as you withdraw the needle from the microelectrode. This will prevent bubbles from forming in the microelectrode.
 - Make sure the microelectrode is filled to the top. You do not want a bubble at the end of the microelectrode when it is put in the microelectrode holder.
2. Fill the microelectrode holder with the same 3M KCl solution used for the microelectrode. This solution completes the electrical connection between the glass microelectrode and the intracellular probe. Make sure the gold-plated socket (pin jack) on the back of the plastic microelectrode holder is screwed tightly into the holder. If the pin jack is loose, the 3M KCl solution can leak out of the electrode holder and make contact with both the pin jack and the pin of the intracellular probe. If the different metals used to make the pin jack, the pin on the intracellular probe, and the pellet or wire in the electrode holder are all in contact with 3M KCl, then, reactions can take place among the different metals. These reactions can cause an off-scale shift in the baseline of the recording.
3. If you are using an microelectrode holder with a silver wire in place of a Ag-AgCl pellet, the holder does not need to be filled with 3M KCl. However, the pin jack needs to be screwed tightly into the holder. In this type of holder, the silver wire goes into the back of the glass microelectrode and makes contact with the solution in the microelectrode. This electrolyte can migrate up the wire and into the back of the holder. If the pin jack on the holder is loose, the 3M KCl can move past the rubber gasket, that normally seals the lumen of the holder from the gold-plated socket, and create the same problems mentioned in Step 2.

4. If you are using a microelectrode holder with an Ag-AgCl pellet, fill the holder with 3M KCl.
 - Loosen the plastic cap of the microelectrode holder to take pressure off the rubber gasket behind the cap.
 - Gently lower the needle of the filling syringe into the lumen of the cap, through the gasket, and into the lumen of the holder until the tip of the needle is close to the Ag-AgCl pellet.
 - Fill the microelectrode holder with 3M KCl, until a drop forms on top of the cap.

Note: Pushing the glass microelectrode into the holder too vigorously, or while the cap is tight, may result in the microelectrode breaking.

- Place the back of the microelectrode into the lumen of the cap. Carefully push the microelectrode through the rubber gasket that sits behind the cap, and into the lumen of the microelectrode holder. The lumen of the microelectrode holder steps down to a smaller diameter, so the back of the glass microelectrode will stop on the edge of this smaller lumen.
 - Gently tighten the cap of the holder. Tightening the cap too much will crack the glass microelectrode.
 - Push the gold-plated socket of the microelectrode holder onto the pin of the intracellular probe.
6. Carefully position the microelectrode tip over the preparation. Use the micromanipulator's vertical controls to move it until the tip is in the saline overlying the preparation.
 7. To complete the circuit, an indifferent electrode needs to be attached to the alligator clip on the black wire of the intracellular probe. The best material for the indifferent electrode is a coil of Ag-AgCl wire. Place the coil of wire in the bath solution surrounding the crayfish tail. Make sure no other metal, only the Ag-AgCl coil is in contact with saline solution.
 8. Check that both electrodes are in the saline in the prep dish. Click Start to begin recording.
 9. With both electrodes in the dish, the trace should be set to zero. Stop recording and make sure the cursors are positioned around a straight stretch of the trace. Choose Units > Set Offset from the channel menu. Set the section between the cursors to zero in the dialog that opens. Click OK.
 10. Check the resistance of your glass microelectrode before you push the microelectrode into muscle fibers. The resistance of the microelectrode tip is a measure of its diameter; the smaller the tip, the greater the resistance. Press the button on the electrode test module, located in the middle of the cable for the intracellular probe, for a few seconds. Measure the deflection of the trace; it will deflect 100mV for every megohm of resistance (10 mV per megohm times the 10X gain of the input amplifier). A good microelectrode for this preparation should have a resistance between 10 and 20 megohms.
 11. The trace on the screen may have a small wave or ripple through it. This is noise from devices in the room that operate on 60Hz AC current. The probe is picking up these currents, but they can be reduced by grounding metal objects like the microscope or light source to any grounded point on the iWorx unit. Turning off and unplugging the light source, when not needed also reduces this noise.

Experiment AN-12: Crustacean Neuromuscular Junction

Exercise 1: Spontaneous Synaptic Potentials

Aim: To record spontaneous synaptic potentials from the fibers of a slow flexor muscle (SFM).

Approximate Time: 30 minutes

Procedure

1. Look through the microscope as you use the controls of the micromanipulator to move the tip of the intracellular microelectrode over a bundle of muscle fibers. Position the tip above a healthy-looking fiber. *Unhealthy fibers are opaque, beaded, or twitching.*
2. Type SFM Fiber 1 in the Mark box to the right of the Mark button.
3. Click Record to begin recording and click the mark button to mark the recording.
4. Once the tip of the microelectrode is close to the membrane of a healthy muscle fiber, watch the recording of the membrane potential on the computer screen. When the microelectrode tip touches a muscle fiber membrane, a small deflection of the recording, either up or down, may occur.
5. Penetrate the membrane of the muscle fiber by either:
 - Pushing the microelectrode tip through the membrane using the controls of the micromanipulator; or,
 - Gently tapping the base of the micromanipulator to create a small amount of vibration in the microelectrode tip, which will aid in the penetration of the membrane.

Warning: *Do not lower the tip into the muscle fiber more than is necessary to avoid breaking the tip*

6. As the tip of the microelectrode penetrates the membrane, the recording displayed on the computer screen deflects downward rapidly. When this happens, stop moving the electrode! The tip of the microelectrode is inside the muscle fiber.

Note: *Sometimes, the initial drop in voltage will not be abrupt. The voltage may drift down to a lower level as the tissue seals around the tip of the electrode. Count this more negative value as the resting potential, if it is at a steady level.*

7. Click AutoScale to view the voltage levels before and after the microelectrode penetrated the membrane.
8. Repeat Steps 2 through 7 to record the resting membrane potentials from nine additional muscle fibers. Check the resistance of the electrode frequently; a low resistance means that the tip is broken and it will damage the muscle fibers.
9. Click Stop to halt recording.
10. Select Save in the File menu.

Data Analysis

1. Scroll through the data file and locate the section of the recording made when the microelectrode penetrated the muscle fiber membrane.
2. Use the Display Time icons to adjust the Display Time of the Main window to display a fifteen-second section of the recording when the microelectrode penetrated the membrane on the Main window. This section of data can also be selected by:
 - Placing the cursors on either side of the fifteen-second section of the recording, and
 - Clicking the Zoom between Cursors button on the LabScribe toolbar to expand or contract the fifteen-second recording to the width of the Main window.
3. Data can be collected from the Main window or the Analysis window. If you choose to use the Analysis window, click on the Analysis window icon in the toolbar.
4. The mathematical functions, V2-V1 and T2-T1 should appear on screen. Values for V2-V1 and T2-T1 on each channel are seen in the table across the top margin of each channel, or to the right of each graph.

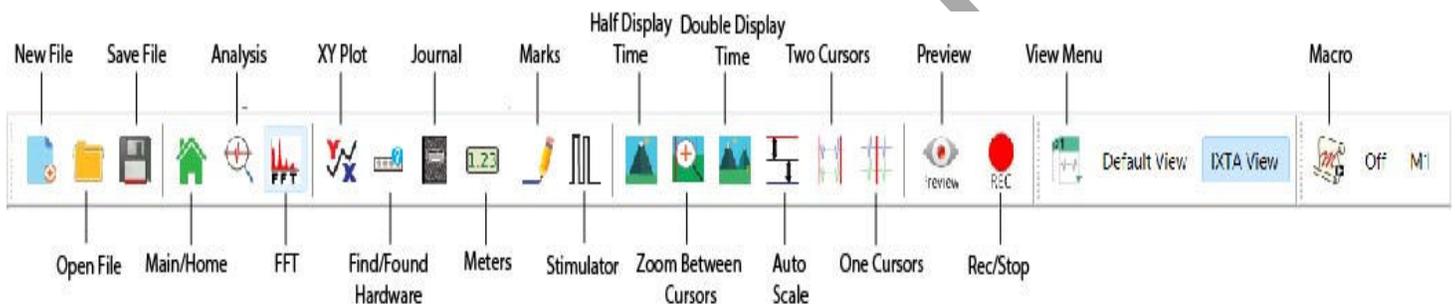


Figure AN-12-L1: The LabScribe toolbar.

5. Once the cursors are placed in the correct positions for determining the change in voltage (V2-V1) as the tip of the microelectrode enters the muscle fiber, the value for V2-V1 can be recorded in the on-line notebook of LabScribe by typing the names and values of the parameters directly into the Journal.
6. The functions in the channel menu of the Analysis window can also be used to enter the name and value of the voltage change from the recording to the Journal. To use these functions:
 - Place the cursors at the locations used to measure the membrane potential.
 - Transfer the name of the parameter to the Journal using the Add Title to Journal function in the Membrane Potential channel menu.
 - Transfer the value for the parameter to the Journal using the Add Ch. Data to Journal function in the Membrane Potential channel menu.
7. On the Membrane Potential channel displayed in the Analysis window, use the mouse to click on and drag a cursor to the baseline recorded before the microelectrode penetrated the fiber. Drag the other cursor to the voltage level that results from the microelectrode being in the fiber.

8. Record the value for the voltage change ($V_2 - V_1$) in the Journal using the one of the techniques described in Steps 5 or 6, and in Table AN-12-L1.
9. Divide the value for $V_2 - V_1$ by the gain of the Model 3100 electrometer to obtain the membrane potential. For example, a voltage difference ($V_2 - V_1$) of 320 mV recorded by the Model 3100 with a gain of 10X is equal to a membrane potential of 32 mV:

$$320 \text{ mV} / 10\text{X Gain} = 32 \text{ mV}$$

10. Place a section of data recorded while the microelectrode was in a fiber on the Main window.
11. Use the AutoScale and the Display Time functions to zoom-in on the voltage recorded while the microelectrode was in the fiber.
12. Examine this section of the recording to determine if any synaptic potentials are present. These potentials usually have actual amplitudes of 5mV or less.

Questions

1. What was the range of values measured for the resting membrane potentials of the fibers?
2. Do some of the values fall outside the range of the mean + 2 Standard Deviations (S.D.)?
3. Why do resting membrane potentials recorded from different fibers vary?
4. Can lower values for the membrane potentials be attributed to the state of health of the muscles or the ease at which the fibers were penetrated?

Table AN-12-L1: Resting Membrane Potentials (E_m) of Slow Flexor Muscle Fibers.

	Voltage Change (ΔV) Recorded, in mV	$\Delta V / \text{Gain} = E_m$, in mV
Fiber 1		
Fiber 2		
Fiber 3		
Fiber 4		
Fiber 5		
Fiber 6		
Fiber 7		
Fiber 8		
Fiber 9		
Fiber 10		
Mean		

Exercise 2: Action Potentials in Motor Neurons

Aim: To record motor nerve action potentials from the SBIII nerve.

Approximate Time: 30 minutes

Procedure

1. Use the second micromanipulator to position the tip of the suction electrode near the SBIII nerve of the slow flexor muscle fibers from which the membrane potentials were recorded.
2. Check the size of the tip of the suction electrode. It should be the same size or only slightly larger than the diameter of the nerve.
3. Without moving the tubing, pull back on the plunger of the syringe, that is connected to the tubing of the suction electrode, to pull the end, or a loop, of the nerve into the suction electrode.
4. Turn on the audio monitor optionally connected to the output of the NA-100 NeuroAmp. The action potentials in the motor neurons should be heard as a series of clicks.
5. Type Motor Neurons in the Mark box to the right of the Mark button.
6. Click Record to begin recording and click the mark button to mark the recording.
7. To improve the display of the action potentials in the SBIII nerve on the Main window, Autoscale the recording and minimize the 60 Hz noise by using the digital filters of the LabScribe software.
8. If the spontaneous rate of firing from the motor neurons is too slow, brush the sensory hairs on the lateral edge of the same or adjacent tail segments to increase the firing frequency.
9. Continue to record for two minutes.
10. If only two or three different types of action potentials are detected, use a suction electrode with a smaller tip or record from a different SBIII nerve.
11. Click Stop to halt recording.
12. Select Save in the File menu.

Data Analysis

1. Inspect the recording made from the SBIII nerve to determine the number of different types of action potentials present. Use these parameters to distinguish the different types of action potentials recorded from the SBIII nerve:
 - **Amplitude (Amp):** Measure the amplitude of an action potential by placing one cursor on the baseline before the action potential, and the second cursor on the peak of the potential. The value for the V2-V1 function on the Nerve Action Potential channel is the amplitude.
 - **Duration (Dur):** Measure the duration of an action potential by placing one cursor at the onset of the action potential, and the second cursor on the point where the potential returns to the baseline. The value for the T2-T1 function on the Nerve Action Potential channel is the duration.

- Rise Time (Rise): Measure the rise time of an action potential by placing one cursor at the onset of the action potential, and the second cursor on the peak of the potential. The value for the T2-T1 function on the Nerve Action Potential channel is the rise time.
 - Fall Time (Fall): Measure the fall time of an action potential by placing one cursor on the peak of the potential, and the second cursor on the point where the potential returns to the baseline. The value for the T2-T1 function on the Nerve Action Potential channel is the fall time.
 - Frequency (Freq.): Measure the frequency of a type of action potential by placing one cursor on the peak of an action potential, and the second cursor on the peak of the successive potential. The value for the T2-T1 function on the Nerve Action Potential channel is the period of the wave type. The inverse of the period is the frequency.
 - Polarity (Pol.): note whether the waveform of the action potentials goes up (positive) or down (negative) on the screen.
2. Enter information about the types of action potentials recorded in the Journal and Table AN-12-L2.

Table AN-12-L2: Properties of Action Potentials Recorded from SBIII Nerve.

AP Type	Amp. (mV)	Dur. (msec)	Rise (msec)	Fall (msec)	Freq. (Hz)	Pol. (+, -)
1						
2						
3						
4						
5						
6						
7						

Questions

1. How many different types of action potentials did you detect?
2. What membrane properties affect the shape and size of action potentials?

Exercise 3: Action and Synaptic Potentials

Aim: To correlate the activity of action potentials in the SBIII nerve with the activity of synaptic potentials in the muscle fibers.

Approximate Time: 30 minutes

Procedure

1. With the suction electrode attached to the SBIII nerve and a microelectrode placed in the membrane of a slow flexor muscle fiber, record the presynaptic action potentials and the postsynaptic membrane potentials at the same time.
2. Record from several muscle fibers since most muscle fibers are not innervated by every motor neuron. The synaptic potentials from a particular muscle fiber are usually the same size and shape every time an action potential from a particular motor neuron occurs.

Questions

1. Is one particular type of nerve action potential associated more closely with postsynaptic potentials more than any other type of action potential?
2. Are particular types of action potentials associated with particular sizes of postsynaptic potentials? For example, are the largest action potentials always followed by largest excitatory post synaptic potentials (EPSP) or by the smallest EPSPs?
3. At least one of the motor neurons in the SBIII nerve is inhibitory. Can you tell which type of action potential from the SBIII nerve produces inhibitory postsynaptic potentials (IPSP)? Does this type of nerve action potential produce IPSPs in all muscle fibers?

NOTE – Exercise 4 needs the settings file CrustaceanNeuromuscularJunction-B to be loaded. Keep this lab open to continue the experiment with the new settings.

Exercise 4: Evoked Synaptic Potentials

Aim: To evoke and record postsynaptic potentials in slow flexor muscle fibers while stimulating the SBIII nerve.

Approximate Time: 30 minutes

This exercise requires the loading of a new settings file, Experiment AN-12B: Evoked Synaptic Potentials. To load that file, follow this procedure.

Keep this document open to complete the exercise.

Start the Software

1. Pull down the Settings menu again. Select the CrustaceanNeuromuscularJunction-B settings file from Animal Nerve.
2. Continue using this lab protocol, Experiment AN-12: Crustacean Neuromuscular Junction, to complete Exercise 4: Evoked Synaptic Potentials.

Procedure

1. If it has not already been done, cut the SBIII nerve at the point where this nerve enters the ventral nerve cord. Pull the cut end of the nerve into the tip of the suction electrode by pulling back on the syringe attached to the end of the suction tubing.
2. Locate the C-BNC-P2 stimulator cable.
3. Attach the BNC connector of the C-BNC-P2 stimulator cable to the adapter on the stimulator outputs or directly to the Stim1 BNC connector on the IXTA.
4. Connect the lead wire for the electrode inside the lumen of the suction electrode to the red pin connector of the C-BNC-P2 stimulator cable. Connect the lead wire for the electrode wrapped the tip of the suction electrode to the black pin connector of the C-BNC-P2 stimulator cable.
5. Click the Stimulator Preferences icon on the LabScribe toolbar (AN-12-L1) to open the stimulator control panel on the Main window.
6. Check the settings on the stimulator control panel against the settings for this portion of the exercise that are listed in Table AN-12-L3. To make changes to the stimulus parameters:
 - Click on the arrow buttons to the right of the window that displays the value of the parameter to increase or decrease the value; or,
 - Type the value of the parameter in the window next to the label of the parameter.
 - Click the Apply button to effect the change to the stimulator.
7. Click the Record button to stimulate the SBIII nerve and record from the postsynaptic slow flexor muscle fiber.
8. If a response did not occur in the muscle fiber as recorded on the Membrane Potential channel, increase the stimulus amplitude. Remember to click the Apply button to finalize changes.
9. Continue to increase the stimulus amplitude until a synaptic potential occurs. Note the amplitude at which this potential occurred.
10. Continue to increase the stimulus amplitude and record the response of the fiber. As the stimulus amplitude is increased, the amplitude of the synaptic potential will increase in a stepwise manner. These steps correspond to activation of different axons in the nerve.

Questions

1. At what stimulus amplitude did the muscle fiber first generate a synaptic potential?
2. At what stimulus amplitude did the amplitude of the synaptic potential reach a maximum level?
3. How many times larger is the stimulus that delivered the maximum response than the stimulus that delivered the first measurable synaptic potential?

Table AN-12-L3: Settings on the Stimulator Window of the Preferences Dialog that Configure the iWorx System for Exercise 4.

Function	Units/Title	Setting
Stimulus Mode		Pulse
Stimulator Start		With Recording
Time Resolution	msec	0.01
Toolbar Step Frequency	Hz	1
Toolbar Step Amplitude	Volt	0.01
Toolbar Step Time	Variable	0.01
Delay	Sec	0.002
Amplitude (Amp)	Volts	0.010
Pulses (#pulses)	Number	1
Pulse Width (W)	msec	0.1
Time Off (T Off)	msec	0.9
Time Off Amplitude	Volts	0
Holding Potential	Volts	0