Experiment AM-10: Summation, Tetanus, and Fatigue in an Intact Nerve-Muscle Prep

By: RJ Cooper, Ph.D., Margaret A. Weck, D.A., and Dayton J. Ford, Ph.D.: at the St. Louis College of Pharmacy. Adapted from: Hoff, H.E. and L.A. Geddes, Experimental Physiology (1965)

Updated by Dr. Abiche Dewilde, Postdoc, U Mass Lowell (2021)

Work Sample Lab

Equipment Required

PC or Mac Computer

IXTA, USB cable, IXTA power supply

FT-302 Force transducer

C-BNC-PN2 Needle-type stimulating electrodes

C-BNC-SE Sleeve-type stimulating electrodes

Ringstand and clamps

Muscle tension adjuster

Thread

Frog board

Dissection tray

Glass dissection hooks

1cc tuberculin syringe with needle

Amphibian Ringer's solution (see Appendix)

1% Tubocurarine solution in Ringer's

Force Transducer and Stimulus Electrode Setup

1. Locate the FT-302 force transducer and its male DIN-DIN cable.

Figure AM-10-S1: The FT-302 force transducer.

2. Connect the force transducer to the data recording unit by inserting either end of the male DIN8 extension cable into the connector on the FT-302 transducer. Plug the other end of the same cable into Channel A5.

Figure AM-10-S2: FT-302 force transducer connected to IXTA.

3. Place the force transducer in the tension adjuster on the ringstand. Turn the positioning knob on the adjuster to place the transducer in the center of the range of movement of the positioner.

Calibration of the FT-302 Force Transducer

1. Type **No Weight** in the Mark box. Click Record and then the mark button to attach the comment to the recording. Record for ten seconds with no weight hanging from the arm or hook of the transducer.
2. Type **5 grams** in the Mark box. Hang a 5 gram weight on the arm or hook of the transducer. Click the mark button. Record for ten more seconds.
3. Click Stop to halt the recording.

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4. Select Save As in the File menu, and name the file. Choose a destination on the computer in which to save the file. Click on the Save button to save the data file.

Unit Conversion

1. Scroll to the beginning of data when no weight was attached to the force transducer.
2. Use the Display Time icons on the LabScribe toolbar to adjust the display time of the Main window to show the complete calibration data on the Main window.
3. Click the Double Cursor icon so that two vertical cursors appear on the Main window. Place one cursor on the flat section of data collected when no weight was attached to the force transducer, and the second cursor on the flat section of data collected when the 5 gram weight was attached to the transducer.
4. To convert the voltages at the positions of the cursors to correct values, use the Simple Units Conversion dialogue window. Click V2-V1 on the force channel, then select Units, and select Simple.
    - Put a check mark in the box next to Apply units to all blocks. Notice that the voltages from the positions of the cursors are automatically entered into the value equations.
    - Enter "Zero" in the corresponding box to the right of the voltage recorded when no weight was attached to the transducer. Enter "5" in the box to the right of the corresponding voltage recorded when the 5 gram weight was hung on the hook of the transducer.
    - Enter the name of the units, grams, in the box below the weights. Click on the OK button in the lower right corner of the window to activate the units conversion.

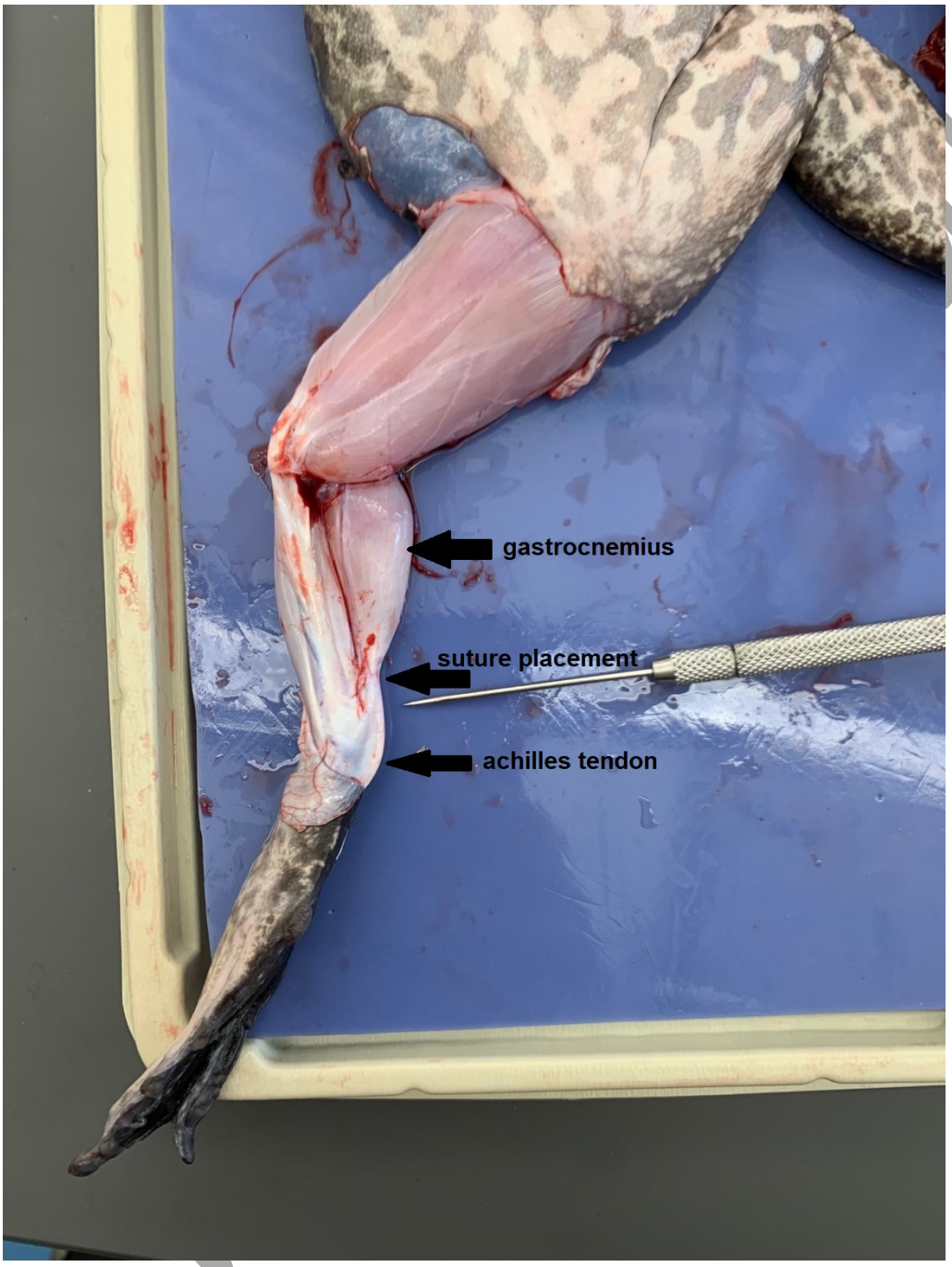
In the 10 gram range, the FT-302 will deliver approximately 75 mV/gram at x1 gain and approximately one tenth of that in the 100 gram range. The FT-302 is now ready for use.

Frog Preparation

Warning: The muscle preparation used in this experiment is functional for a limited period of time. If the muscle is bathed periodically in Ringer's solution, it will work for about four hours. To conserve time, complete all the exercises in the experiment before analyzing the data.

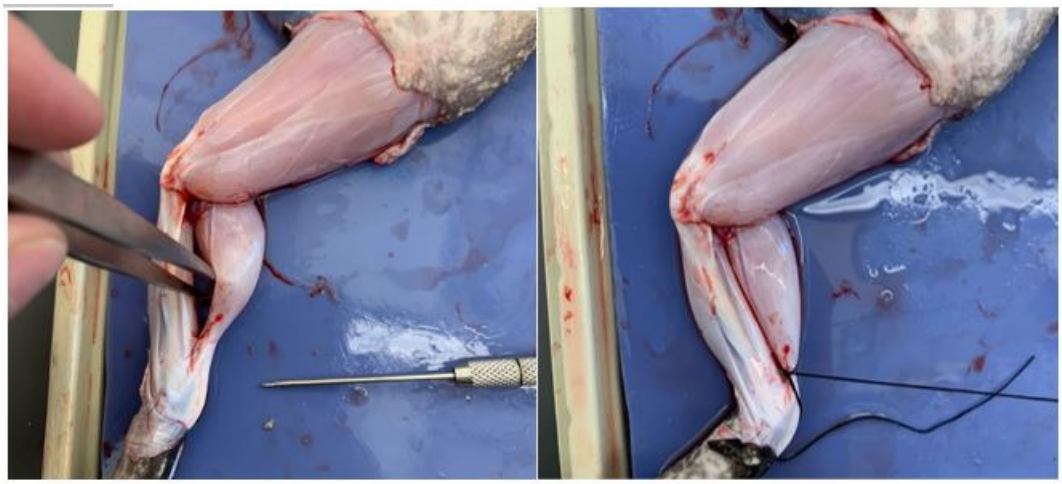
1. Place a frog in ice water for 15 minutes. Double pith the frog as soon as it is removed from the ice water.
2. Take off the skin off one leg. Cut around the thigh of the leg, until you have completely detached the skin from the torso. Slowly disconnect the skin from the leg by cutting the connecting facia. Remove the skin all the way down to the foot.

IWorx Sample Lab



3. Separate the muscle from the underlying tissues. Tie a thread securely around the Achilles tendon. Bisect the tendon between the thread and the foot. Try to leave enough of the tendon distal to the thread, so the thread will not slip off when it is stretched, or when the muscle contracts.

Work

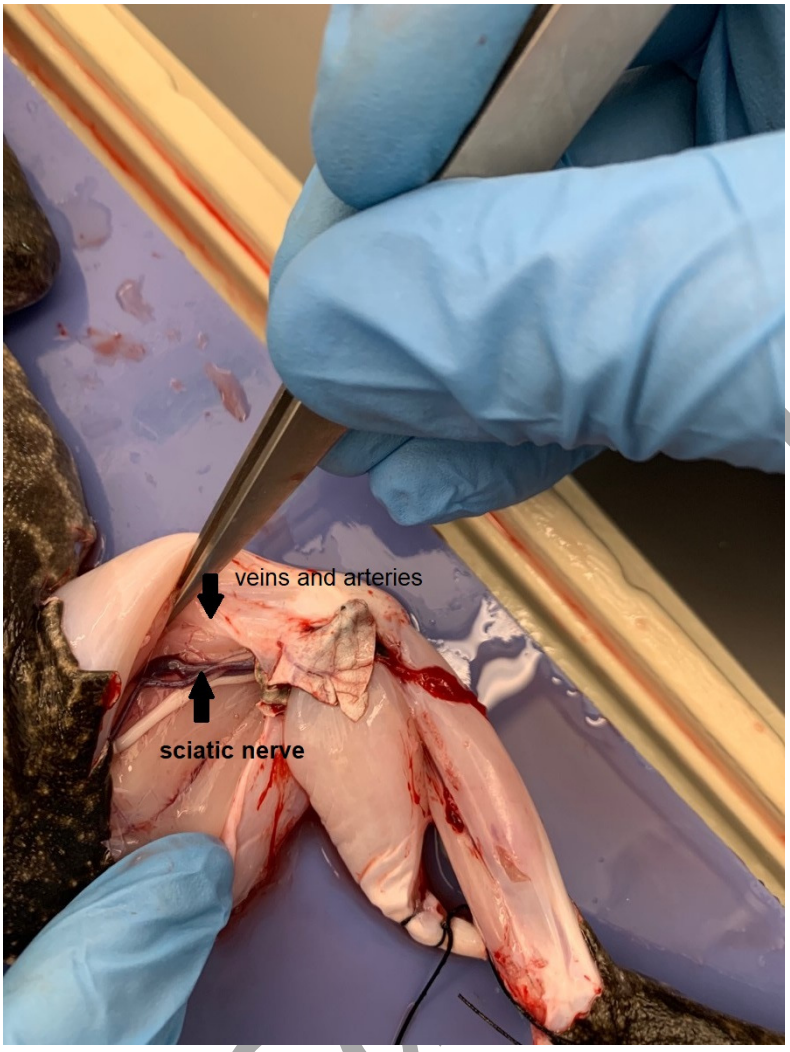


Note: Moisten the exposed limbs of the frog with Ringer's solution every five minutes or so.

4. Place the frog on its belly. Locate and isolate the sciatic nerve in the thigh region. **NEVER TOUCH THIS NERVE WITH METAL.** Cut very careful and probe with the glass Pasteur pipette until you are sure you can cut without touching the nerve.
5. This nerve extends from the spinal cord to various muscles of the leg and foot. With the frog lying on its ventral surface (belly), separate the dorsal muscles of the thigh along a natural separation that extends lengthwise along the thigh.
6. Gently retract the muscles to expose the nerve, lying close to the femur. The nerve may be identified as a creamy white, cord-like structure of uniform diameter, running near and parallel to the femoral artery and vein.

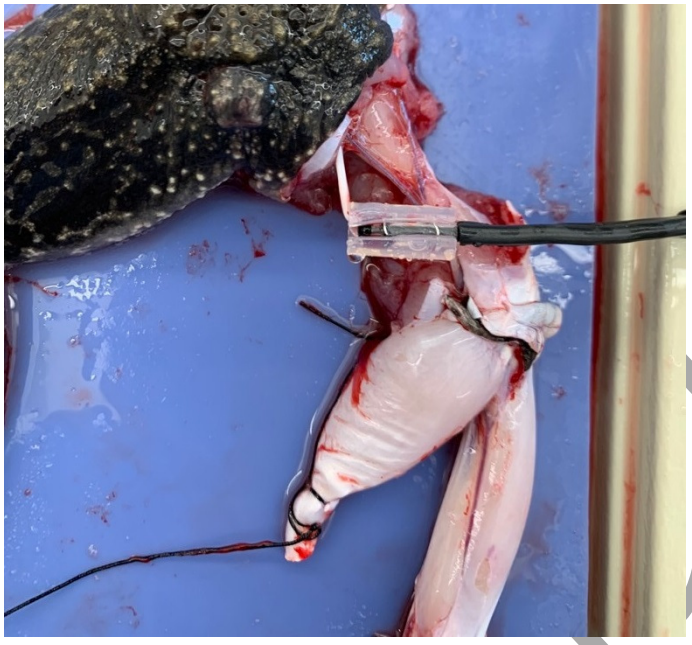
Warning – Do not confuse this nerve with a silvery-shiny structure – that is the tendon of one of the thigh muscles. Avoid stretching or otherwise damaging the nerve. Keep the tissue moist with the Frog Ringer's solution.

IworX Sample



7. The nerve will need to be carefully separated from the surrounding tissues, for a distance of about 2 cm (almost 1 in.). **This should be done with a glass probe.** Do not touch the nerve with any object that is made of metal; this will render the nerve useless!!!
8. You can remove muscle around the sciatic nerve to get easier access to it, BUT BE CAREFUL! Work with your glass probe and cut well away from the nerve when dissecting the muscle away.
9. Open the split in the retaining sleeve of the sleeve electrodes assembly and carefully place the sleeve around the nerve, then slip the electrode over the nerve before securing the frog to the frog board or wax-filled dissection tray. The sleeve electrodes will be used later.

IworX Sc Lab



10. Place the frog on the board or the tray in the prone position (belly down). If you are using a frog board, attach the knee of the prepared leg in the holder on board. If you are using a wax-filled dissecting tray, the knee can be held in place by pushing down two T-pins, one on either side of the knee.

Warning: Take care not to pinch or place a pin through the sciatic nerve in the knee region!

11. Tie the free end of the thread on the Achilles tendon of the muscle to the transducer:
12. Make a loop, using a non-slip knot, on the end of the thread and place the loop over the 0-10g hook of the transducer.

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13. Adjust the height of the tension adjuster to lift the muscle vertically to an angle that is perpendicular to the rest of the frog leg. Move the tension adjuster to align the attachment point on the transducer over the tendon of the muscle.
14. Two types of stimulating electrodes will be used to create twitches in the gastrocnemius muscle. Sleeve electrodes are placed on the sciatic nerve for stimulating the muscle through the nerve and pin electrodes are placed into the muscle itself for direct stimulation.
15. Obtain the C-BNC-SE sleeve electrodes and C-BNC-PN2 needle-type electrodes.

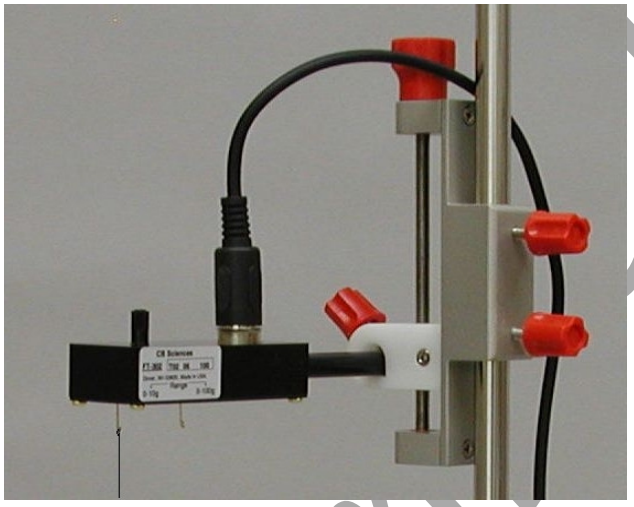

Figure AM-10-S3: FT-302 held in a muscle tension adjuster on a ringstand.

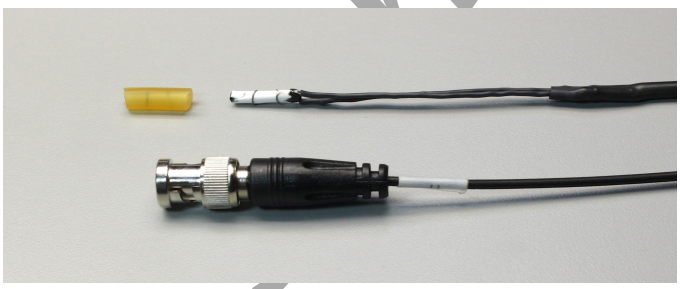

Figure AM-10-S4: The C-BNC-SE sleeve-type stimulating electrode.

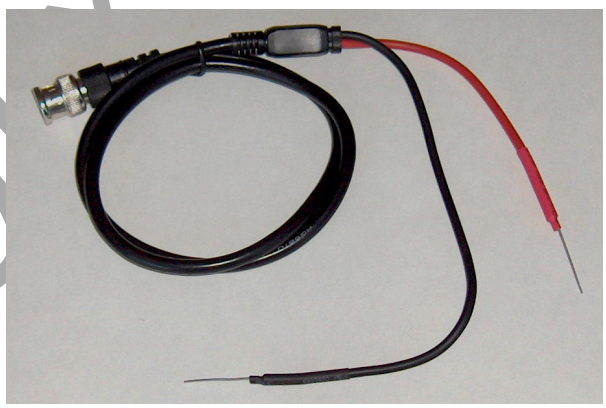

Figure AM-10-S5: The C-BNC-PN2 needle-type stimulating electrodes.

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Figure AM-10-S6: A FT-302 force transducer and an C-BNC-PN2 needle-type stimulating electrodes attached to IXTA.

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Exercise 1: Twitch Threshold Determined by Direct Stimulation

Aim: To determine the threshold stimulus of the muscle when it is stimulated directly with single pulses of constant duration.

Approximate Time: 15 minutes

- **Plug in the Pin stimulator into the SI port. Leave the sleeve stimulator on the nerve but not plugged into the iWORX box**
- **DO NOT PIN THE FOOT, pin the skin around the knee**

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

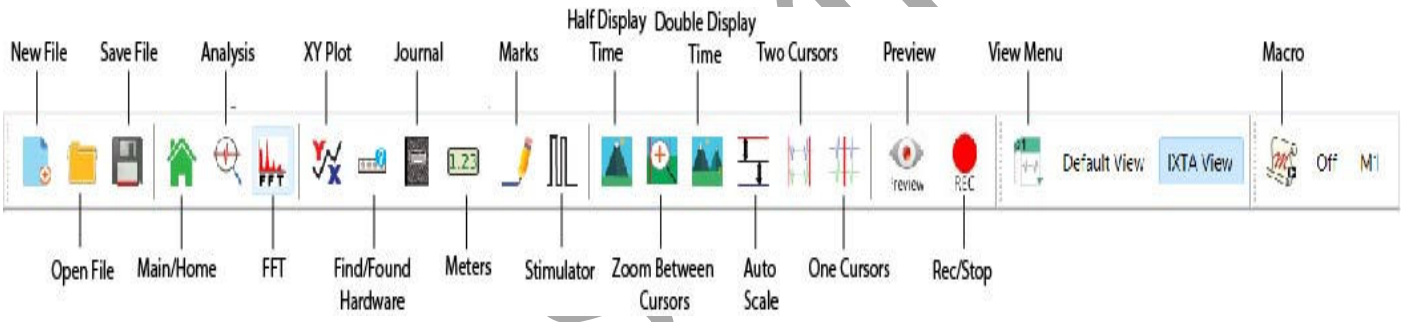

Figure AM-10-L1: The LabScribe toolbar.

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 0.000 V;
   - the number of pulses (#pulses) to 1;
   - the frequency (F(Hz)) to 1;
   - and, the pulse width (W) to 5 ms.
   - Click APPLY in the upper left of the control panel
3. The value for a stimulus parameter can be changed by either of two methods: 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 finalize the change in any stimulus parameter.

iWorx Sample Lab

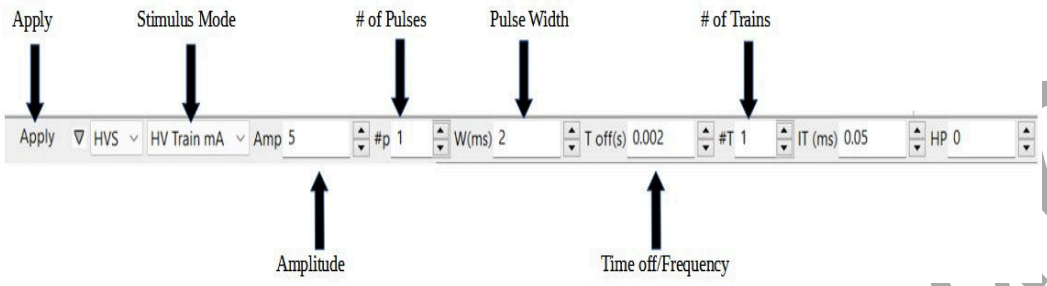

Figure AM-10-L2: The stimulator control panel

4. Type **0.000V** in the Mark box. Click Record to stimulate the nerve with 0.000V and then click the mark button to attach the comment to the recording.
5. Apply a light load to the muscle by raising the force transducer with the tension adjuster until the trace moves a few mV above the baseline. If you are using a FT-302 force transducer, the initial baseline can be adjusted to 0 mV by rotating the offset knob on top of the FT-302 transducer.
6. Once a light load is applied to the muscle, Click on the Stop.
7. Change the stimulus amplitude (Amp) to 0.1V using one of the techniques described in Step 2. Click the Apply button on the Stimulator control panel to effect the change in amplitude.
8. Type **0.1V** in the Mark box. Click Record to stimulate the nerve with 0.1V and then click the mark button to attach the comment to the recording. Stop the recording as soon as the muscle twitch is finished, or after a couple of seconds if no twitch is detected.

Note: Be sure to have at least one member of your lab group LOOKING at the specimen at all times. It is possible for contractions to occur and not be recorded. Click AutoScale to make sure the recording is displayed properly. Check the tautness of the thread going from the tendon to the transducer, the transducer itself, and check the connection from the transducer to the iWorx recorder.

9. Determine the threshold voltage for direct muscle stimulation by increasing the stimulus voltage in 0.1V increments. **DON'T SKIP A VALUE and DO NOT EXCEED 1V.** Remember to click the Apply button on the Stimulator control panel each time it is changed. Record and mark the muscle response as described in Steps 3 and 7.
10. Once the threshold voltage has been determined, test the muscle's response to direct stimulation at additional stimulus voltages. Increase the stimulus amplitude in 1.0V increments, up to 5 V. Record the muscle response and mark the recording as described in Steps 3 and 6.
11. Select Save As in the File menu, type a name for the file. Click on the Save button to save the data file.
12. Frog Nerve Muscle-Contractions - https://youtu.be/apccrZLA8v4

iWorx Sample Lab

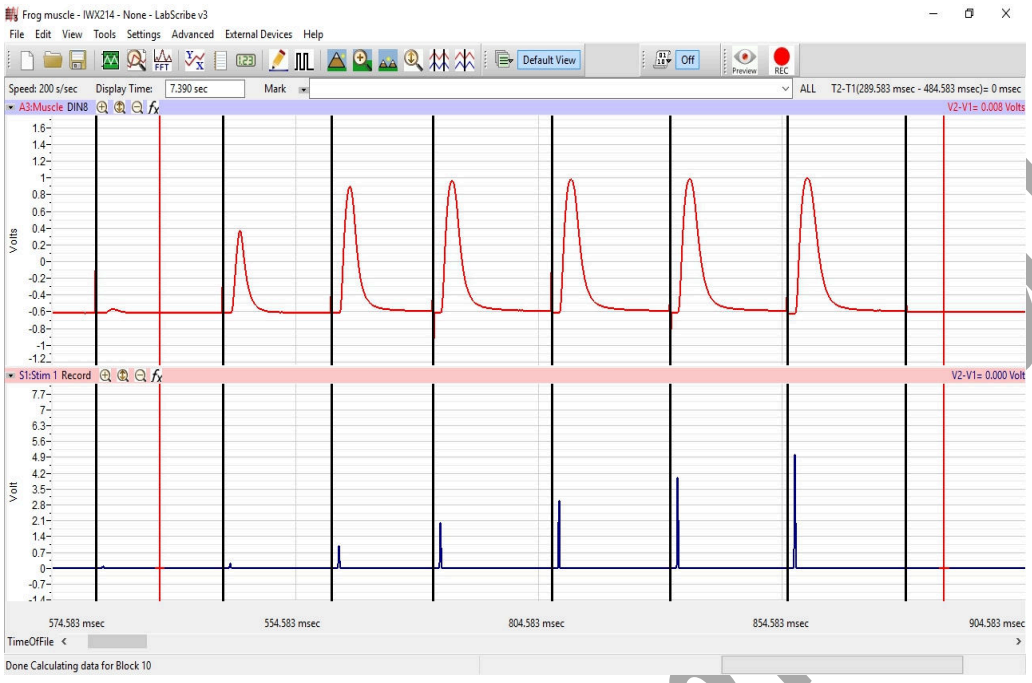

Figure AM-10-L3: Muscle twitches resulting from direct stimulation of the muscles.

Exercise 2: Twitch Threshold Determined by Nerve Stimulation

Aim: In this exercise, the muscle will be stimulated by action potentials generated in the nerve attached to the muscle. The nerve will be stimulated by single pulses of constant duration.

Approximate Time: 30 minutes

1. On the iWorx box, disconnect the BNC connector of the pin electrodes used for direct stimulation of the muscle. Leave the pin electrodes in place in the muscle.
2. On the iWorx box, connect the BNC connector of the sleeve electrodes to the BNC-banana adapter on the stimulator output of the IWX/214 or to the BNC output of the stimulator on the front of the IXTA.
3. Reset the pulse amplitude (Amp) to 0.000V. Remember to click the Apply button on the stimulator control panel to finalize the change.
4. Repeat Exercise 1 while stimulating the gastrocnemius muscle through the sciatic nerve with single stimulus pulses of increasing amplitude. Label the recording at each voltage attempted.
5. Determine the threshold voltage for nerve stimulation by increasing the stimulus voltage in 0.1V increments. Above threshold, test the muscle's response to nerve stimulation in 1.0V increments.
6. Click the Save button to save the file.

iWorx Sample

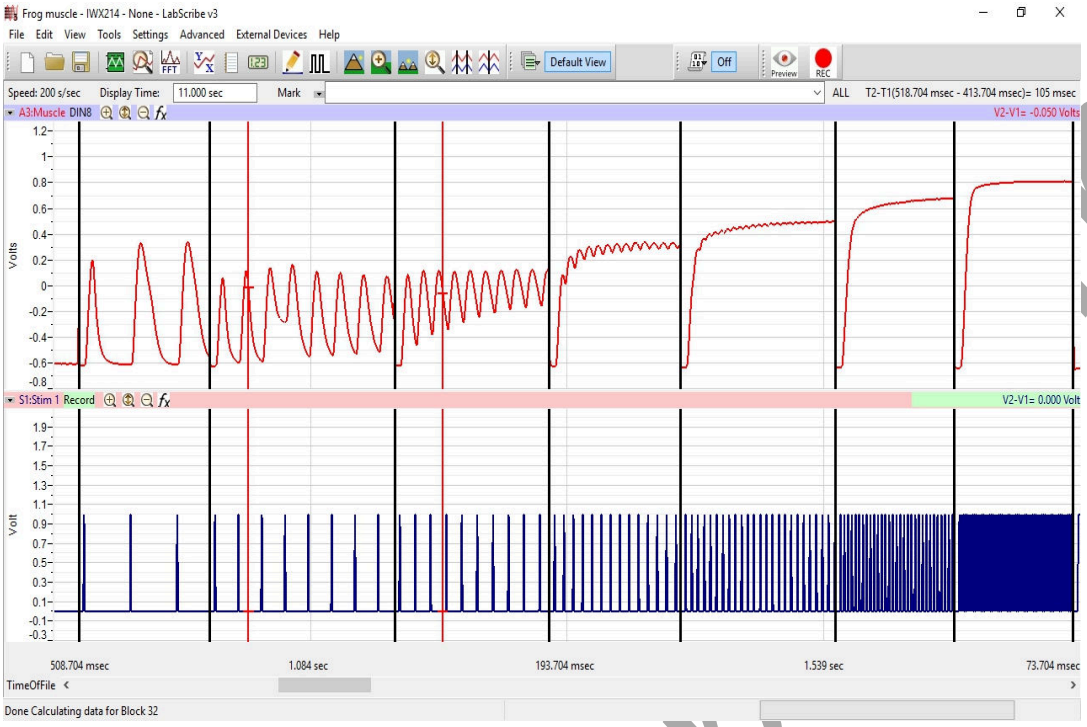

Figure AM-10-L4: Muscle twitches resulting from nerve stimulation.

Exercise 3: Effect of Stimulus Frequency on Twitch Amplitude

Aim: In this exercise, the muscle will be stimulated by action potentials generated in the nerve attached to the muscle. The nerve will be stimulated for short periods at different stimulus frequencies.

Approximate Time: 30 minutes

1. On the iWorx box, leave the BNC connector of the sleeve electrodes attached to the BNC-banana adapter on the stimulator output of the IWX/214 or on the stimulator output on the front of the IXTA.
2. Set the stimulus frequency (F(Hz)) to 2 Hz, the number of pulses (#pulses) to zero (0), and the pulse amplitude (Amp) to the lowest value that still gave strong contractions in the previous exercise. This voltage will usually be in the range of 0.400V to 1.000 V, but your frog may be different. Keep the pulse width (W) at 5 ms. Remember to click the Apply button on the Stimulator control panel with each change.

Warning: Since the number of pulses (#pulses) is set to zero (0), the stimulus pulses will be delivered continuously at the frequency selected. It is important to stop recording once you see the effect of stimulating at a certain frequency.

3. Type 2 Hz in the Mark box to the right of the Mark button. Click Record to stimulate the nerve and click the mark button to mark the recording. Stop the recording after 5 seconds
4. Change the stimulus frequency to 4 Hz. Repeat Step 3. Continue the recording for stimulus frequencies of 5, 10, 25, 50, and 100 Hz.

iWorX Sample

5. Click the Save button to save the file.
6. When you analyze the data for this exercise, note the frequency at which wave summation begins, the frequencies at which incomplete and complete tetanus occur, and the shape of the contraction waves and any indication of fatigue.
7. Frog Nerve Muscle-Tetanus - https://youtu.be/5dyuVMr6oa8

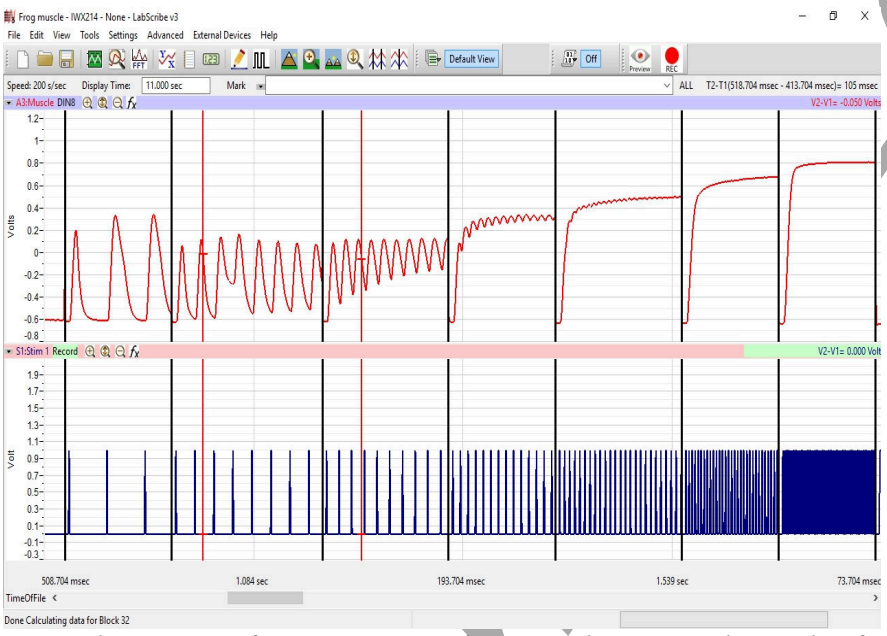

Figure AM-10-L5: Muscle response from nerve stimulation with increased stimulus frequencies.

Exercise 4: Fatigue with Repeated Stimuli

Approximate Time: 45 minutes

1. On the iWorx box, disconnect the sleeve electrodes from the stimulator output, and connect the pin electrodes to the stimulator. Leave the sleeve electrodes in place on the nerve.
2. Set the Display Time to 20 sec, the stimulus frequency to 4 Hz, the number of pulses (#pulses) to zero (0), and pulse amplitude (Amp) to an adequate value between 0.4V and 1 V. Remember to click the Apply button.
3. Type 4Hz-Direct in the Mark box to the right of the Mark button. Click Record to stimulate the nerve with 4 Hz and press the mark button to attach the comment to the recording. Stop the recording after 5 seconds
4. After recording at 4Hz with the pin electrodes, switch to the sleeve electrodes.
5. Type 4Hz-Nerve in the Mark box to the right of the Mark button. Click on the Record button to stimulate the nerve with 4 Hz and press the mark button to attach the comment to the recording. Stop the recording after 5 seconds.

iWorx Sample Lab

6. Change the stimulus frequency to 50 Hz. Remember to click the Apply button on the Stimulator control panel.
7. Type 50Hz-Nerve in the Mark box to the right of the Mark button. Click Record to stimulate the nerve with 50Hz and press the mark button to attach the comment to the recording. Continue to stimulate through the nerve until the force of the muscle contraction drops to an amplitude that is only 25% of the maximum force at the beginning of this tetanic contraction.
8. Continue to record after the contraction force of the muscle has dropped below 25% of maximum. Quickly disconnect the BNC connector of the sleeve electrodes from stimulator, and reconnect the BNC connector of the pin electrodes to the stimulator.
9. Stimulate the muscle directly for 3 seconds, with the same voltage, duration, and frequency that was used to fatigue the muscle.

Note: *If no contraction is seen while using these stimulus parameters, increase the stimulus amplitude until a contraction is measured.*

10. Continue to record after the direct stimulation of the muscle. Quickly disconnect the BNC connector of the pin electrodes from the stimulator, and reconnect the BNC connector of the sleeve electrodes to the stimulator.
11. Apply the same stimulus used in Step 9 to the nerve for a period of 3 seconds.
12. After you stop the recording, label the recording with marks and notations to indicate the site of stimulation (muscle or nerve) and the stimulus voltage that generated each response.
13. Click the Save button to save the file.
14. When you analyze the data for this exercise:
    - Note the shape of the contraction waves and any indication of fatigue.
    - Note whether the muscle contraction was stronger when the stimulus was applied directly to the muscle or when the stimulus was applied to the nerve. What does this indicate about the location of the fatigue observed during nerve stimulation?

IWorX Sample Lab

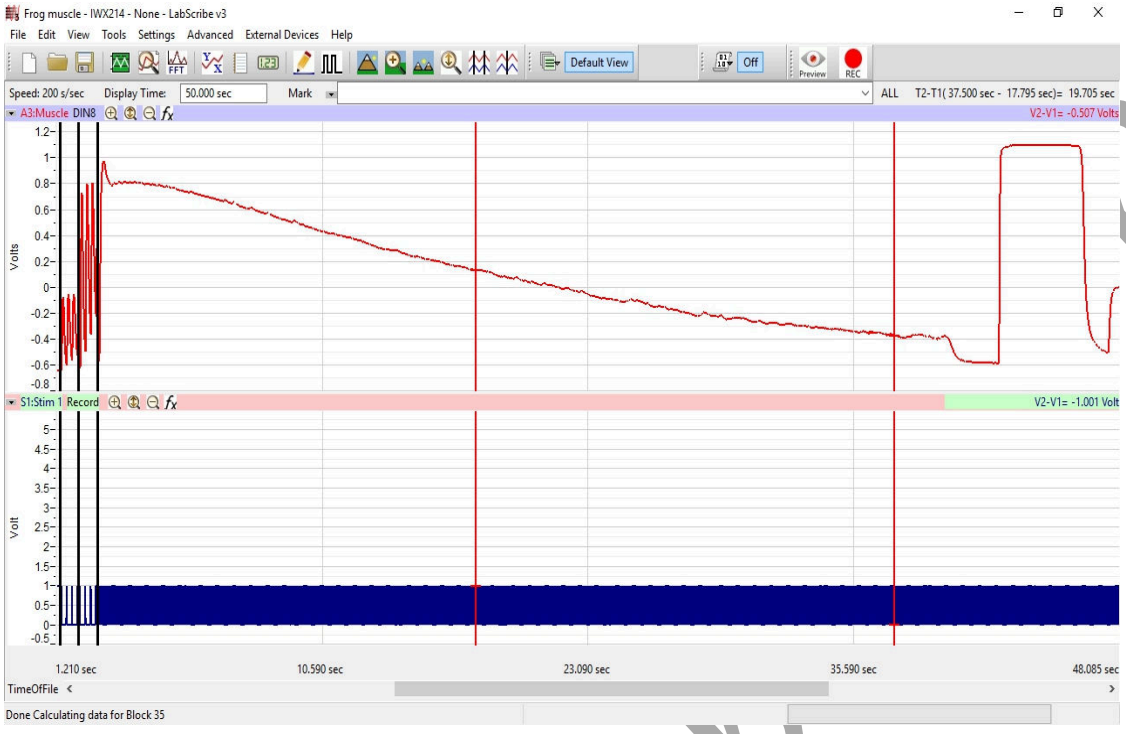

Figure AM-10-L6: Decrease in muscle force (fatigue) with continuous, high frequency stimulation through the nerve innervating the muscle. The period of fatigue occurring during nerve stimulation is followed by a large response by the muscle when it is stimulated directly.

Exercise 5: Myoneural Block

Approximate Time: 45 minutes

1. After completing Exercise 4, allow the frog nerve/muscle preparation to rest for at least 5 minutes. Leave the BNC connector of the sleeve electrodes attached to the stimulator.
2. Set the Display Time to 10 sec, the stimulus frequency (F(Hz)) to 1Hz, the number of pulses (#pulses) to 0, the stimulus duration (W) to 5 ms, and the pulse amplitude (Amp) to 1 V. Remember to click the Apply button on the Stimulator control panel.
3. Click on the Record button and record the muscle's response to nerve stimulation. Stop the recording after 4 seconds. If no response is observed, prepare the other leg of the frog for this exercise.

Note: *If a new frog leg is needed, insert pin electrodes into the gastrocnemius muscle and sleeve electrodes on the sciatic nerve of the leg. Adjust the tension on the muscle for an adequate response. Attach the BNC connector of the pin electrodes to the IWX/214 stimulator.*

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4. Click on the Record button and stimulate the muscle directly for 4 seconds. While continuing to record, change to using the sleeve electrodes and stimulate the muscle through the nerve for 4 seconds. Click on the Stop button to halt the recording. Make sure the contractions following direct stimulation of the muscle and stimulation of the nerve are adequate. Increase the pulse amplitude (Amp), as needed, to create strong muscle contractions.
5. Click on the Record button and stimulate the muscle through the nerve for 4 seconds.
    - While the recording, inject about 0.1 ml of 1.0% turbocurarine solution into the muscle. Use several injection sites to decrease the total deformation of the muscle.
    - Turn off the recording for 10 seconds. Follow the 10 second rest period with a 2 second period of nerve stimulation and recording.
    - Repeat this pattern until little or no muscle response is seen with nerve stimulation. The longer the curare stays on the muscle, the more dramatic the change in force should be.
6. After the muscle's response to nerve stimulation has disappeared, disconnect the BNC connector of the sleeve electrodes from the stimulator. Reconnect the BNC connector of the pin electrodes to the stimulator. Stimulate the muscle directly for 2 seconds and observe the contraction. If there is no response, repeatedly increase the stimulus amplitude and stimulate the muscle until it shows an observable contraction.
7. When a visible muscle contraction is seen, quickly disconnect the pin electrodes from the stimulator and reconnect the sleeve electrodes. Immediately stimulate the muscle through the nerve with pulses that have the same parameters as the ones just used for direct muscle stimulation.

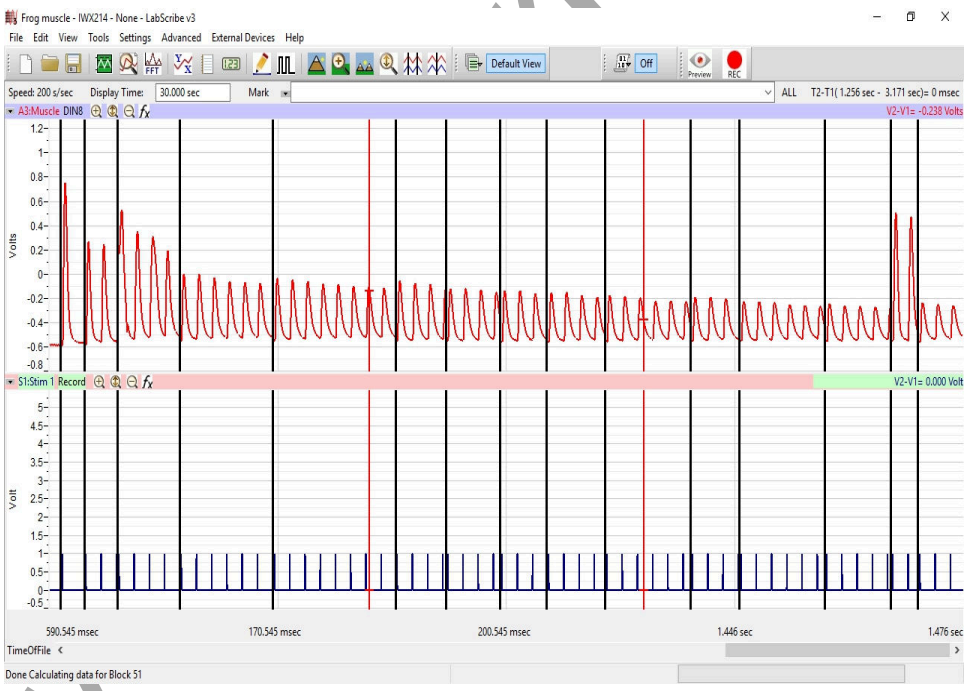

Figure AM-10-L7: Decrease in contraction force with increase in time after the injection of curare into the muscle. The muscle has a large response to direct stimulation even though the response to nerve stimulation is low.

IWorx Lab

8. Label the recording to identify the mode of stimulation and the stimulus parameters used for each response.
9. Click the Save button to save the file.
10. Clean up your area and equipment. Put the frogs and any frog tissue in the designated container. Return all equipment to where it belongs.

Data Analysis

Exercise 1: Twitch Threshold - Direct Stimulation

1. Scroll through the data from Exercise 1 and find the first muscle twitch to be generated by a stimulus pulse. Click the AutoScale button to maximize the size of the muscle twitch on the window. Note the stimulus voltage used to generate this twitch.

Note: *At stimulus voltages that are below the threshold of the muscle, the amplitude of the muscle twitch is zero.*

2. Use the Display Time icons to adjust the Display Time of the Main window to show the stimulus pulse used to generate the twitch and the complete twitch on the Main window. The stimulus pulse and the twitch can be selected by:
    - Placing a cursor before the stimulus pulse, and a cursor after the muscle has completely relaxed; and
    - Clicking the Zoom between Cursors button on the LabScribe toolbar to expand the display of the stimulus pulse and the twitch 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.
5. Maximize the height of the trace on the Muscle Contraction Channel by clicking on the AutoScale All button on the toolbar.
6. Once the cursors are placed in the correct positions for determining the amplitude of the muscle twitch, the value for V2-V1 can be recorded in the on-line notebook of LabScribe by typing the name and value directly into the Journal, or on a separate data table.
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 muscle twitch.
    - Transfer the name of the mathematical function used to determine the amplitude to the Journal using the Add Title to Journal function in the Muscle Contraction channel pull-down menu.

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- Transfer the value for the amplitude to the Journal using the Add Ch. Data to Journal function in the Muscle Contraction channel pull-down menu.

8. On the Muscle Contraction channel, use the mouse to click on and drag the cursors to specific points on the recording to measure the muscle twitch amplitude, which is the difference between the baseline tension of the muscle and the tension at the peak of the twitch. To measure this parameter, place one cursor at the beginning of the twitch, and the second cursor on the peak of the twitch. The value for the V2-V1 function on the Muscle Contraction channel is the muscle twitch amplitude.
9. Record the value in the Journal using the one of the techniques described in Steps 6 or 7, and on Table AM-10-L1.
10. Repeat Steps 2 through 9 to find the muscle twitch amplitudes of the other muscle twitches recorded in this exercise. Record the values for V2-V1 in the Journal and on the table.
11. Determine the force (in g) of each contraction by dividing its twitch amplitude by the sensitivity of the transducer used in the experiment. Note: when used with an IWX/214 or an IXTA:
    - the FT-302 has an output of 100 mV for each gram of weight.
    - the FT-104 has an output of 6.5 mV for each gram of weight.

Exercise 2: Twitch Threshold - Nerve Stimulation

1. Use the same techniques used in Exercise 1 to determine the amplitudes of muscle twitches generated by nerve stimulation.
2. Record the values in the Journal using the one of the techniques described in Exercise 1, and on the table.

Questions - Exercises 1 and 2

1. Prepare a graph showing the relationship between the stimulus amplitude and the strength of the muscle contraction for the stimuli applied to the nerve and directly to the muscle.
2. Are the results consistent with an all-or-none response of a muscle cell that is stimulated with a pulse with an amplitude that is equal to or greater than the threshold amplitude of the muscle cell? Explain the reasoning for your conclusion.

Hint: *Are muscles made of single or multiple muscle cells?*

Exercise 3: Stimulus Frequency and Twitch Amplitude

1. Use the same techniques used in Exercise 1 to determine the amplitudes of muscle twitch or response generated at different frequencies.

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2. Determine the following values:
    - Force of contraction of a single twitch when stimulus frequency was 2 Hz.
    - Force of the maximum muscle response to a stimulus frequency of 50 Hz. To measure this force, place one cursor on the baseline tension and the other cursor on the highest muscle response that occurred during stimulation.
    - Tetanus-Twitch Ratio:

        $$\frac{\text{Maximum Muscle Force in Tetanus}}{\text{Force in a Single Twitch}} = \text{T-T Ratio}$$

3. Record the values in the Journal using the one of the techniques described in Exercise 1.

Question - Exercise 3

How do the forces of skeletal muscle contractions relate to stimulus frequency?

Exercise 4 - Fatigue

Use the same techniques used in Exercise 1 to determine the following values:

- Maximum contractile force with continuous nerve stimulation at 50 Hz and _____ V.
- Time to fatigue to 25% of maximum contractile force with continuous nerve stimulation at 50 Hz and _____ V.
- Contractile force at end of continuous nerve stimulation .
- Initial contractile force, after fatigue, using direct muscle stimulation.
- Initial contractile force, after fatigue, using sciatic nerve stimulation.

Question - Exercise 4

Based on the results of this exercise, where do you conclude the major site of fatigue is located? Explain the reasons used to your conclusion.

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Table AM-10-L1: Muscle Response to Different Stimulus Amplitudes Delivered by Two Modes.

	Stimulus Applied Directly to Muscle		Stimulus Applied to Sciatic Nerve	
Stimulus Amplitude (V)	Amplitude (mV)	Force (g)	Amplitude (mV)	Force (g)
0.0				
0.1				
0.2				
0.3				
0.4				
0.5				
1.0				
2.0				
3.0				
4.0				
5.0				
Twitch Threshold (V)				

Exercise 5 - Myoneural Block

Use the same techniques used in Exercise 1 to determine the following values:

- Contractile force before the injection of curare, triggered by nerve stimulation with an amplitude of _____V.
- Time after the injection of curare for the contractile force to decrease to 20% or less of the force before the injection, triggered by nerve stimulation with an amplitude of _____V.
- Contractile force at the time measured above.
- Contractile force triggered by direct stimulation right after the response from nerve stimulation was 20% or less.
- Contractile force triggered by nerve stimulation right after direct stimulation measured above.

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Questions - Exercise 5

1. Why was the stimulation of the muscle through the nerve at the end of this exercise necessary?
2. Based on the results of this exercise, where do you conclude the site of action of curare is located? Explain the reasons used to your conclusion.

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