Analysis of AP/axon classes and PSP on the basis of AP amplitude

In this analysis manual, we aim to measure and analyze AP amplitudes recorded with a suction electrode and synaptic potentials recorded with a sharp electrode in a muscle fiber. The suction electrodes and sharp electrode recordings were be obtained simultaneously so that we can match synaptic responses with the action potentials that evoke them.

Before you start:

HOW TO RUN COMMAND? Most of the analysis commands have been written for you in a NoteBook called Analysis Script.
To run these commands, you need to **highlight** the commands, hit Return while holding Ctrl key.

There are lines started with "//". These are comment lines you should read so you know what to look for in the graphs that appeared after you run your command.

STEP 1: check stability.

Highlight all 4 lines and run them, there should be a graph which looks like this (Fig.1):

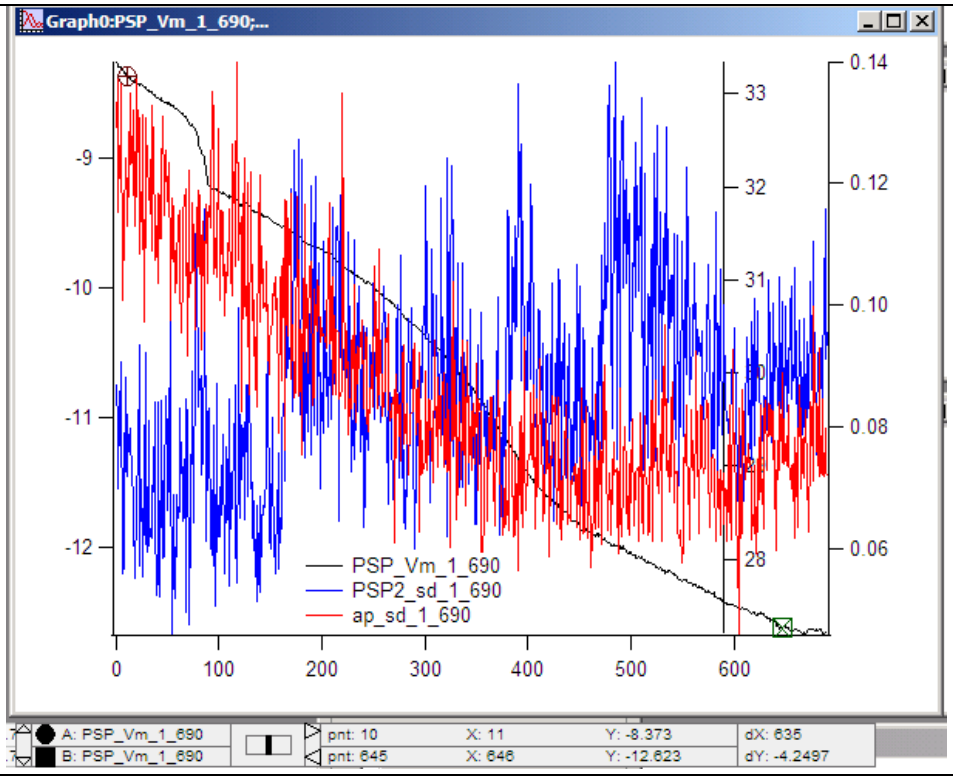


Fig.1

Here, the X-axis is trace numbers. You should have 200. (Students in my lab sometimes run 700-1000 traces.) The lines in the graph are color coded: black for muscle resting V_m, blue for the noise level of muscle fiber and red for noise level of the third nerve.

There are three Y axis: left for muscle Vm, right for noise level of muscle fiber, right middle for noise level for axon. Noise level means the standard deviation of each of the 200 traces, a good indicator if the nerve or muscle increased/decreased its activity in response to the drug or neuromodulator.

In general, if muscle V_m changes for less than 10 mV, then you are ok.

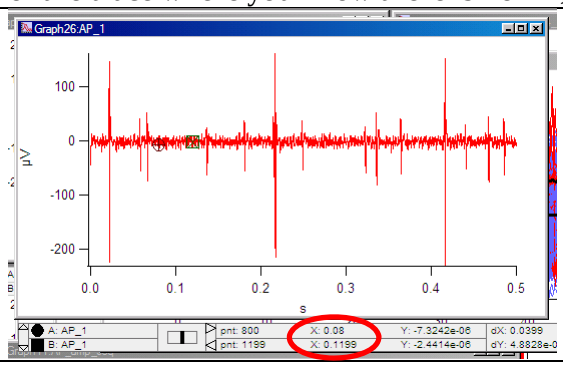
STEP 2: set noise level.

Run the top 3 lines first. Igor will spin away for a few minutes. Let it be. No graph will be displayed.

Run the next three lines; there should be a graph that pops up (Fig.2).
There are three steps in this analysis.

First, we need to set a threshold, typically 3 times the background noise. Signals with amplitudes higher than the threshold are considered real. You need to drag the two cursors to a section of the trace where you know there is no AP, see Fig.2.

Fig.2



Execute the next line, which starts with "Wavestats..."
You should then see a printout below your command: v_sdev= xxxxx
This is the standard deviation of the baseline noise level.

STEP 3: Spike sorting

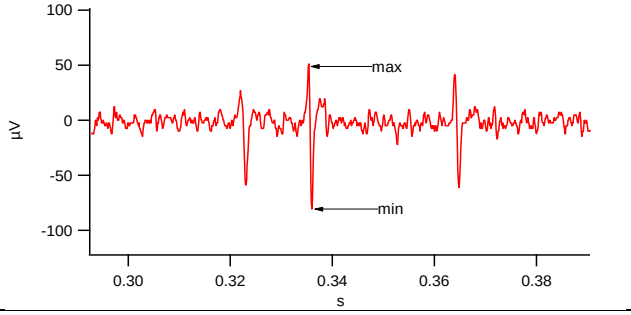
Run the single line starting with "AP_peak_search_..."

Go to have a cup of coffee, it will take ~10 to 30 minutes. The software is sorting through all 200 traces and pick out every one of the APs recorded by your suction electrode. In some preparations, there will be more than 10,000 events.

Note: This step is computationally intensive, your computer will run hot. Make sure that your laptop is plugged in and placed in a well-ventilated place, i.e. on desk but no on bed or sofa where air vent may be blocked.

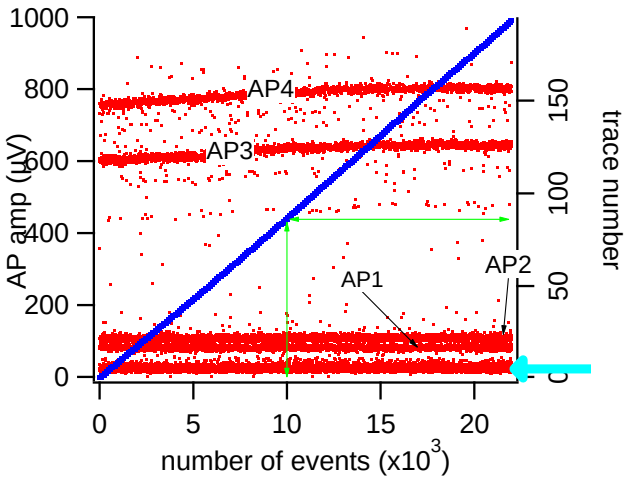
A little more details about what this sorting program is doing. This program will use the V_sdev*3 as threshold, find spikes above the threshold and measure the amplitudes of action potentials, by taking the difference between the maximum and the minimum of each AP (Fig.3).

Fig.3



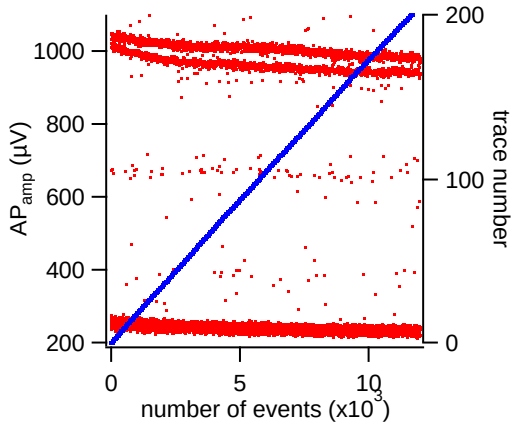
At the end of "AP_peak_search_select("ctrl",sel_first,sel_last)", you should see a plot of AP amplitudes found in the two hundred traces you have collected (Fig.4). The left Y-axis is the AP amplitude and X-axis is the AP number. (There are more than 2000 in this experiment!) The right axis corresponds to the blue line, which indicates the trace numbers, from 1 to 200. For example, the 10,000th event (vertical green arrow) was detected in trace 86 (horizontal green arrow). The graph shows that the amplitudes cluster around 5 levels (data bands), suggesting potentially 5 axons with different amplitudes. (It turns out that the lowest one, the blue arrow, is not real.)

Fig.4



Sidebar:

It is important to check the plot of AP amplitude in figure 4 carefully. Sometimes the 3rd nerve gradually or suddenly slips out of the suction electrode tip and the amplitude plot may look like the graph shown in the right instead. In this case, although the data bands are clear, there is obviously a continuous decline in all the bands. The nerve appears to have slipped out of your suction electrode gradually. We cannot analyze data that are drifting continuously. We can select a subset of data ranges within which AP amplitudes are relatively stable, from event 8000 to 12000 for example. The AP event range of 8000-120000 corresponds to traces 140-200. Later when we analyze AP1 to AP4 individually, we should use only traces 140-200.



STEP 4: Analysis of individual AP

Run the first 3 lines. They are meant to define variables and strings to be used later. No need to read deep meaning into them.

You next need to **enter three numbers** before we run the next macro.
The first number is peak_num. As a convention, we will name the smallest AP as AP1. If we start with AP1, we should make peak_num=1 by overwrite the "?" under the comment "//Need to enter peak_num, ap_peak_low, ap_peak_high"

The second and third numbers are determined after you position the round and square cursors: For the analysis of AP1, we need to tell the software the range of AP amplitude acceptable as AP1. We accomplish this by placing the round cursor in the lower boundary of the data band corresponding to AP1 and the square cursor in the upper boundary (Fig.5A and B). (You can zoom in the region to position the cursors precisely by drawing a square at the area of interest and right clicking, then choose any of the expansion options you like. To zoom out, just click Ctrl-a.)
This may be obvious but just to remind you, the numbers you should read are the Y-values. Smaller Y value for ap_peak_low and larger Y value for ap_peak_high (Fig.5B circle). Just to be methodical, you should enter the numbers exactly as shown in the cursor strip.

Wait there are two more numbers you may want to check. The line starting with "cut_and_show_...." is the macro that will pull out all the APs with amplitude within the range defined by ap_peak low and high. The macro will also "cut out" traces in the muscle recordings, to isolate EPSPs evoked by AP1.

There are two numbers (1,200) at the end of the macro command. They define the traces you will use to pick out AP1. The default is 1,200 because that is the number of traces you have in your file. However, sometimes not all our traces are equally good. For example, inspection of Figure 1 may suggest that only traces 100 to 200 have stable muscle V_m. If so, the last two numbers should be 100,200. Our discussion in Fig.4 led us to the decision that trace 140 to 200 cover a window within which AP amplitudes were stable. If your data look perfect, you can set the trace range as 1,200. In this case, you will have the benefit of very large sample size.

Hight the four lines starting with peak_num and execute them.

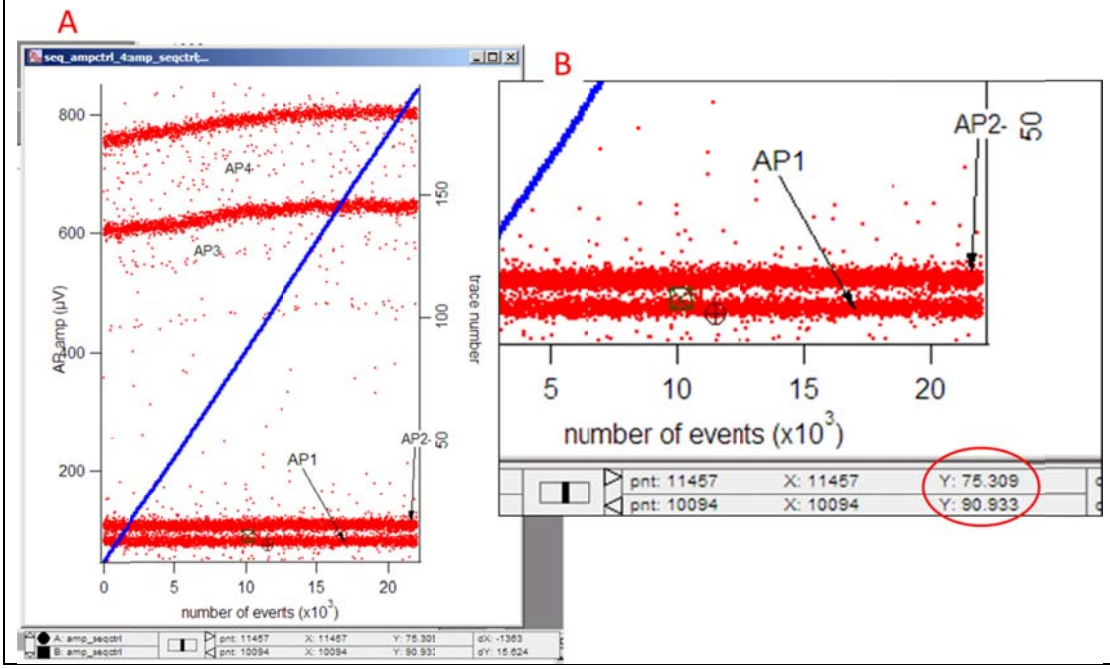
For AP2, **you should copy and paste the four lines you used for AP1** and change peak_num to 2 and reposition the cursors and update ap peak low and high for AP2.

It is **strongly recommended** that you **don't overwrite** what you have done for AP1 so you can easily check the parameters you have used for AP1 later.

Don't forget to change the peak_num for each AP, otherwise the APs and PSP selected for the last AP peak_num will be overwritten.

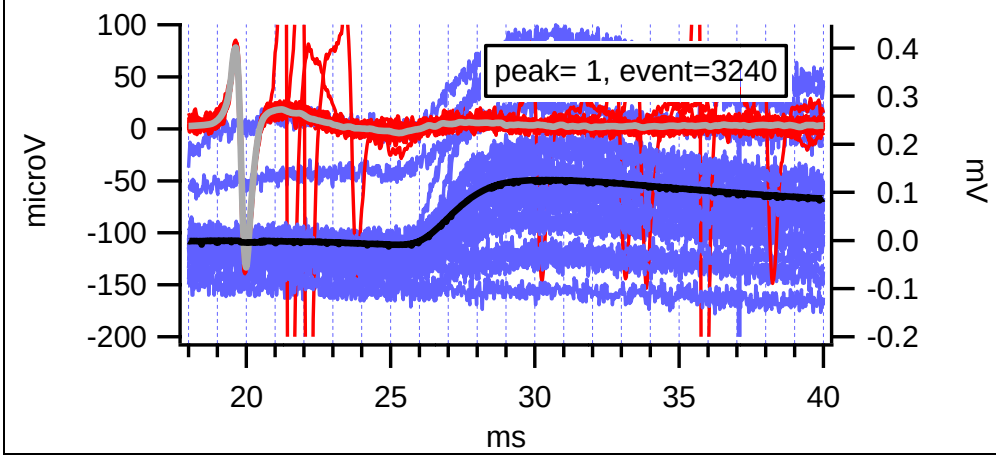
In most preparations, we analyze APs from 4 axons that fire at high frequency and give us a good sample size.

Fig.5



For each of the APs you pick, you should get a graph like this:

Fig.6



The grey trace is the averaged AP from 3240 trials, the black averaged EPSP. (The AP is aligned at the 20ms location.) Red and blue traces are randomly selected AP and EPSP traces from the 3240 events. Some of the red traces have ugly transients shooting up and down. Those are APs from other axons that happen to fire shortly after AP1 fires.

Don't be alarmed if your black and blue traces look flat. In this case, it means that the axon you selected did not make synaptic contact with the muscle fiber you have recorded from. The data will still be useful as a reference for our project.

This graph will be live while you are running the "cut_and_show_PSP..." command. It puts somewhat of a load on the computer as the graph needs to be updated from time to time but I left this feature in so you can watch the process of data selection.

Note: this macro may run for a while, **just watch it and don't click on any windows**. (Sorry about being so blunt, if you click other windows, the program will start to

append those blue and red traces in windows for which they are not intended). Watching traces appending to the graph becomes tedious after a while, you are allowed to walk away!
Further notes: You are most likely to have to run the same analysis for before and after a drug or neuromodulator. In other words, you would want to compare AP1 before and after an experimental manipulation. **To avoid confusion, you should give a different peak_num after drug/modulator.** For example, AP1 after drug should be given peak_num=11 and become AP11. Peak_num must be a numerical value.

STEP 5: Further analysis of individual APs.

Now that we have isolated individual APs, we are ready to make measurements of the following parameters: AP amplitude, AP maximum, AP minimum, AP duration, EPSP amplitude, EPSP delay. Of these parameters:
EPSP amp and AP amp will appear on the 1st graph,
EPSP amp and EPSP delay 2nd graph,
AP max and AP min 3rd graph,
AP amp and AP dur 4th graph,
EPSP amp histogram 5th.

First execute the first two lines, to define variables.

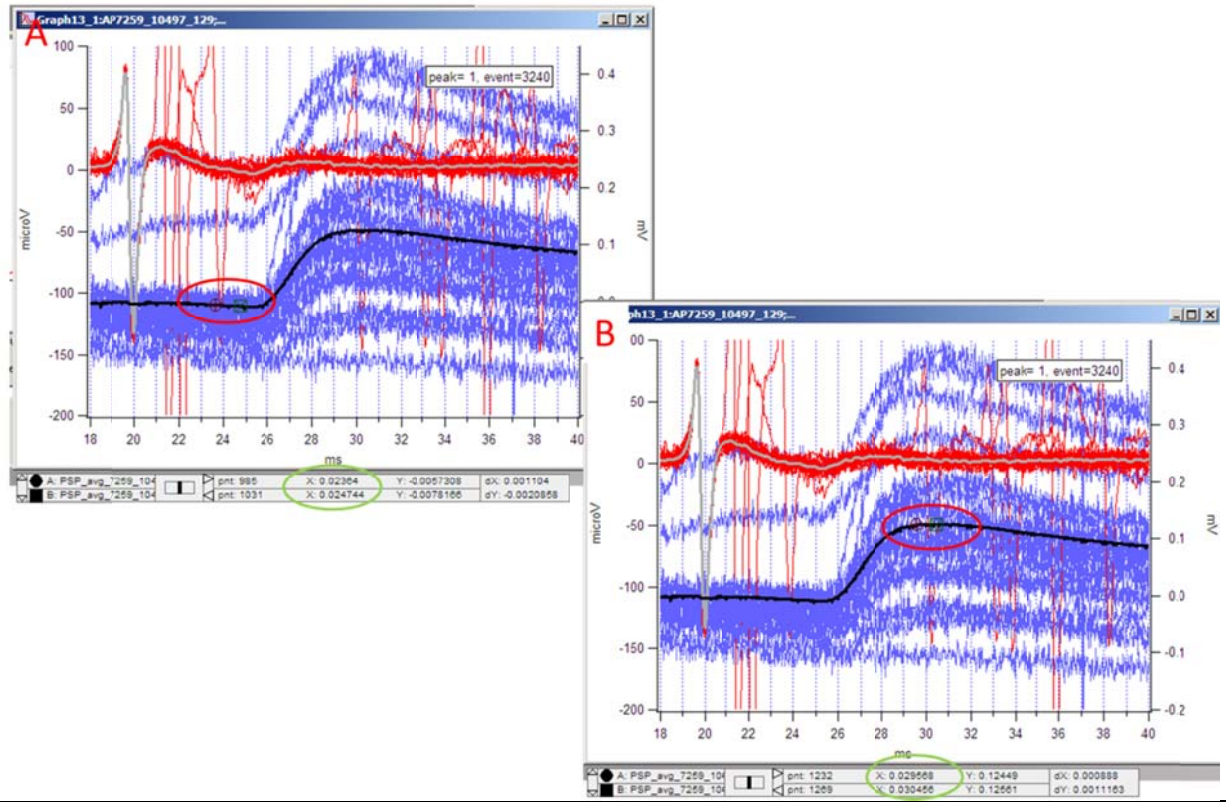
We next need to tell the macro which AP are we analyzing and how many APs have been detected as AP1. For this information, just go back to the graph and the legend box should have the info. The example in Fig.6 says this is AP1 and there are 3240 traces. Enter the corresponding number from your data in num_traces= and peak_num=. (peak_num=1 and num_traces=3240 in this case.)

We then need to tell the macro where to make measurements for EPSP amplitude. We need to define where the baseline is and where the peak of EPSP is. For the measurement of EPSPs associated with AP1, we should go back to the graph window for AP1 (Fig.6).

We define the EPSP baseline by placing the cursors in a time window after the AP and before EPSP takes off (Fig.7A red circle). We then need to read the **X-values** (Fig.7A green circle) and enter into bfrom and bto. The macro will take all the data points between bfrom and bto and average them. The average value will be the baseline. Next, you need to move the cursors to the top of EPSP (Fig.7B red circle). The cursor values on X-axis (green circle) will be entered as sfrom and sto.

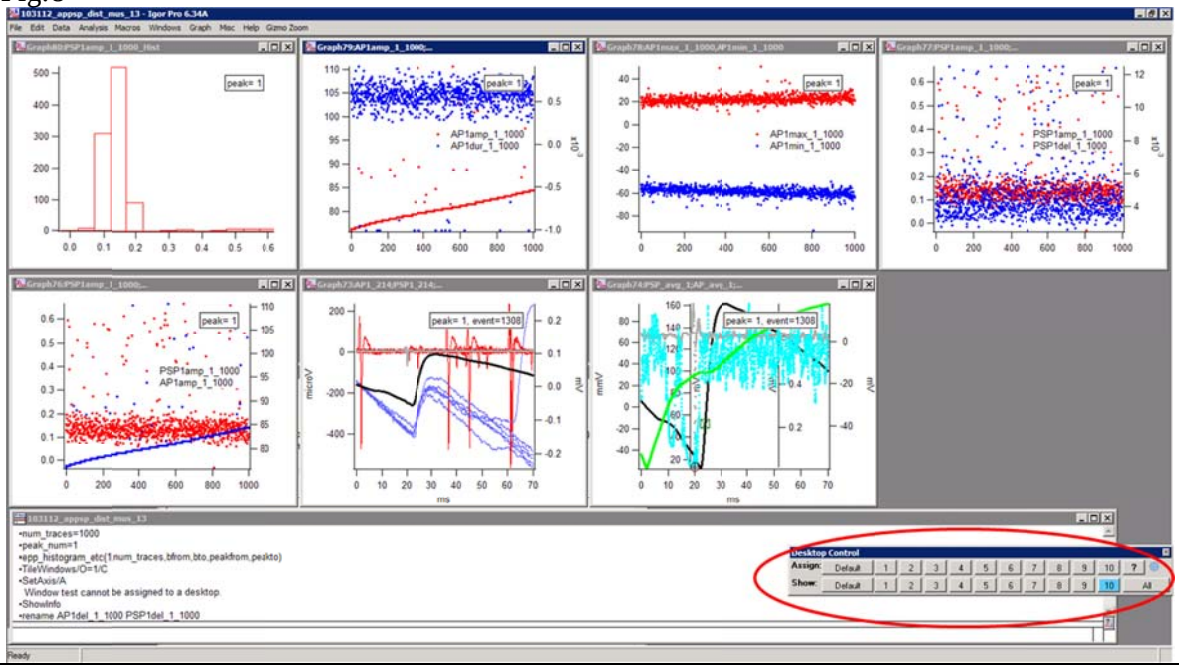
Now we are ready to execute the macro.
Highlight all 7 lines, from "num_trace= " to "epp_histogram_etc(...)". (Note that you **must execute all 7 lines, including the ones with numbers you just entered**, so the macro knows which AP/EPSP to measure etc.)

Fig.7



When the epp_histogram_etc finishes analysis, you will have a busy desktop (Fig.8). All the traces on the graph are labeled in the legend. You will need to repeat this analysis for your experimental file, after drug/modulator, and compare them.

Fig.8



*****Now is the time to stare at the data and think!**

A few tips for further analysis and comparison.

I. Save your experiment from time to time as you go through each steps of your analysis. The file get quite big, don't be alarmed.

II. To help organizing your graphs, it would be useful to put figures from each axon on a separate desktop. To do this you should download a plug in for Igor from:

http://www.igorexchange.com/project/ACL_WindowDesktops

Extract the files and put all of them in Igor Procedures folder within Igor Pro Folder.
Relaunch Igor, Misc➔Desktop Control Panel.
The control strip will show up (Fig.8 red circle).

III. You are likely to want to have results from control and experimental files of the same axon, say AP amp of AP1, to be side by side so you can compare them properly. One simple way to do this is the following:
1. click on the graph you would like to compare so it is on top.
2. graph➔packages➔save graph.
3. all the info in your graph will be saved in a text file.
4. open a new Igor experiment and drag the text file into Igor, you will see the graph. (Mac users will have to right click the text file and use "Open with" option.)

Do the same for control and drug experiments then you will have both graphs in the same Igor file and the comparison will be easier.

IV. Sometimes you would want to display two traces on the same graph to really compare them.
1. Right click on the 1st trace you would like to compare, select "Copy Display Command" from the context menu (Fig.9 arrow 1).
2. Paste the command in the command window at the bottom of the monitor (Fig.9 arrow2) then hit return.
3. A new window will appear with the trace you select.
4. go to the second graph where you want to select a trace to be displayed in the same window as the first one. Go through the same steps, right click➔copy display command➔paste in the command window.

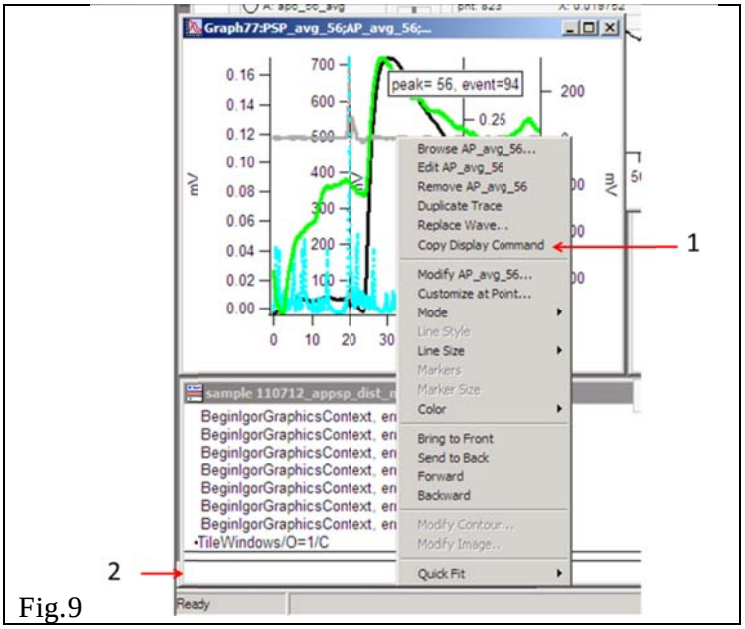


Fig.9

5. **However**, you now need to delete the "display" and replace it with "append", don't hit return yet.
6. Click on the graph window you would like the trace to append to and hit return.