

Program on Igor Pro for the Automatical Analysis of Amperometric Spikes

by

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TABLE OF CONTENTS

1. Aim and purposes of the program.
2. Computer and software requirements.
3. How to acquire and store files.
4. How program works.
5. Getting started.
6. Spike analysis.
 - 6.1. Selection of data files.
 - 6.2. Digital filters.
 - 6.3. Spike identification criteria.
 - 6.4. Start the analysis.
7. Spike view.
8. Galleries.
9. Displaying data.
10. Statistical analysis of data.
11. Troubleshooting. On-line help.

1.-Aim and purposes of the program.

This software has been created for the full-automatical analysis of secretory spikes resulting from amperometric detection. It calculates the kinetic parameters of spikes and produces histograms for their characterization.

The program permits noise analysis, digital filtering, spike identification and kinetic characterization. In addition, it allows to modify the type and levels of digital filtering and the spike identification criteria. It also provides an easy

way to correct spikes automatically identified and to pool the results from a group of experiments.

2.-Hardware and software requirements.

Our program can be used on Power Macintosh and PC-based platforms (Pentium and above). The speed of processing will logically be increased with the use of faster computers. This program only runs on Igor Pro software. You can purchase this directly from the company (<http://www.wavemetrics.com>).

The program has been created, compiled and tested using Igor Pro 3.13 for Macintosh and 3.11 for Windows 95/98. It is most probably that it will continue to work with the newer versions of Igor Pro.

Optimal RAM requirements are over 16 Mb although it probably can work with less. If you want to modify the RAM memory assigned to Igor Pro in a Mac, please look for and open the Igor Pro folder, click once on Igor Pro program icon and then selects "Information" in "File" option. There you can modify the amount of memory assigned. We recommend assigning a value of above 10 Mb.

Data acquisition can be assessed by many programs provided that they can produce files in recognized data format by Igor (i.e. Igor binary format, delimited text, general text, and so on). We currently use Labview® (National Instruments) which directly produces Igor binary files. In the case that Igor binary format is not used (i.e. general text), the files must be converted into this format (.bwav extension for Mac or .ibw extension for PC) prior to proceeding with our analysis program. Text format files must use decimal point instead of comma in real numbers.

User must be ensure that no others macros are loaded on Igor Pro when the analysis program is run, because conflicts related to object names could appear.

3.-How to acquire and store files.

Data files have to be saved unpacked, in other words, each experiment must be stored as a separated file.

For a right organization of the whole information user can make a main folder, called "Amperometry" for instance. Into this, one folder must be created for each different experimental conditions, with a proper name to identify these conditions, "SNP" for instance. Into "SNP", three subfolders will be created. The first one will be made to save all experiment files done under these experimental conditions and the control experiment files. This folder, called "Data_Files" for instance, will contain raw data files with Igor binary format (Exp01.bwav, Exp02.bwav, etc.). The second one, called "Result_Files" for instance, will be made to save the result files obtained by the program for every raw data file processed. Into this folder, the program automatically creates one subfolder for each experiment data file, in which the result files of that experiment will be saved. The third one, called "Tested_Files" for instance, will be made to save the result files tested visually by the user. Into this folder, the program automatically makes one subfolder for each tested experiment. Finally, user can make two spike galleries, one for control experiments and second for experiments made under conditions. These two galleries will be saved with Igor experiment format, producing two files that must be placed into the "Tested_Files" folder. A gallery will group all the spikes obtained under the same conditions for applying statistical analysis. It must be noted that once all experiments have been tested, the "Result_Files" folder can be erased.

The program saves a file, into the "Result_Files" and "Tested_Files" folders, called "Parameters_Exp01" for "Exp01" file, "Parameters_Exp02" for "Exp02" file, and so on, where all the parameters used for the analysis of the experiment are stored. These are the types of filters used, the spike identification criteria, and so on. Therefore you can, of anytime, know these values utilized in the processing.

4.-How program works.

Program converts raw data into files containing spike kinetic characteristics. For a description of the filter, spike identification algorithms and spike classification used please refer to:

Segura, F., Brioso, M.A., Gómez, J.F., Machado J.D. and Borges, R. (2000) Automatic Analysis for Amperometrical Recordings of Exocytosis. *Journal of Neuroscience Methods*. **103**, 151-156.

5.-Getting started.

- a) Prepare the folder structure as it has been explained above.
- b) Double click on the program and the computer will run automatically Igor Pro and load the program in memory as an Igor procedure.
- c) Close procedure window, choosing "Hide" option in the dialog box, not kill.
- d) The program offers three main options, which appear under the "Macros" option: "Spike analysis", "Spike view" and "Galleries". User must select the first one, "Spike Analysis".
- e) User will be able to resize all the windows (panels, graphs, tables, ...) to match them to the graphic resolution of the used system. This can be made resizing and moving each window to the desired position, and then closing it with the proper button (the small cross in the right top corner in PC or the small square in the left top corner in Mac). In this moment, Igor shows the "Close Window", where only "Replace" button must be pressed. After that, user has to save the modified procedure, choosing "Save Procedure" option in "File" menu of Igor Pro. From now, when the program is run again, the new size and position of each window will be used.

6.-Spike analysis.

Now you will see the main panel (Fig. 1).

You can get on-line help about the program functions by pressing "Help" option of Igor Pro. Besides that, help about all panel fields can be got having activated the "Show balloon" option and placing the mouse on the desired field in Mac platforms or pressing "F1" key after placing the mouse on the desired field in PC platforms.

This panel is divided in three main parts: selection of data files, choosing of filters and specification of spike identification criteria (Fig. 1).

6.1.-Selection of data files.

In this first part of the panel user has to fill the following fields:

DATA FOLDER: type the full path of the folder where raw data files are stored. The "<>" button can be used to select the folder with the mouse.

RESULT FOLDER: type the full path of the folder where the result files obtained by the program will be saved. The "<>" button can be used to select the folder with the mouse.

Selecting data and parameters...

DATA FOLDER:
iMac 14:Amperometry:SNP:Data_Files: <>

RESULT FOLDER: **Several folders**
iMac 14:Amperometry:SNP:Result_Files: <>

Comment: SNP

DATA FILE NAME: All files ▼

Scale (pAM): 100

Buttons: Add Kill Run

FILTERS: Test on file Id# 0

FIR1: 500-850 Hz

FIR2: 350-600 Hz

Smooth 15

Others Custom Smooth

Coefs: iMac 14:Filters:C800_1000Hz.bwa

DEFINING NOISE:

Smth. 1stDer: 35

1D.Threshold: 4

Test on file Id# 0

Figure 1. The main panel used in the first part of the program, "Spike analysis". It is divided in three parts (see text).

Several folders: When this check field is clicked, the result files of each data file will be saved in a different subfolder. These subfolders will be automatically created into the "Result Folder" typed previously. The subfolder names are created with the "Comment" field value (see below) plus the data file name. When the check field is not clicked, the result files of all processed experiments will be saved in the "Result Folder". The result files of each experiment are recognized because the name of each result file is created with the name of the experiment (data file name) plus the name of the result file. For instance, the result file called "X_Peak_Exp05" stores the peak times of the experiment called Exp05.

Comment: type a comment which is used to make the subfolder name (see above, "Several folders") where result files are saved. It helps to identify the experiments, i.e. SNP, Control, Histamine, etc.

Id#	Folder	Name	Comment
0	iMac 14:Amperom	Exp01.bwav	SNP
1	iMac 14:Amperom	Exp02.bwav	SNP
2	iMac 14:Amperom	Exp03.bwav	SNP
3	iMac 14:Amperom	Exp04.bwav	SNP
4	iMac 14:Amperom	Exp05.bwav	SNP
5	iMac 14:Amperom	Exp06.bwav	SNP
6	iMac 14:Amperom	Exp07.bwav	SNP
7	iMac 14:Amperom	Exp08.bwav	SNP
8	iMac 14:Amperom	Exp09.bwav	SNP
9	iMac 14:Amperom	Exp10.bwav	SNP
10			

Figure 2. **The table with the list of files to be processed.** The program displays this table on the right of the main panel (Fig. 1). In this case, it is shown an example with 4 files selected (see text).

DATA FILE NAME: choose the raw data file and then press "Add" button. The file will be added to the list of experiments to be processed when "Run" button is pressed. The raw data file must be located in "Data Folder" typed previously. User can select "All files" option and then press "Add" button to choose all the files located in "Data Folder".

Add: press this button once for each data file. This will be added to a list of files which are to be processed. They are shown in an Igor table which is displayed by the program just on the right of the previous panel (Fig. 2).

Kill: press this button to remove from the list of files the data file selected in "DATA FILE NAME" field. In "All files" option is selected in "DATA FILE NAME", all files will be erased from the list.

Scale (pA/V): type here the conversion scale from volts to picoamperes. The value typed in this field depends on the gain applied in the acquisition process of the amperometric signal.

6.2.-Digital filters.

The second part of the panel, called "FILTERS:" (Fig. 1), is used to choose the digital filters to be applied to all data files when these are processed. Digital filtering is usually required for spike analysis. You will be able to choose the type of filter or a combination of them. This operation is carried out before the program starts to find spikes. User can choose the following filters:

FIR1: click in this check to activate a low-pass FIR filter, with a cut frequency of 500 Hz and a stop frequency of 850 Hz.

FIR2: click in this check to activate another low-pass FIR filter, with a cut frequency of 350 Hz and a stop frequency of 600 Hz.

Smooth: click in this check to activate a binomial smooth filter, included in Igor Pro program. This is another low-pass filter. It is required the smoothing factor to use with this filter. Please refer to the Igor Pro manual for a description of the smoothing filtering properties.

Others/Custom Smooth: click in this check to activate an Igor Pro custom smooth, a particular FIR filter. In "**Coefs:**" field user has to introduce the path and name of the file containing this filter coefficients.

Test on file Id#: click in this button to test the results of the selected filters applied on the data file typed in "**Id#**" field. Program will filter the data file and display a graph including both the original and the filtered signal (Fig. 3). It will be filtered the data file whose value of the Id# column of the table containing the file list to be processed is equal than the value of Id# field typed here. User will be able to test if the selected filters are proper before starting the analysis.

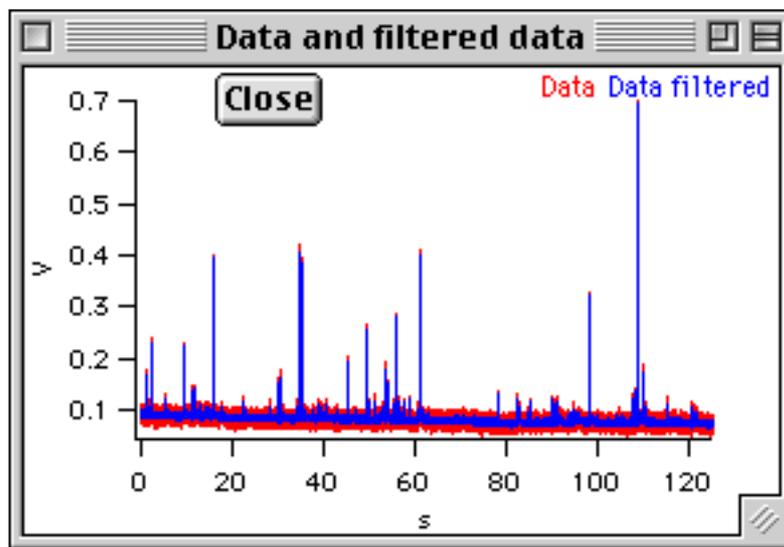


Figure 3. **User can test the filtered data using this graph.** It is shown pressing the "Test on file #id" button in the second part of the main panel (see text).

The two files containing the coefficients of the first two filters, FIR1 y FIR2, must be located in the folder where Igor Pro program is installed (usually Igor Pro Folder, the default folder of Igor installation process). These two files can be downloaded from our web page:

<http://webpages.ull.es/users/rborges>

6.3.-Spike identification criteria.

The third part of the panel, called "DEFINING NOISE:" (Fig. 1), is used to choose the spike identification criteria. The following fields are required:

Smth.1stDer: type here the factor of a binomial smooth filter which is applied to the first derivative of the signal. When the signal is differentiated, the width of the basal noise increases. This filter reduces that noise and helps for the localization of spikes, which begins finding maxima in the first derivative signal. This filter does not modify the kinetic characteristics of spikes, because it is only used to find maxima of spikes.

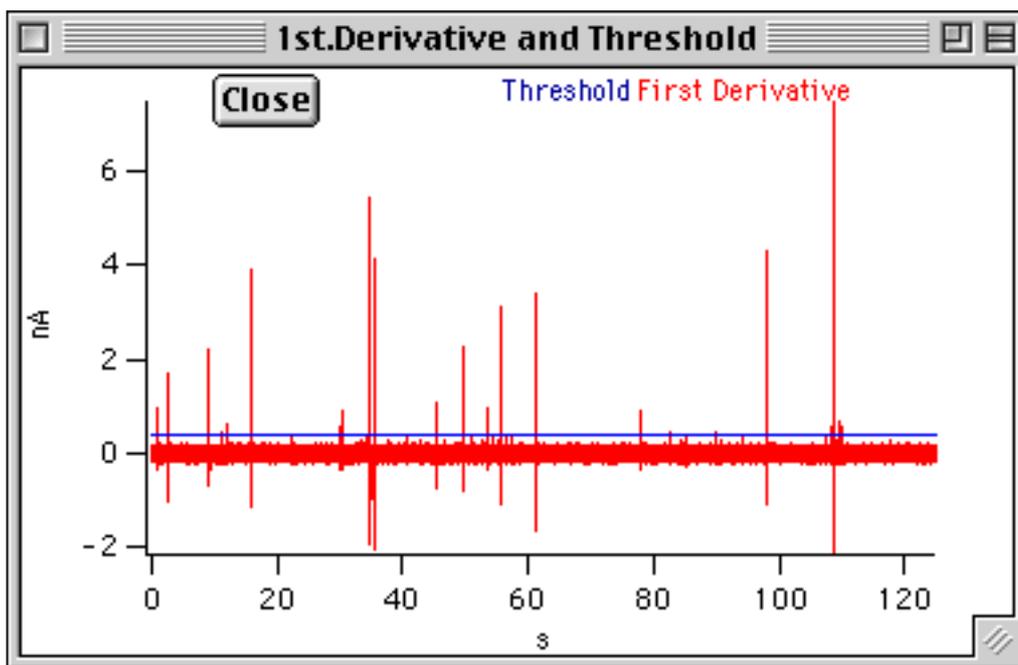


Figure 4. **Graph used to test the defined threshold to find spikes.** This graph is shown when the "Test on file #Id" button in the third part of the main panel is pressed (see text).

1D.Threshold: type here a value that will be multiplied by the noise standard deviation of the first derivative signal, which has been calculated by program. This multiplication gives a threshold. A period of first derivative above this threshold means the presence of a spike. A bigger value of this field will result in a fewer amount of spikes found, but these located spikes will be higher or faster.

Test on file Id#: click in this button to test, before starting the analysis, the result threshold calculated with the value typed in the previous field "1D.Threshold". It will be tested on the data file typed in "Id#" field (see the

previous "Test of file Id#" field for more explanation). Program will display a graph including both the first derivative signal and the threshold (Fig. 4). User will be able to test if the defined threshold is right before starting the analysis. The first derivative signal in this graph is calculated after applying to signal all the digital filters selected in the second part of the panel and the previous smooth filter (Smth.1stDer) to the first derivative signal.

6.4.-Start the analysis.

Once all previously mentioned fields have been filled or selected, user can start the analysis of the whole list of data files by pressing "**Run**" button. The status of the analysis is shown in the "Processed files..." window, displayed by the program (Fig. 5).

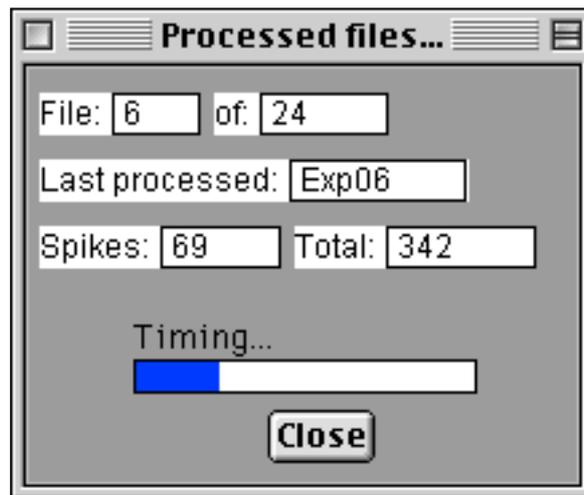


Figure 5. **Window informing about the status of the analysis process** (see text).

Each raw data file (experiment) produces 12 result files, which are saved into the "RESULT FOLDER" typed (see above). The result files of each experiment can be stored in a different subfolder, activating the "Several folders" check field (see above). The subfolder name is created with the "Comment" typed (see above) plus the experiment name (raw data file name), for instance "SNP_Exp12". The names of the result files are made with two parts: the first one indicates what it contains; and the second one is the

experiment name to facilitate its identification. For instance, the experiment called "Exp19" produces the following 12 result files:

- **Data_Exp19**: the processed raw data file with the selected filters.
- **Parameters_Exp19**: the values of the panel fields used by the automatic analysis.
- **Noise_Data_Exp19**: the noise data of this experiment after the filtering.
- **X_Beginning_Exp19**, **X_Final_Exp19**, **X_Peak_Exp19**: the initial, final and peak times respectively of all the spikes found in this experiment.
- **pA_Exp19**, **T_Half_Exp19**, **pC_Exp19**, **pC_Third_Exp19**, **M_Half_Exp19**, **T_Peak_Exp19**: the kinetic spike parameters I_{max} , $t_{1/2}$, charge (Q), $Q^{1/3}$, slope in the rise and time to peak respectively.

7.-Spike view

The researcher can check the spikes found by the previous automatic analysis and manual corrections can be introduced along this second part of the program, which appears in the "Macros" menu labeled "Spike view".

The first panel displayed asks for the folder where the experiment has been saved and the experiment name (data file name, without the extension) (Fig. 6). This panel contains the following fields:

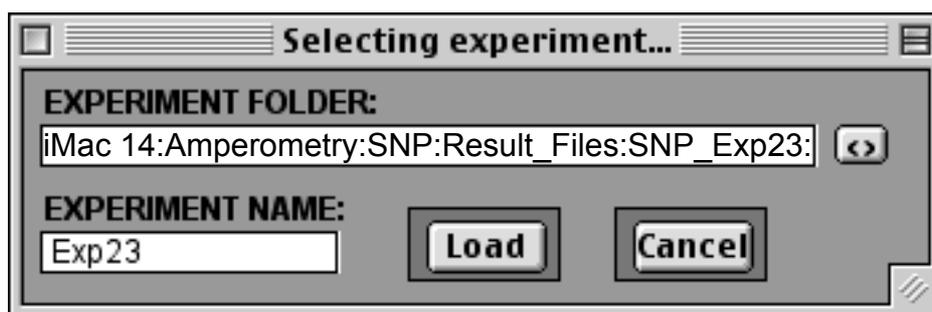


Figure 6. **The initial panel of the second part of the program.** This panel is used to select the experiment to be checked (see text).

EXPERIMENT FOLDER: Type in this field the full path of the folder where experiment result files were saved. This folder should now contain the 12 result files. The "<>" button can be used to select the folder with the mouse.

EXPERIMENT NAME: Type in this field the name of the experiment or the original raw data file previous to the analysis, without extension, where raw data was saved.

Load: With this button, all result files from the folder and experiment typed are loaded in memory and four new windows will appear for the visual revision and manual corrections of spikes automatically found.

One of these four windows is the "Analysis Panel" (Fig. 7). This new panel includes the following fields:

EXPERIMENT NAME: In this box the current experiment name is displayed.

Spikes: Number of spikes found within this experiment.

Noise data: Click in this check in order to show/hide alternatively the information about the segment of noise located previously in the spike analysis.

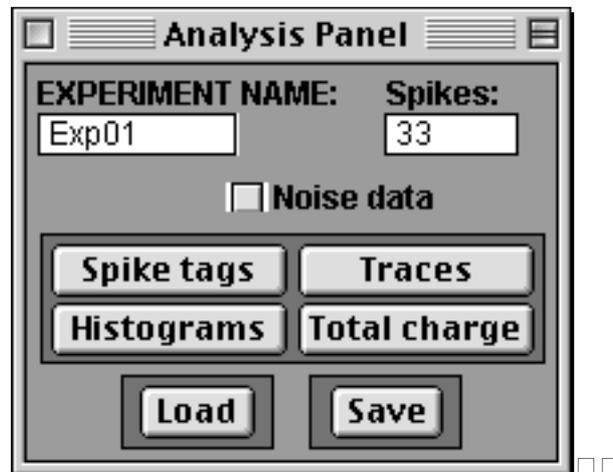


Figure 7. The "Analysis panel" of the second part of the program (see text).

Spike tags: Click in this button for show/hide tags alternatively to the spikes in the graph on the right (see below) of this panel.

Traces: Click in this button to display graphs, because they could have been closed with Igor Pro options.

Histograms: Click in this button to see histograms obtained from spike kinetic parameters.

Total charge: Click in this button to see the cumulative charge either considering only the identified spikes or considering the whole signal.

Load: Load a new experiment for its revision.

Save: Save corrected results. You can save them in the same folder where result files were stored, overwriting them, or in a different folder, where the 12 corrected result files would be stored.

Another graph shown in this second part of the program is used to display the spikes one by one for reviewing its beginning and final points and correcting them if it is necessary (Fig. 8). Each spike is showed with its beginning, peak and final points tagged, as well as its kinetic parameters quantified. You can use the following fields in this graph:

Dots/Line: Change the graph to dots or line between dots mode.

Add: Add a new spike. The A and B cursors have to be placed on the beginning and final points of the new spike to be added, then press "Add" button.

Modify: In the case that the beginning or the end of the displayed spike are located in a wrong time, they can be modified by dragging the cursor(s) to the desired point(s) before pressing Modify. All kinetic parameters are automatically recalculated.

Erase: Erase the shown spike.

Param.: Draw 4 lines on the shown spike corresponding to the I_{max} , $t_{1/2}$, time to peak and slope in the rise parameters.

<< Show the previous spike.

>> Show the next spike.

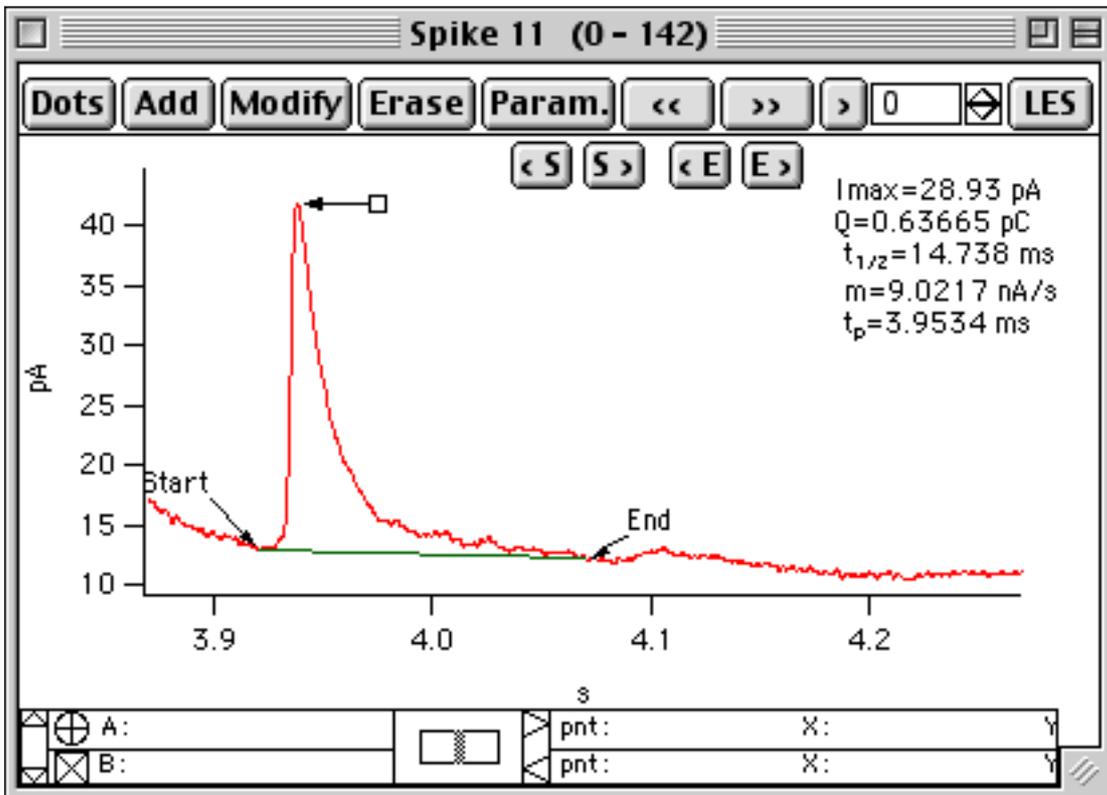


Figure 8. **This graph is used to review the spikes.** Manual corrections can be applied (see text).

> Show the spike whose order number has been typed in the field on the right before pressing this button ">".

LES: Restore the last erased spike.

The following four buttons are used to place the spike beginning or final with a more precise way:

<S The spike beginning is moved backwards five points.

S> The spike beginning is moved forwards five points.

<E The spike final is moved backwards twenty points.

E> The spike final is moved forwards twenty points.

The 3.2 version of the program allows to modify the beginning and final spike by clicking on the spike, just on the point where you want to locate the new beginning or final. This new method is more faster but you need Igor Pro 3.13 or newer.

The third window used in the spike revision is shown on the right of the previous one and displays the same spike in a different scale (Fig. 9). It helps for a better identification of the beginning and final spike.

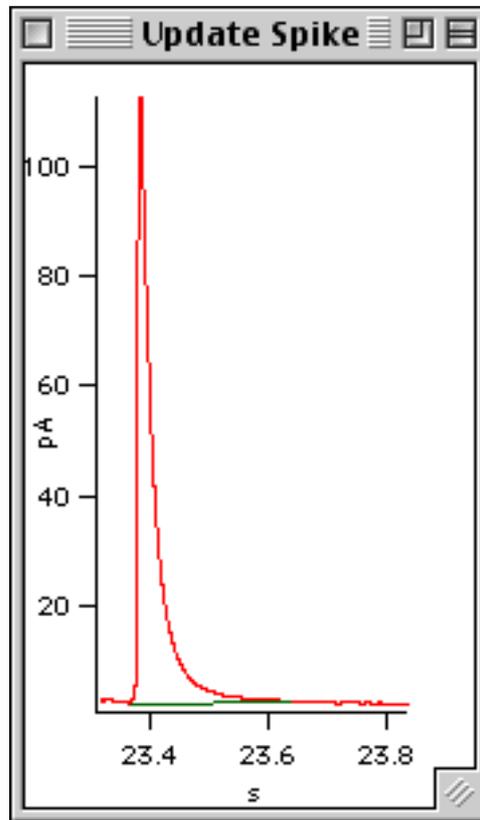


Figure 9. **The spike displayed in a different scale.** This example shows the same spike included in the figure 8 but in a different scale (see text).

The last window displayed in this part of the program shows the whole record or the part amplified by user (Fig. 10). There are two buttons:

Add: add a new spike. The A and B cursors have to be placed on the beginning and final points of the new spike to be added, then press "Add" button.

A-Kill-B: delete a group of undesired spikes after placing A and B cursors. It is used when a lot of spikes were found due to high oscillations of noise.

When all the spikes have been reviewed, you can save the corrected results pressing "Save" button of the "Analysis panel" (see above) and then

typing the full path of the folder. This can be the same folder where the initial result files were stored by the initial automatic analysis, overwriting them, or a different one, for instance "Tested_Files", which will be created automatically in the case that it does not exist. The programs saves 12 result files (see above).

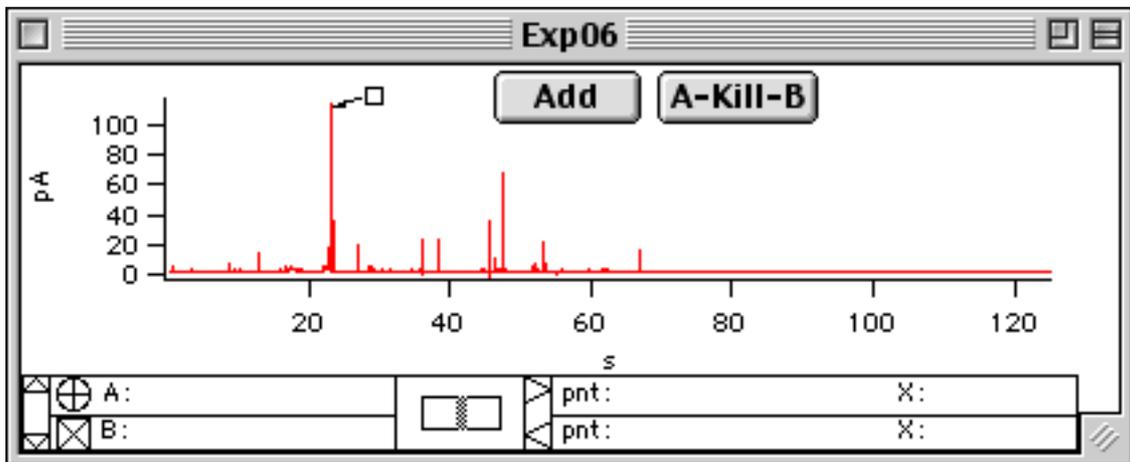


Figure 10. **The whole record is too displayed in this part of the program.** The spike shown in the two previous graphs is tagged in this one (see text).

8.-Galleries.

This third and last part of the program has been created to pool spikes from different experiments obtained under the similar conditions. This allows to get statistical information from a significant number of spikes. This phase can be run clicking the "Galleries" option in the "Macros" menu.

The first shown panel is used to create a new gallery of spikes, where user has to type the folder where corrected result files were saved and the experiment name (data file, without extension) (Fig. 11).

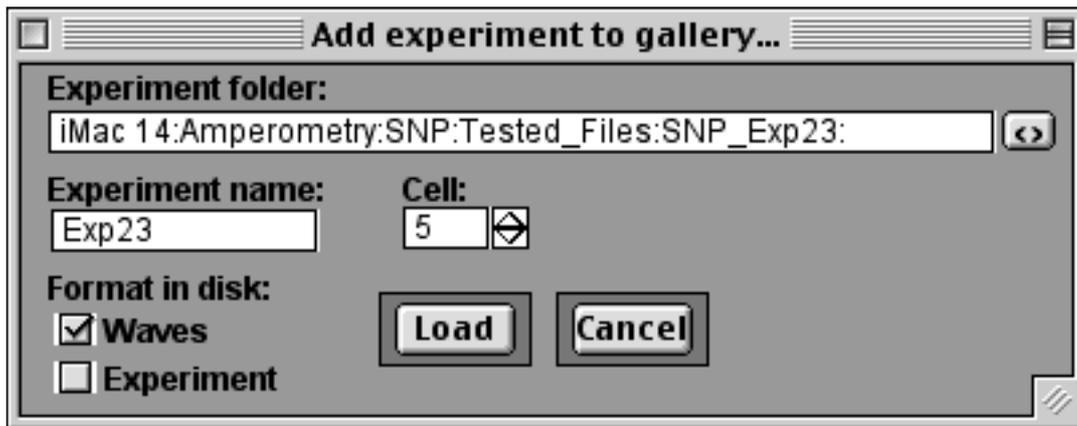


Figure 11. Panel used to add the spikes of an experiment to the gallery. This panel is displayed to create a new gallery or to add a new experiment to an already created gallery (see text).

The corrected result files could have been saved as "Igor experiment" format, using the "Save experiment" option from "File" menu of Igor Pro, instead of the "Save" button of our program, explained in the previous part, "Spike view". The "Igor experiment" format is a file format where all data in memory are saved as a single file. If this option has been used, you must click in the "**Experiment**" check field of the panel. In other case, user must click in the "**Waves**" check field. In "**Cell**" field user can introduce the cell number generating each experiment. This can be used in the final statistical analysis. When "Load" button is pressed, a new gallery has been created and opened, containing initially the spikes from the typed experiment. At this moment a second panel is displayed. This is called the "Gallery" panel (Fig. 12). It includes the following buttons:

Add: Add the spikes from another experiment to the opened gallery. It is shown the same panel explained previously to create a new gallery (Fig. 11). This button deactivates the "**Cell**" check (see below), so the new added spikes will be considered when this check is activated again.

Table:

Show: Show a table with the kinetic parameters from all spikes included in the gallery until that moment.

Close: Close the previous gallery table.

Histograms:

Show: Make and display histograms from pooled spikes.

Close: Close histogram windows.

Layout:

Show: Make histograms from pooled spikes and put them in a single sheet with Igor Pro "Layout" format, facilitating their printing. You can modify this sheet and introduce decorations and legends before printing.

Close: Close layout sheet.

Pict: Save in hard disk the previous layout as a "PICT" format image.



Figure 12. The "Gallery" panel is used in the creation of a gallery of spikes (see text).

Spikes:

Show: It displays a window containing the spike number 0 of the gallery. You can use several buttons to pass and review the gallery spikes.

Close: Close the previous window.

Close All: Close all windows from the opened gallery, except the panel containing this button, the "Gallery" panel.

Start: Create a new gallery. A warning window is shown informing that if you continue by making a new gallery without saving the opened gallery, this will be erased. The "No" button in the window warning can be pressed and then save the opened gallery using the "Save experiment" option from the "File" menu of Igor Pro. After that, you can press the "Start" button again and then choose "Yes" option. The initial panel of this third part of the program is displayed (Fig. 11).

The gallery created in this way will contain a number of spikes proceeding from several cells. If user wants each cell has the same contribution to the final statistical analysis, the " **Cell**" check can be used. When this check is activated, a new panel is shown (Fig. 13).

This panel offers two possibilities:

All Spk/Cell: This option can be used to calculate and display mean, median and standard deviation by cell of every kinetic parameter, including all spikes of each cell. These three values are calculated of every kinetic parameters when "Run" button is pressed. In this way, the cell, instead of the spike, is considered as n in the statistical analysis. All tables shown are easily exportable ("Copy" and "Paste") to statistical programs. These tables can be closed clicking in "Random Spike" option or deactivating the "Cell" check.

Random Spike: This option can be used to create a subgallery of spikes, which includes a number of spikes, randomly selected, of every cell. This number must be typed in "N.Random Spk:" field and then press "Run" button to create the subgallery. When a subgallery has been created, the options (Tables, Histograms, Layout, Spikes) of "Gallery" panel (Fig. 12) are applied to this subgallery. Mean, median and standard deviation by cell of every parameter are calculated, including the spikes of the subgallery, and displayed in tables. These tables can be closed clicking in "All Spk/Cell" option or deactivating the "Cell" check.

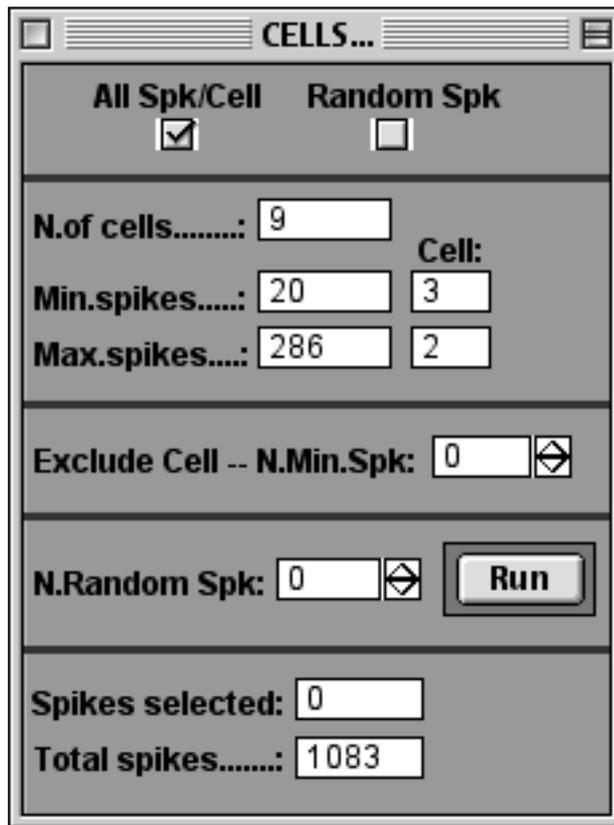


Figure 13. The "CELLS" panel is used to make statistical analysis considering the cell as n (see text).

In both cases, user will be able to exclude cells with a low number of spikes, typing the desired value in "N.Min.Spik:". All cells whose number of spikes is smaller than the typed value are excluded.

It is possible to return to the initial situation desactivating the "Cell" check again, and all panels and tables will be closed. In this situation, the gallery includes all spikes of all experiments added.

9.-Displaying results.

The program allows to present results as histograms, tables, etc. This is very convenient as a working page resulting from a working day or for the internal use within your lab. These options are offered in the Gallery window. However, Igor Pro is not intended as a graphical software. Using the "Copy" and "Paste" options you will be able to export graphs to another graphical or programs (Canvas, Adobe Photoshop, Adobe Illustrator, Power Point, etc).

10.-Statistical analysis of data.

Igor Pro allows the calculation of some descriptive statistic parameters with the "Wave stats..." option into the "Analysis" menu. However, most statistic programs accept Igor Pro tables. We use the "Copy" and "Paste" options. Be sure that commas and periods are appropriately used.

11.-Troubleshooting. On-line help.

As it was mentioned, you can use the on-line help (Show help balloons in Mac or <F1> key in PC, after placing mouse on the desired field). For Igor Pro specific problems, please refer to the Igor Pro manual.

Some old versions of IGOR could have problems to calculate the FFT provided they required a power of 2 number of points, please referred to the IGOR manual.

Our program can enter in conflict with other Igor Pro macros loaded in memory. By that reason, we recommend to close all macros opened, with "Kill" option, before starting the running of our analysis program. You can use the "New experiment" option into the "File" menu to start with the program being sure that no macro is loaded.

Mistakes typing the full path of a file are very common. Please use the ":" character between folders. In case that the problem persists it could occur when files are stored in a different disk (Zip, magneto-optical units, etc). Try to save them directly in your hard disk.

If you have all data saved as an "Igor experiment" format in a single file, you must unpack this experiment file prior to use the analysis program.

Please try to fix the problem by yourself. You can, however, contact us (<http://webpages.ull.es/users/rborges>).