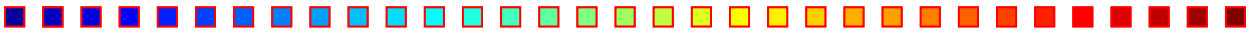
ScatterSlice v 1.0 Tutorial: IL-2 and CD25



Revised: February 2013

Introduction

This tutorial is intended as an example of practical usage of ScatterSlice, using the demo data files provided. Before following through this demo, please see the User Guide for information on installation of the package. The User Guide contains many of the same figures, but is intended to provide a complete description of the program's functions. This tutorial contains specific information regarding the "Demo Data" files provided with the program.

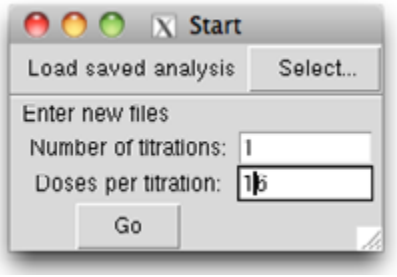
Experimental Background

In the experiment analyzed in this tutorial, cells were treated with a dose-response titration of IL-2 for 10 minutes, prior to fixation in paraformaldehyde, permeabilization in methanol and processing for staining and flow cytometric analysis. The cells were stained with antibodies to a chain of the IL-2 receptor (CD25 or IL-2Rα) a second marker (research is pending, so it will remain nameless), and an antibody to phospho-STAT5 (pSTAT5), the major downstream mediator of IL-2 signaling. The purpose of this experiment is to quantify the effect of CD25 on IL-2 sensitivity, measured through EC_{50} and amplitude. This experiment is similar to those performed in Cotari & Voisinne et al. Science Signaling 2013.

Running ScatterSlice

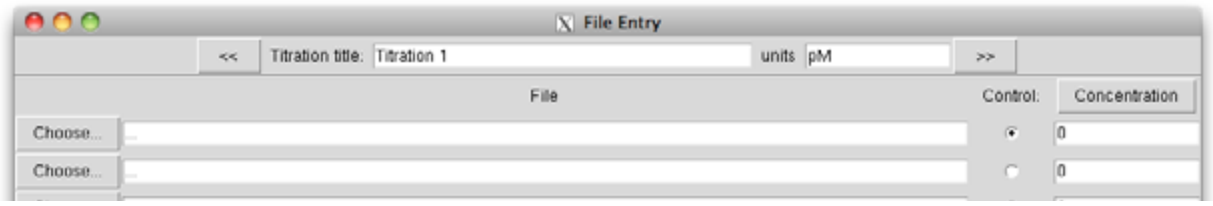
Start

In this example, we have one titration of IL-2 in 16 doses. In the "Start" window, enter 1 and 16, respectively. Then click "Go."

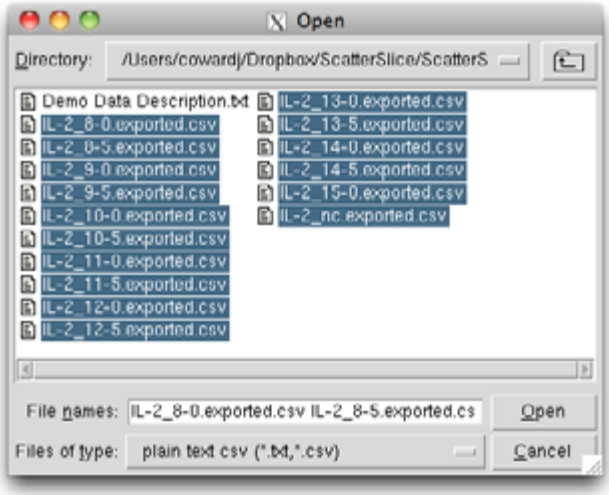


File Entry

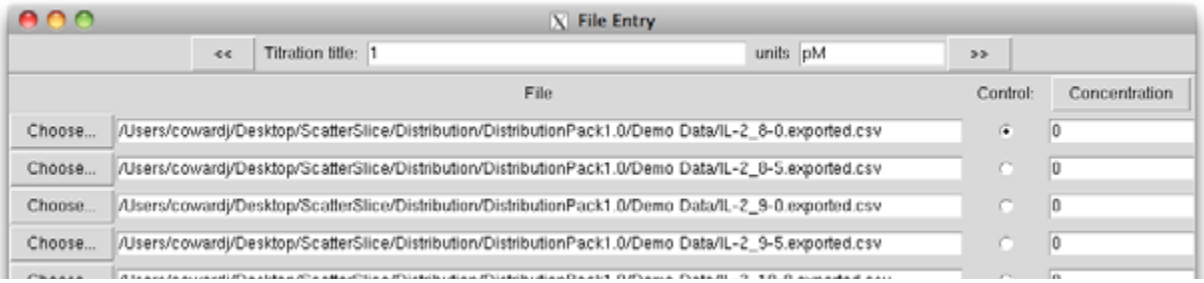
The next window is where you'll choose the files, name the titration and set the doses.



1. Click the "Choose..." button on the top row. To fill in multiple files at once, navigate to the ScatterSlice Demo Data folder, select the top dose (IL-2_8-0), then hold shift and click the bottom dose (IL-2_nc). These denote 10^{-8} M IL-2 and "no cytokine," respectively. Naming the 0 dose as "IL-2_nc" keeps the files in alphabetical order, making multi-file import much easier.



After selecting files, they will populate the window as shown below.



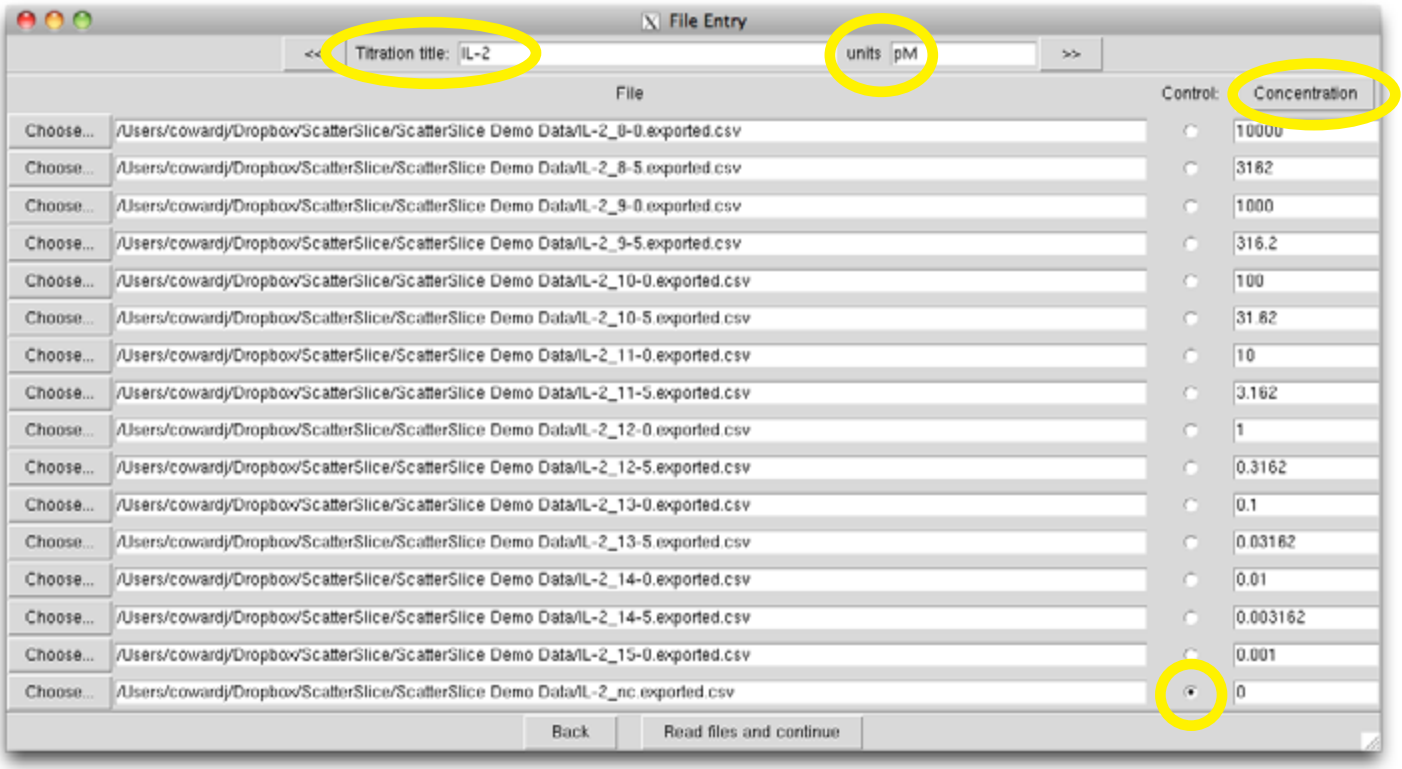
2. Next, fill in the important information. If we had multiple titrations, we would use the "<<" and ">>" buttons to move between titration sets.

Titration Title: "IL-2". This will be used to title graphs and identify exports.

Units: "pM." This results in a midpoint around 1, which is best for fitting and display.

Control: Select the "nc" file.

Concentration: Click the "Concentration" button to automatically populate concentrations (see next page).



Concentration

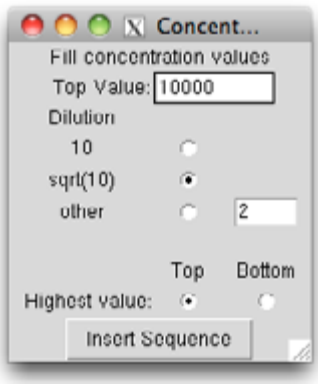
This dialog box allows you to specify the doses that will be used across all of your titrations. It is possible to fill all the titrations first, then subsequently change values for individual groups.

Top Value "10000" (10^{-8} M in pM)

Dilution sqrt(10). This titration was made in half-log increments, i.e. a serial dilution by 3.16.

Highest value "Top". The highest value is at the top of the file choice window.

Insert Sequence Clicking this button will fill the values specified, as shown at right.



After filling in all important information, use the ***Read Files and Continue*** button to read your data files into memory.

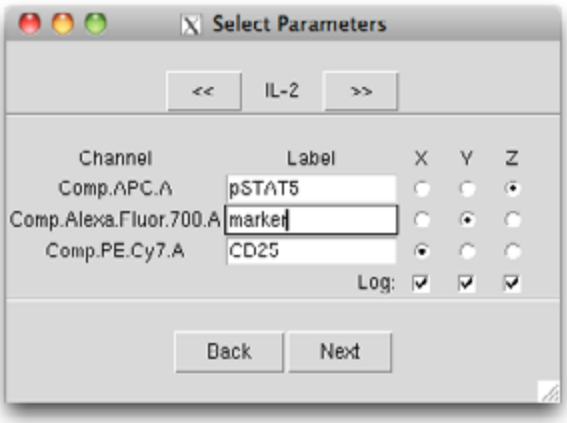
Note: During processing, there will not be an hourglass or pinwheel indicating that the computer is working. Instead, the last button you clicked (in this case Next) will remain depressed. Depending on the size of your files and the speed of your computer, this may take a few seconds. To decrease the reading time, export from FlowJo only the columns you will use in your analysis.

Select Parameters

1. Labeling

The ***Channel*** column is the header of each column in the raw data, as exported by FlowJo.

Modify the ***Label*** column to reflect the values shown at right.



2. Analysis Setup

X, Y, Z: Select X, Y and Z as shown. pSTAT5 is our output; that will change with dose. CD25 (IL-2Ra) is on the X axis, an anonymous marker occupies the Y axis.

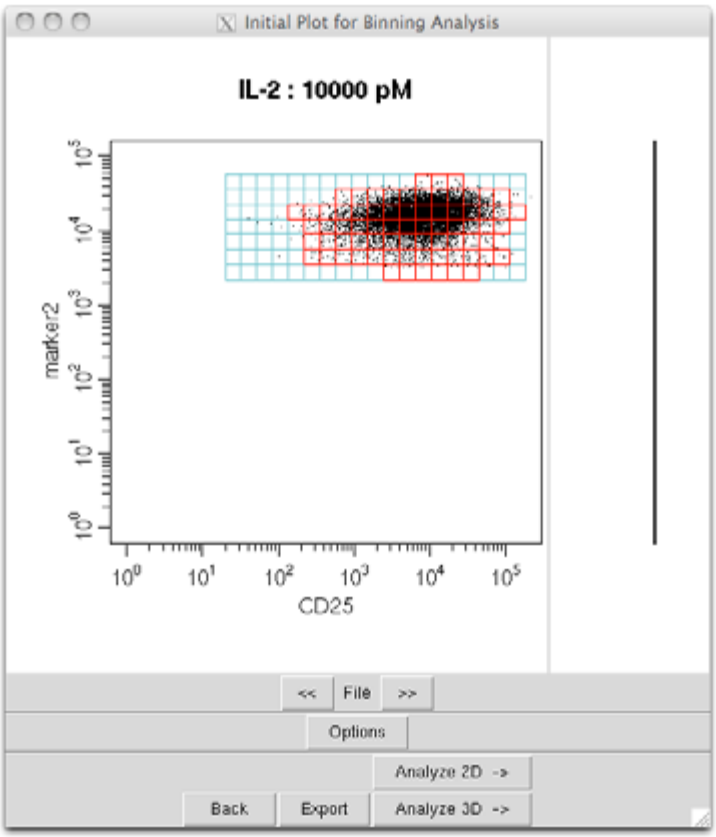
Log: All of our parameters will change in log scale, so leave the "Log" boxes checked. It is rare that these will need to be changed; DAPI staining for DNA, or FSC for size are the most common uses of linear scale.

Next: Performs binning and moves to initial plotting of data. This can be a memory-intensive process, especially for large files. Be prepared to wait one second for every 10^5 total cells to be binned (on a computer with 2 GB RAM). Freeing memory by closing other programs will speed the process.

Initial Plot

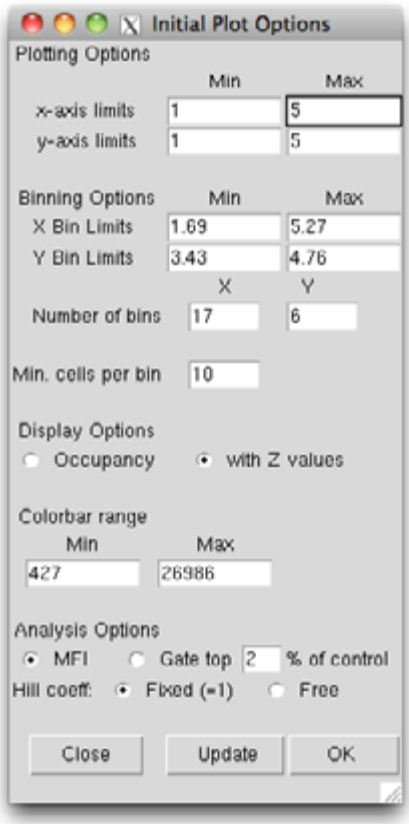
This window allows you to set up binning and display parameters and to inspect the phospho-responses before fitting. Shown at right is the default binning and display.

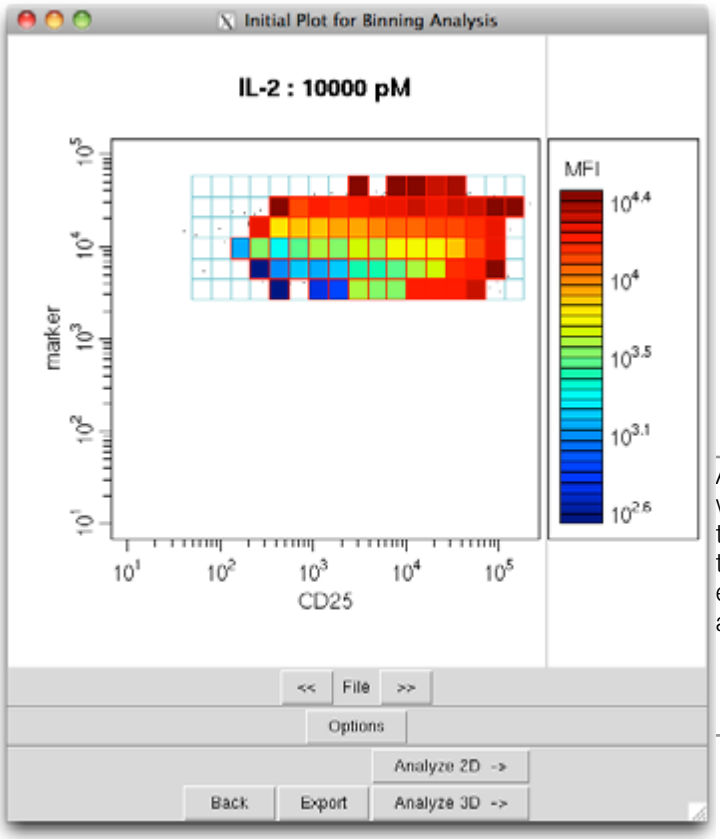
In this case, the window is too large for the data, so we're going to modify the ***Options***. (See next page)



Initial Plot Options - specify binning and analysis parameters.

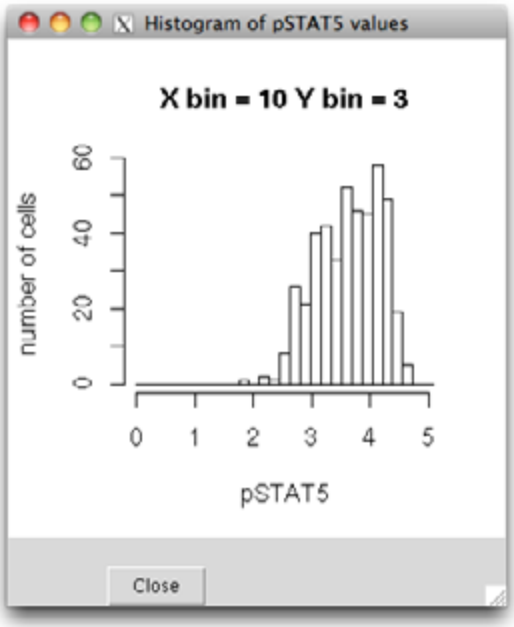
We'll leave the binning options alone, but change the plotting options to shrink the window. We'll also select "with Z values" so that we can see the pSTAT5 levels in each of the bins.





After setting the options above, the Initial Plot window looks like this. If you're interested in the plot for each dose in your titration use the ***Export*** button. This opens up a dialog to export a PDF showing binning and Z levels across all concentrations.

Clicking on a bin will pop up a histogram of the Z parameter in a new window. The title indicates which bin is represented, counting from the lower left.



After examining the data files, click ***Analyze 3D*** to fit curves within each bin. The InitialPlot window will remain open so that you can use the ***Analyze 2D*** function later.

3D Analysis

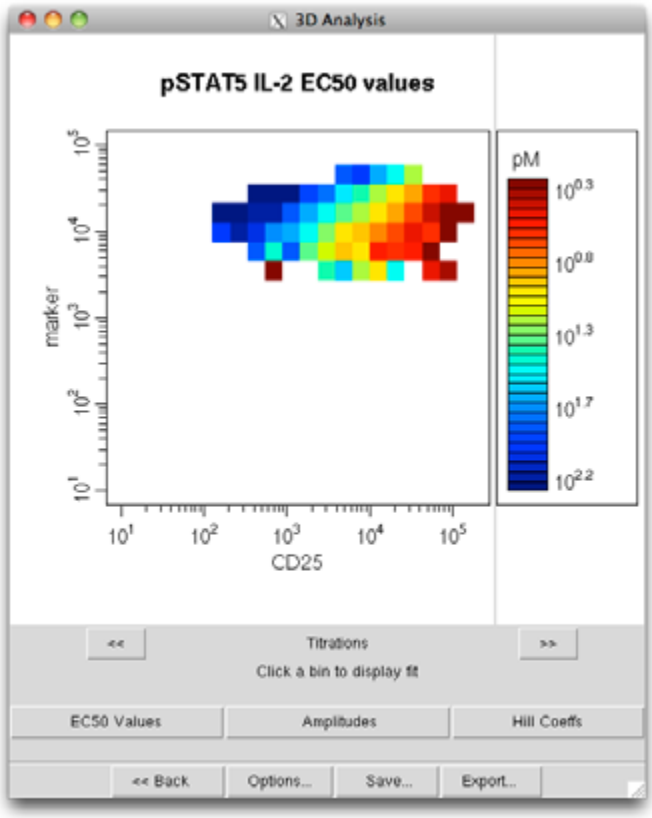
After clicking 3D analysis (and potentially waiting patiently for a minute or two, if you have many cells) displays the first output: a graph of ***EC50 values***. Select ***Amplitudes*** or ***Hill Coeffs***: to display the respective graphs.

Color Bar: The color bar shows that this plot includes values from $10^{0.3}$–$10^{2.2}$ pM, (2–160 pM). The lower values are shown in red, indicating higher sensitivity.

Save: Stores all data and settings. This will allow you to return to exactly this point by reloading this analysis in the first window.

Export: Opens a dialog to export fit values as text files and images as PDF files.

Options: Click on options to open the window below.



Clicking within any bin opens a plot of the concentration and Z values used for fitting, overlaid with the fit curve.

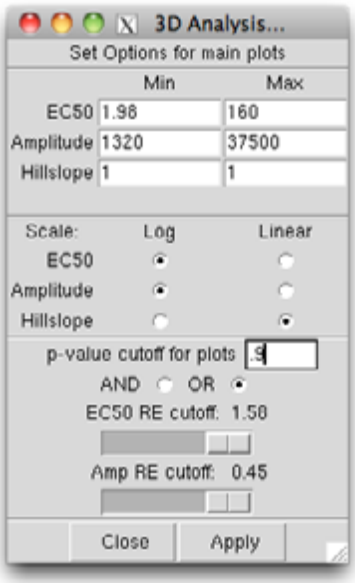
3D Analysis Options - Settings to adjust how fits are displayed

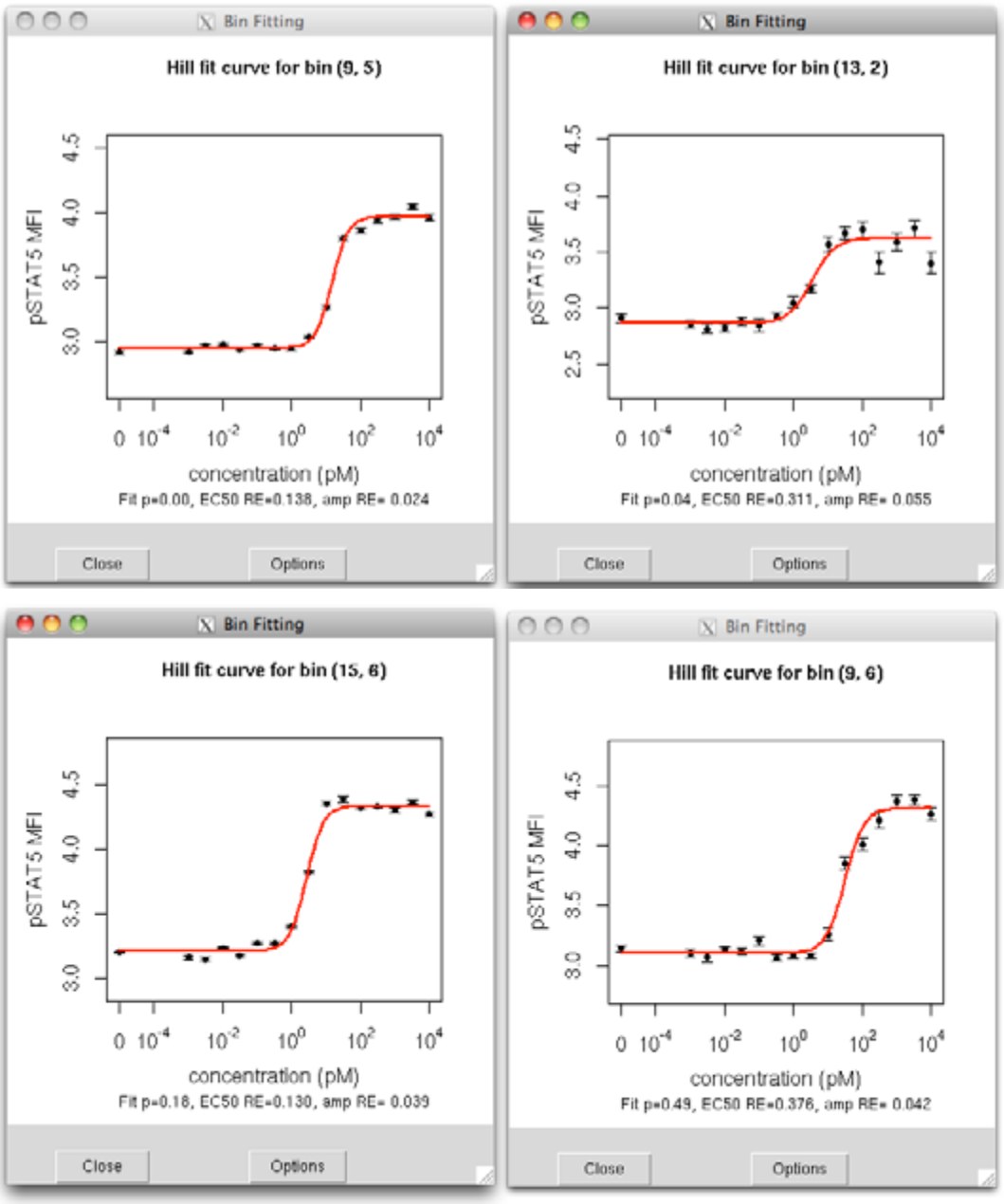
EC50, Amplitudes, Hillslope Min/Max: sets minimum and maximum display values. Any values above the maximum will be displayed the same as the maximum (i.e. dark red), likewise for the minimum.

Scale: log or linear color scaling

p-value cutoff and RE cutoffs: General measures of goodness of fit. Do not attempt to set the p-value cutoff to 0.05; it will exclude much more of your data than you would anticipate. See the following page for further discussion.

The "3D Analysis" graph shown at the top of the page has been adjusted appropriately, such that the color of the bins spans the entire range of the color spectrum, without saturation at either the upper or lower bound.



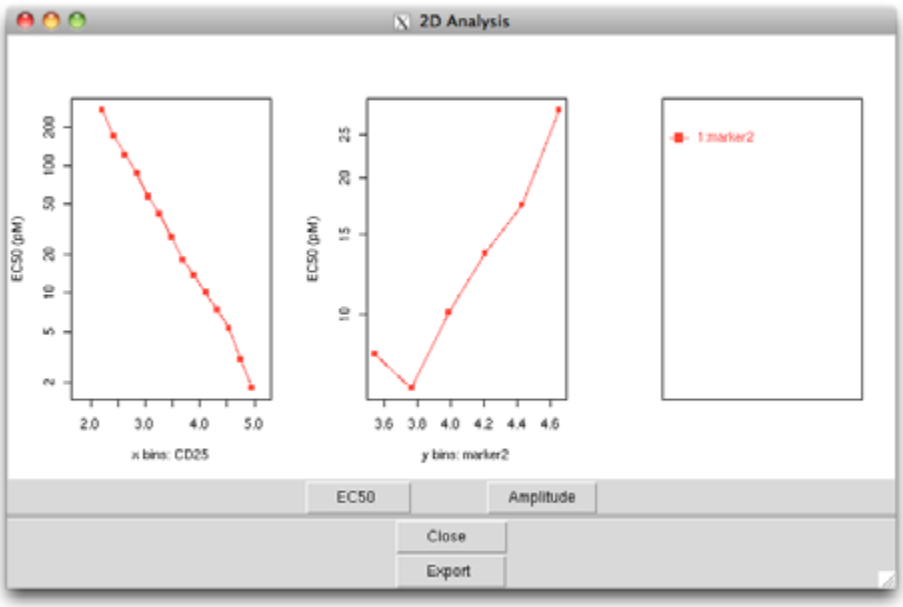


"Goodness of fit"

The above graphs show the result of clicking various bins shown on the previous page to evaluate goodness of fit within each bin. The top two fits would be judged statistically significant with a p-value cutoff of 0.05, whereas the bottom two fits would not. In (A), the curve passes through most of the points, so it's clear why this is significant. In (B), the points that the curve misses are noisy, leading to less penalty on the p-value. The bottom two curves provide equally valid estimations of EC_{50} and amplitude (by eye), but because the points missed by the curve have small errors, the p-value for the fit is relatively large. For this reason, the p-values and RE values should be used as general measures to reduce noise, but should not be used to place strict cutoffs on the EC_{50} or amplitudes.

2D Analysis

Going back to the InitialPlot window and clicking the ***Analyze 2D*** button will bring up the plot at right. From here, your best bet is to ***Export*** the data and use a program such as Graphpad Prism or MS Excel to tailor the graph to your liking.



The exported file will be named by the titration names, followed by "Analysis in 2D.txt". Each file contains a number of data tables, and is easily readable by Microsoft Excel (or R). The headers of the columns for each table are (using the data at right as an example).

	A	B	C
1	Values exported from ScatterSlice		
2	Tue Feb 26 17:14:54 2013		
3			
4			
5	MFI values represent bin centers		
6			
7			
8	ec		
9	x	1:CD25	
10	1.79529412	NA	
11	2.00588235	NA	
12	2.21647059	268.973602	
13	2.42705882	168.550488	
14	2.63764706	120.963842	
15	2.84823529	86.0063509	
16	3.05882353	56.8958081	
17	3.26941176	41.3633459	
18	3.48	27.2491999	