



PESTLE USER MANUAL

pes•tle | 'pe-səl, 'pes-tl

noun

a handheld tool for mashing data into a finely-ground collection for proper use in SPICE

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1 OVERVIEW

Pestle is a software application that formats and re-codes data generated in FlowJo for use in SPICE. This documentation assumes that you are familiar with the use of SPICE; many of the terms used here are defined and explained in the documentation for SPICE. Therefore, you should acquaint yourself fully with the SPICE tutorials and documentation before reading the Pestle documentation.

Pestle is designed to reformat data that is generated in FlowJo's Table Editor. While it is particularly suited for tables output for Boolean Gate combinations, it will work with any table. The statistics (and keyword values) exported by FlowJo are then managed by Pestle, with several distinct operations that can be performed in succession. The resulting output file is properly formatted for import into SPICE, and contains edited variable and statistic names for optimal presentation.



The screenshot shows the Pestle software interface. On the left is a sidebar with icons for Input Data, Column Roles (which is selected and highlighted in blue), Background Subtraction, Matrix Columns, and Category Columns. The main panel is titled "Column Roles" and contains the text "Choose the roles for each column found in your input data." Below this is a table with two columns: "Role" and "Name". The "Role" column has three options: "ignored", "category", and "value". The "Name" column lists various SPICE variable names. At the bottom of the main panel is a "Matrix Preview" section with a legend: Variable (blue square), Value (red square), Collapsed (orange square), Statistic (yellow square), and Ignored (grey square). To the right of the preview are the corresponding SPICE variable names for each role.

Role	Name
ignored	Sample
category	Stimulation
value	Group
ignored	SampleID
category	Lymphs/CD4 T/g+2+T+,Freq.
value	Lymphs/CD4 T/g+2+T-,Freq.
ignored	Lymphs/CD4 T/g+2-T+,Freq.
category	Lymphs/CD4 T/g+2-T-,Freq.
value	Lymphs/CD4 T/g-2+T+,Freq.
ignored	Lymphs/CD4 T/g-2+T-,Freq.
category	Lymphs/CD4 T/g-2-T+,Freq.
value	Lymphs/CD4 T/g-2-T-,Freq.

Matrix Preview	
Variable	Lymphs/CD4 T/g+2+T+,Freq. of
Value	Lymphs/CD4 T/g+2+T-,Freq. of
Collapsed	Lymphs/CD4 T/g+2-T+,Freq. of
Statistic	Lymphs/CD4 T/g+2-T-,Freq. of
Ignored	Lymphs/CD4 T/g-2+T+,Freq. of
	Lymphs/CD4 T/g-2+T-,Freq. of
	Lymphs/CD4 T/g-2-T+,Freq. of
	Lymphs/CD4 T/g-2-T-,Freq. of

The input to Pestle is the text file that FlowJo creates when you choose to save a Table to a file. The contents of the table can contain any number of descriptive variables that provide information about each sample, such as demographic data. The table must contain one or more columns of data that are derived from statistics such as frequencies, fluorescence intensities, or other calculations.

Pestle allows you to perform several distinct operations on the data.

First, you can opt to merge in other categorical (descriptive) data about the samples - for example, disease status, age grouping, sex, etc. You can also append additional tables and perform basic text find & replace operations on the merged input.

Second, you can perform background subtraction on any subset of the columns. In this case, you would have samples that are stimulated and those that are controls, and you wish to subtract the value for the control from the stimulated sample.

Finally, Pestle gives you the chance to assign meaningful names to each of the variables (and descriptive values) in the final dataset. The names extracted from the FlowJo

Tables are usually not very descriptive (and often contain much otherwise extraneous information, such as the full gating path), so this provides you an opportunity to better annotate the dataset. This makes for much better visualization and analysis in SPICE.

Thus, Pestle is a tool to port data from FlowJo tables to SPICE. It will typically be used only once for any given table output, whereas SPICE will be used many times to generate different visualizations from that data. However, Pestle provides automation tools so that you can repeatedly and consistently process FlowJo data into SPICE data, and apply that process to any number of different FlowJo Tables.

The rest of this documentation is divided into three sections:

- **Section 2** is a brief introduction into the data analysis process with two tutorials.
- **Section 3** describes the user interface of Pestle: the menus, buttons, and other controls.
- **Section 4** is a description of Pestle's scripting system.

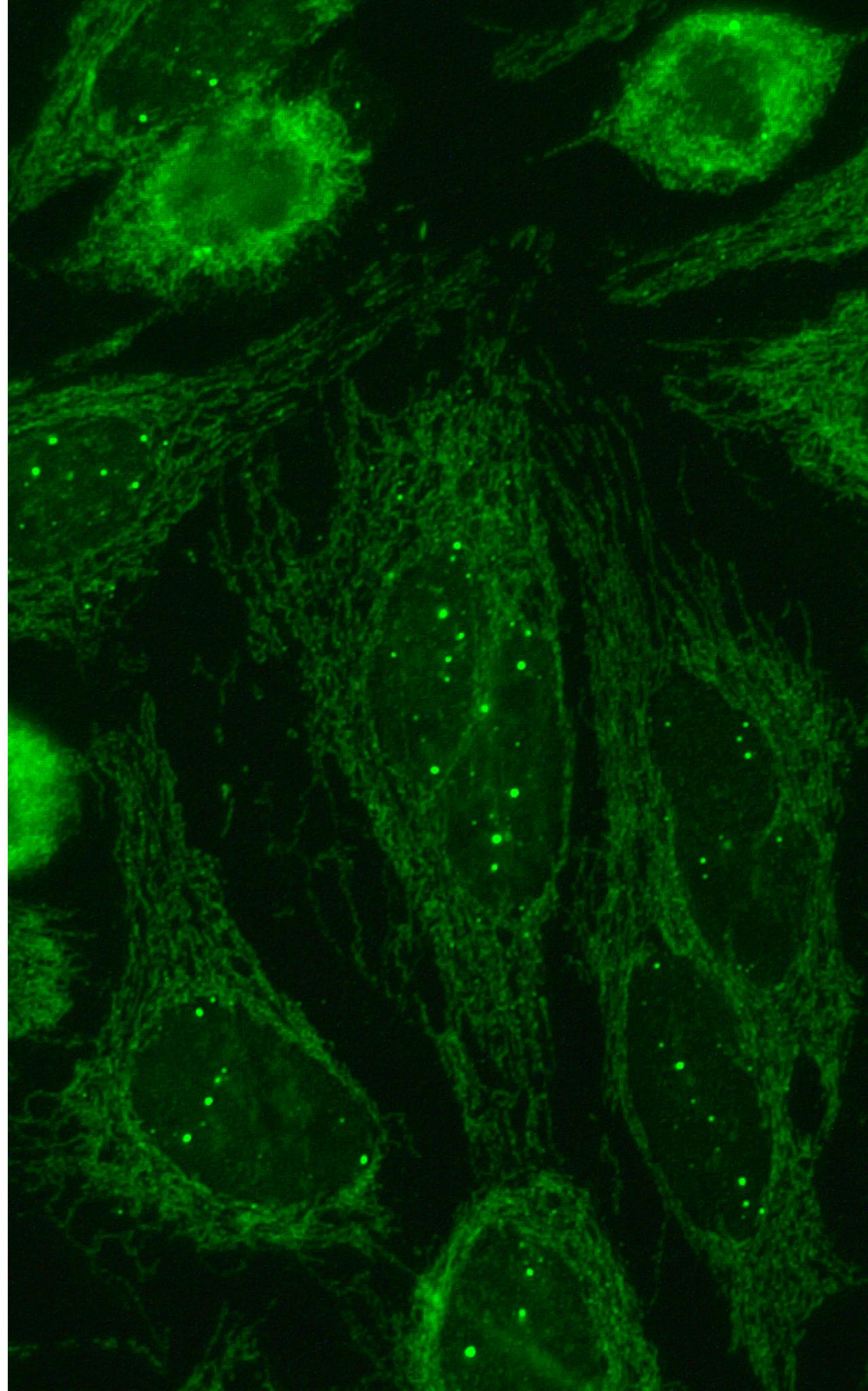
2

DATA ANALYSIS PROCESS

This section is a mixture of a brief overview and tutorials for running Pestle. The first tutorial is a rather simple example, which you can use to familiarize yourself with the operation of the program, and the generation of basic graphs. The more complex example leads you through the process of delving into a dataset to explore a variety of different aspects of an immunological response.

Please read the documentation for SPICE. That documentation fully explains variable roles and will familiarize you with the need for the different kinds of variables in your datasets. You should run the SPICE tutorials before tackling the tutorials here.

In order to analyze data in SPICE, you will need to have data values for display, as well as categorical values which discriminate data into different subsets. Examples of categorical variables could be Sample ID, stimulation, subject sex, and so forth. These variable values will be used



in SPICE to group analyses (for averaging or other aggregating statistics), or to specify overlays, or to select specific subsets for display. In Pestle, you must specify which values are categorical, and which are “values”, if you intend to perform background subtraction of columns.

The input data for Pestle must originate from a FlowJo Table Editor. Pestle is specifically designed to reformat (and process) data that requires background subtraction. Its real utility is for tables that are created for Boolean gate combinations; however, it will work for other tables as well. These tables are considered “Matrix” value tables - again, this is fully described in the SPICE documentation.

Both the input files saved from FlowJo’s Table Editor, and the output files suitable for reading by SPICE are in the folder “Demo Data”. If you properly execute the tutorial, you should generate an output file that is similar to the output file in this folder.

Note: Due to differences between versions 1 and 2 - including removal of certain little-used features and related scripting commands, you’ll notice the output from version 2 doesn’t exactly match some of the older SPICE demo data files.

2.1 Tutorials

Included with this documentation are two tutorials.

The first, a relatively simple tutorial, will lead you through the processes that are most commonly needed for processing FlowJo data into SPICE data. The second exemplifies all of the different operations that Pestle can accomplish.

2.1.1 Simple Example

This example is based on an immunogenicity experiment, in which all combinations of three functional outputs (TNFa, IFNg, and IL2) were measured on CD4 T cells. There were five groups of samples, with three samples in each group. Within each group is a pooled sample that was left unstimulated, for background subtraction. The goal in this process is to generate a SPICE file that has background-subtracted values suitable for analysis.

Launch Pestle by double-clicking on the application. Pestle immediately shows you an “Open File” dialog window, asking you to identify a FlowJo output file. Navigate to the file “simpleFJ.txt” provided with the documentation, and select it. This file can be opened in any text editor, or in Excel or Numbers. You can see what the data table looks like in **Table 2.1.1**.

This file has several columns of data. The first column, always output by FlowJo Tables, contains the file name of the sample. In general, this filename is not useful, and Pestle will ignore it by default (see below). The second, third, and fourth columns are keyword values that were entered for the data samples. They contain categorical

information that identifies characteristics of each sample. In this case, the second column, “Stimulation”, identifies how the sample was treated (either stimulated only with costimulatory antibodies, or with antibodies plus antigenic peptide). The third column identifies the group (there are four samples per group: one control and three positive samples). The fourth column defines a unique “Sample ID” for each sample.

Following these columns are those representing the Boolean matrix of values for each of the gated samples. The first contains the percentages of cells that are positive for each of the three functions, and so forth.

For this example, we want to subtract the background for each sample—the background is contained in the “Costim” sample for each group.



simpleFJ.txt

Sample	Stimulation	Group	SampleID	Lymphs/CD4 T/g+2+T+, Freq. of Parent	Lymphs/CD4						
1: Sample_001_Well_001_A05.fcs	Costim	Untreated	1	0.018	0.00439	0.00219	0.00658	0.053	0.15	0.05	
2: Sample_001_Well_002_A06.fcs	Costim + Peptide	Untreated	2	0.074	0.0024	0.014	0.065	0.1	0.24	0.14	
3: Sample_001_Well_003_A07.fcs	Costim + Peptide	Untreated	3	0.03	0.00189	0.00567	0.011	0.1	0.26	0.12	
4: Sample_001_Well_004_A08.fcs	Costim + Peptide	Untreated	4	0.072	0.00899	0	0.02	0.14	0.17	0.19	
5: Sample_001_Well_005_B05.fcs	Costim	A	5	0.026	0.00217	0	0.00435	0.052	0.22	0.061	
6: Sample_001_Well_006_B06.fcs	Costim + Peptide	A	6	0.27	0.028	0.039	0.067	0.16	0.21	0.21	
7: Sample_001_Well_007_B07.fcs	Costim + Peptide	A	7	0.3	0.027	0.057	0.086	0.16	0.34	0.2	
8: Sample_001_Well_008_B08.fcs	Costim + Peptide	A	8	0.28	0.035	0.049	0.11	0.18	0.2	0.23	
9: Sample_001_Well_009_C05.fcs	Costim	B	9	0.00696	0.00232	0.00232	0.026	0.06	0.17	0.065	
10: Sample_001_Well_010_C06.fcs	Costim + Peptide	B	10	0.26	0.012	0.031	0.031	0.13	0.27	0.22	
11: Sample_001_Well_011_C07.fcs	Costim + Peptide	B	11	0.66	0.019	0.075	0.063	0.15	0.38	0.25	
12: Sample_001_Well_012_C08.fcs	Costim + Peptide	B	12	0.4	0.011	0.052	0.11	0.11	0.2	0.24	
13: Sample_001_Well_013_D05.fcs	Costim	C	13	0.015	0.00857	0.00214	0.013	0.09	0.27	0.062	
14: Sample_001_Well_014_D06.fcs	Costim + Peptide	C	14	0.31	0.00391	0.033	0.057	0.24	0.27	0.28	
15: Sample_001_Well_015_D07.fcs	Costim + Peptide	C	15	0.53	0.011	0.086	0.075	0.29	0.34	0.33	
16: Sample_001_Well_016_D08.fcs	Costim + Peptide	C	16	0.33	0.00977	0.02	0.046	0.32	0.33	0.38	
17: Sample_001_Well_017_E05.fcs	Costim	D	17	0.04	0.00288	0.035	0.2	0.11	0.32	0.11	
18: Sample_001_Well_018_E06.fcs	Costim + Peptide	D	18	0.23	0.012	0.1	0.95	0.13	0.45	0.31	
19: Sample_001_Well_019_E07.fcs	Costim + Peptide	D	19	0.44	0.00486	0.087	0.34	0.14	0.51	0.25	
20: Sample_001_Well_020_E08.fcs	Costim + Peptide	D	20	0.53	0.00681	0.089	0.57	0.092	0.32	0.34	

Table 2.1.1

When loaded into Pestle, you will see the window shown in **Figure 2.1.1a**. Pestle has read in all of the values in this file, and identified the columns. The first task is to make sure that all of the column roles are properly set.

Pestle can perform various tasks and transformations on the input data. Here we will only need to adjust column roles, set up background subtraction, then edit some column definitions.

Most operations can be performed in any order, though some will necessarily invalidate others (adding or removing input data files, changing column roles, etc.). You can navigate the various functions in several ways:

- The tabs or back-and-forward button in the navigation bar on the left edge of the window
- The View menu in the main menu bar
- The keyboard shortcuts found in the View menu.

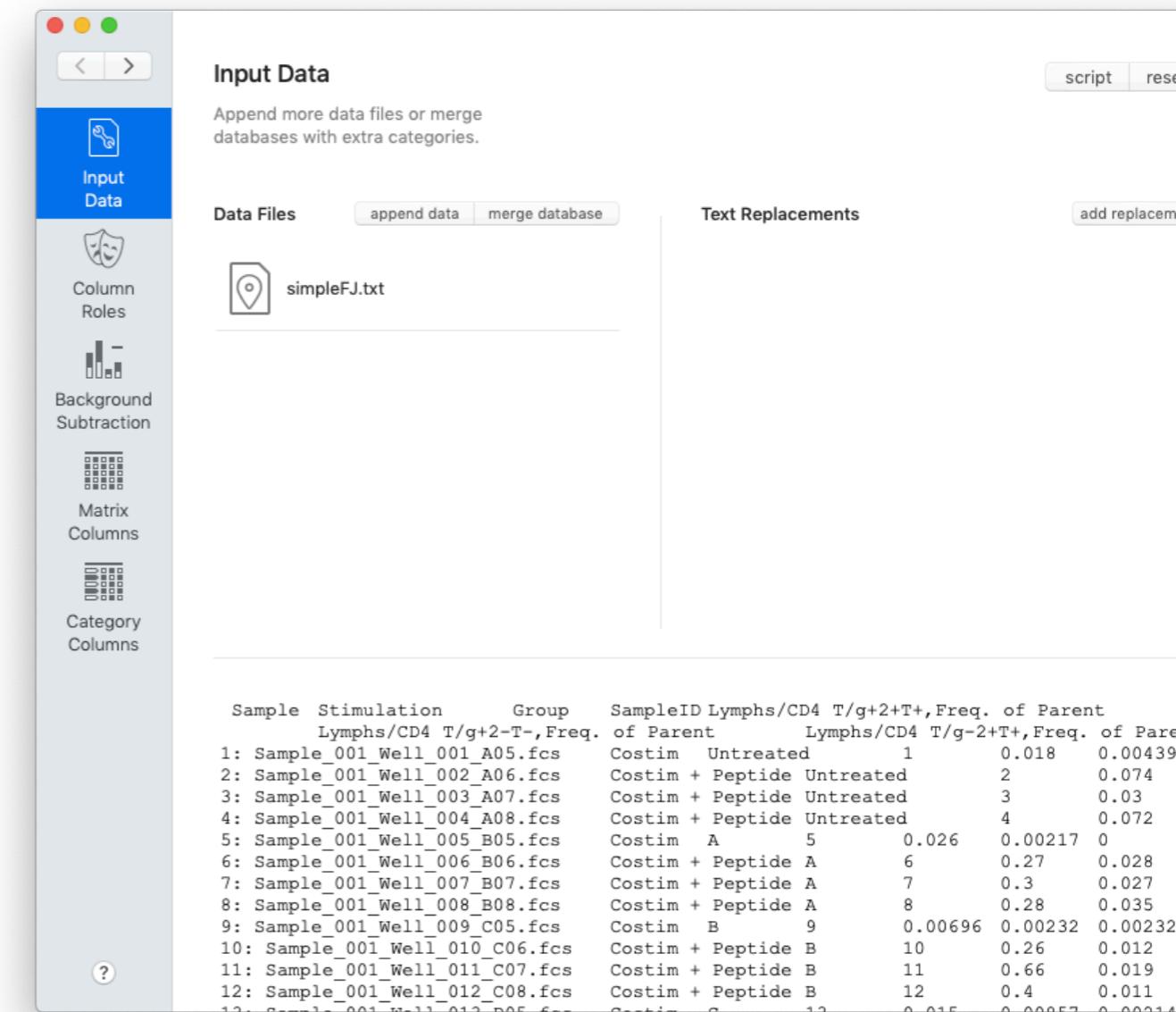


Figure 2.1.1a

Note: Each tab's content has a brief description of its function at the top, along with a control for saving and running scripts, and for resetting the customizations you've applied at that stage. Some tabs have a preview pane at the bottom, showing the results of the operations within that stage or information relevant thereto.

Adjusting Column Roles

Select the Column Roles tab to assign roles to each text column (**Figure 2.1.1b**). Column roles can either be *ignored* (not output or used for any purpose); *category* (descriptive of the groups of data values), or *value* (containing data values to be analyzed quantitatively by SPICE). Only columns that come from Boolean gate combinations in FlowJo should have *value* roles.

Pestle analyzes the data values in the columns, and will initially assign columns a *value* role if all data values in the column appear to be numerical. In this data set the *SampleID* column, which contains a unique numerical identifier, has been assigned a role of *value*, however, this is a categorical column. To fix this, select *category* using the role button next to *SampleID* in the list. The only columns that have *value* roles should be those with the Boolean gates (the last seven, beginning with "Lymphs" in this example).

Once you have set the *SampleID* column to be a categorical column, the Matrix Preview pane has updated to reflect decoded variables and values. The next task is to specify the background subtraction.

Role	Name
ignored	Sample
ignored	Stimulation
ignored	Group
ignored	SampleID
ignored	Lymphs/CD4 T/g+2+T+,Freq. of Parent
ignored	Lymphs/CD4 T/g+2+T-,Freq. of Parent
ignored	Lymphs/CD4 T/g+2-T+,Freq. of Parent
ignored	Lymphs/CD4 T/g+2-T-,Freq. of Parent
ignored	Lymphs/CD4 T/g-2+T+,Freq. of Parent
ignored	Lymphs/CD4 T/g-2-T-,Freq. of Parent

Matrix Preview

Variable	SampleID
Value	Lymphs/CD4 T/g+2+T+,Freq. of Parent
Collapsed	Lymphs/CD4 T/g+2+T-,Freq. of Parent
Statistic	Lymphs/CD4 T/g+2-T+,Freq. of Parent
Ignored	Lymphs/CD4 T/g-2+T+,Freq. of Parent
	Lymphs/CD4 T/g-2-T-,Freq. of Parent

Figure 2.1.1b

Background Subtraction

Select the Background Subtraction tab. You will see the window shown in **Figure 2.1.1.c.**

Initially, background subtraction will not be applied (hence the yellow caution flag). You must click the *enable* switch at the top. This will immediately cause Pestle to display several errors, preventing you from exporting. These errors are expected, however, because you must tell Pestle how to match up the background sample for each of the samples, and on which columns to apply the subtraction.

The errors are descriptive of the missing “steps” and will persist if you navigate away from the Background Subtraction tab until you return and finish setting up the three required steps. You can click the arrow in each error message to jump to the relevant tab (that is, the arrow will take you to the tab on which you must resolve the error).

Note: The red dot that appeared in the navigation pane next to the Background Subtraction tab helps you to identify tabs containing errors that prevent Pestle from generating output.

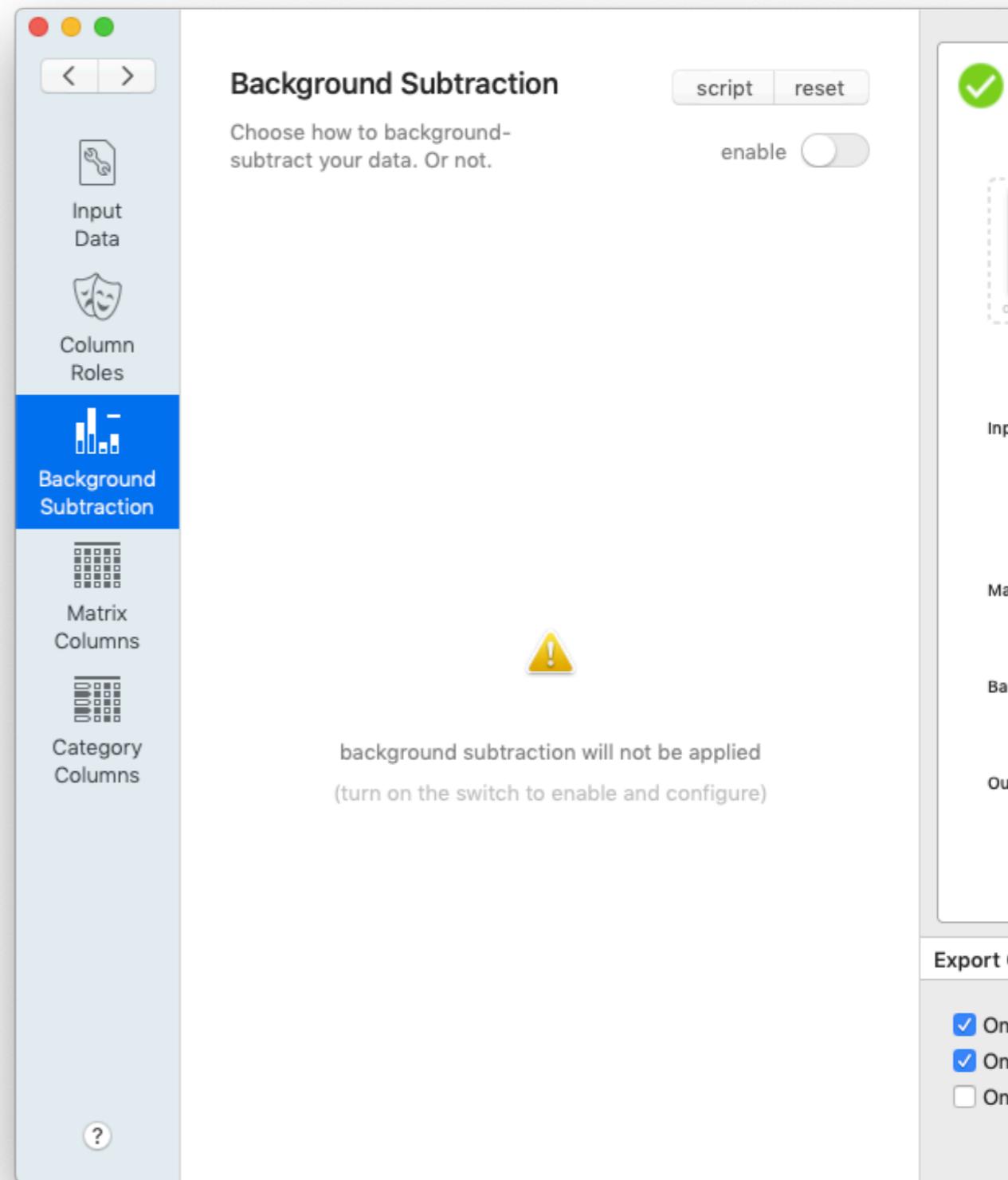


Figure 2.1.1c

Pestle will walk you through the three setup steps, only moving on to subsequent steps after the previous step has been completed. You must have at least two different category columns to proceed.

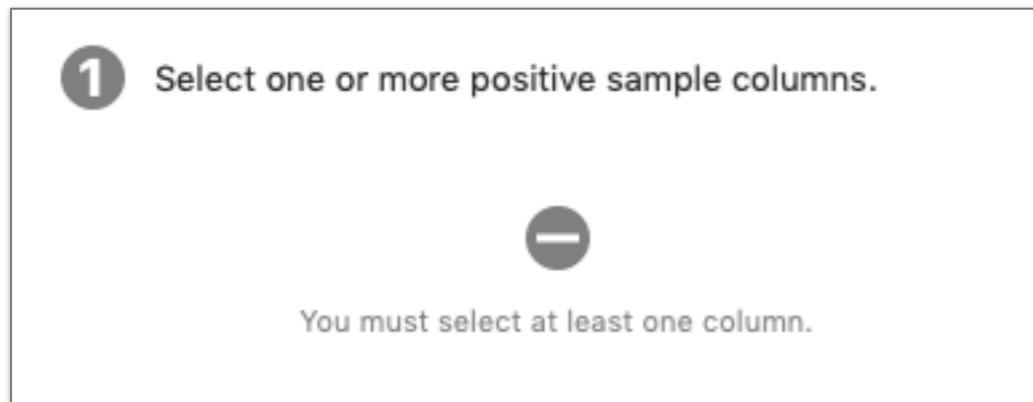


Figure 2.1.1d

The first step (**Figure 2.1.1d**) is to select one or more category columns that identify your positive samples. At least one of these columns will have values that are the same for each sample and its corresponding background control. You may have more than one such column. To complete this step, click the *select* button, select the column or columns (only category columns will appear), then click *choose*. Note that you can use common keyboard shortcuts (such as Command+Click) to select multiple items. In this example, the *Group* column contains the values *A*, *B*, *C*, and *Untreated*. Choose this column.

Upon completion of step 1, step 2 is presented (**Figure 2.1.1e**).

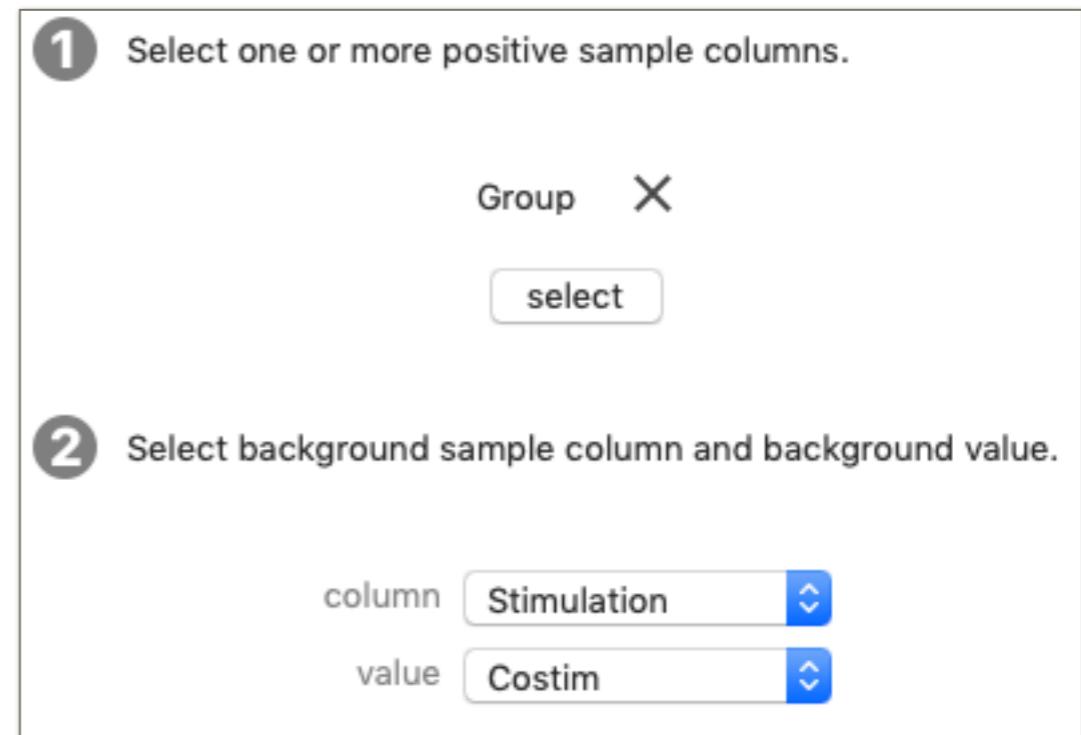


Figure 2.1.1e

Here you must select the column that distinguishes the background samples from the positive samples; in this example, that is the *Stimulation* column. Choose *Stimulation* from the *column* control. To tell Pestle which values of the *Stimulation* column uniquely identify the background samples, choose the value from the *value* control (*Costim*, in this case).

Note: If you have multiple samples that fit the selected criteria to match as background tubes, Pestle will average the data for all of the available tubes and use that as the background value.

3 Select value columns to be background-subtracted.

- Lymphs/CD4 T/g+2+T+,Freq. of Parent
- Lymphs/CD4 T/g+2+T-,Freq. of Parent
- Lymphs/CD4 T/g+2-T+,Freq. of Parent
- Lymphs/CD4 T/g+2-T-,Freq. of Parent
- Lymphs/CD4 T/g-2+T+,Freq. of Parent
- Lymphs/CD4 T/g-2+T-,Freq. of Parent
- Lymphs/CD4 T/g-2-T+,Freq. of Parent

Figure 2.1.1f

Once step is complete, step 3 will appear (**Figure 2.1.1f**). In this step, you must identify which *value* columns should be background subtracted. In this example, all columns should be corrected, so click the *all* button on the *include* control at the bottom of the list. You can use the *include* control and the search field for quick and easy selection modification in addition to clicking the checkbox for individual columns. Figure 2.1.1f shows all columns

selected for background selection, thus completing the setup and clearing all errors. At this point you could generate the SPD file for SPICE (**Figure 2.1.1g**). But wait!

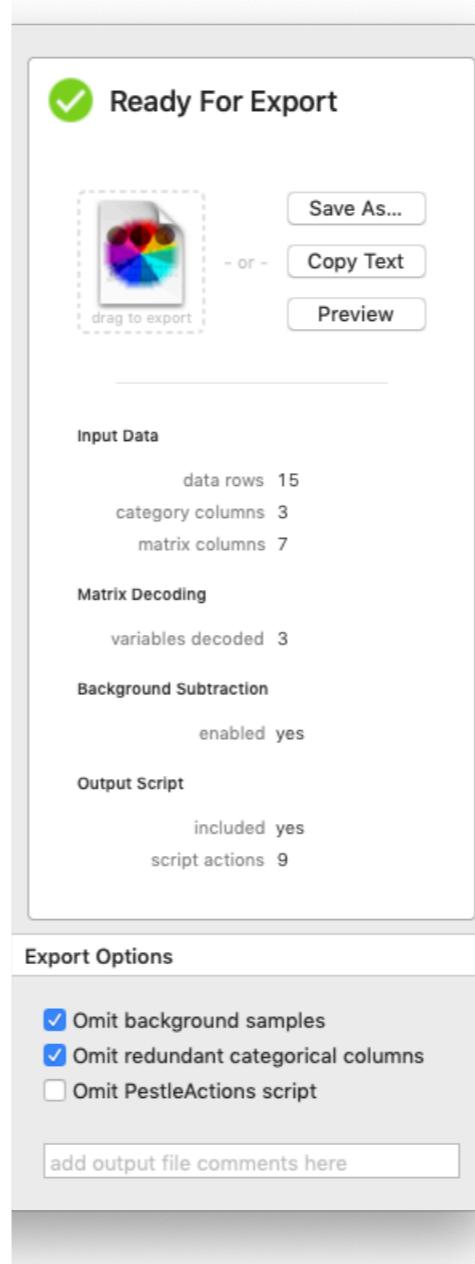


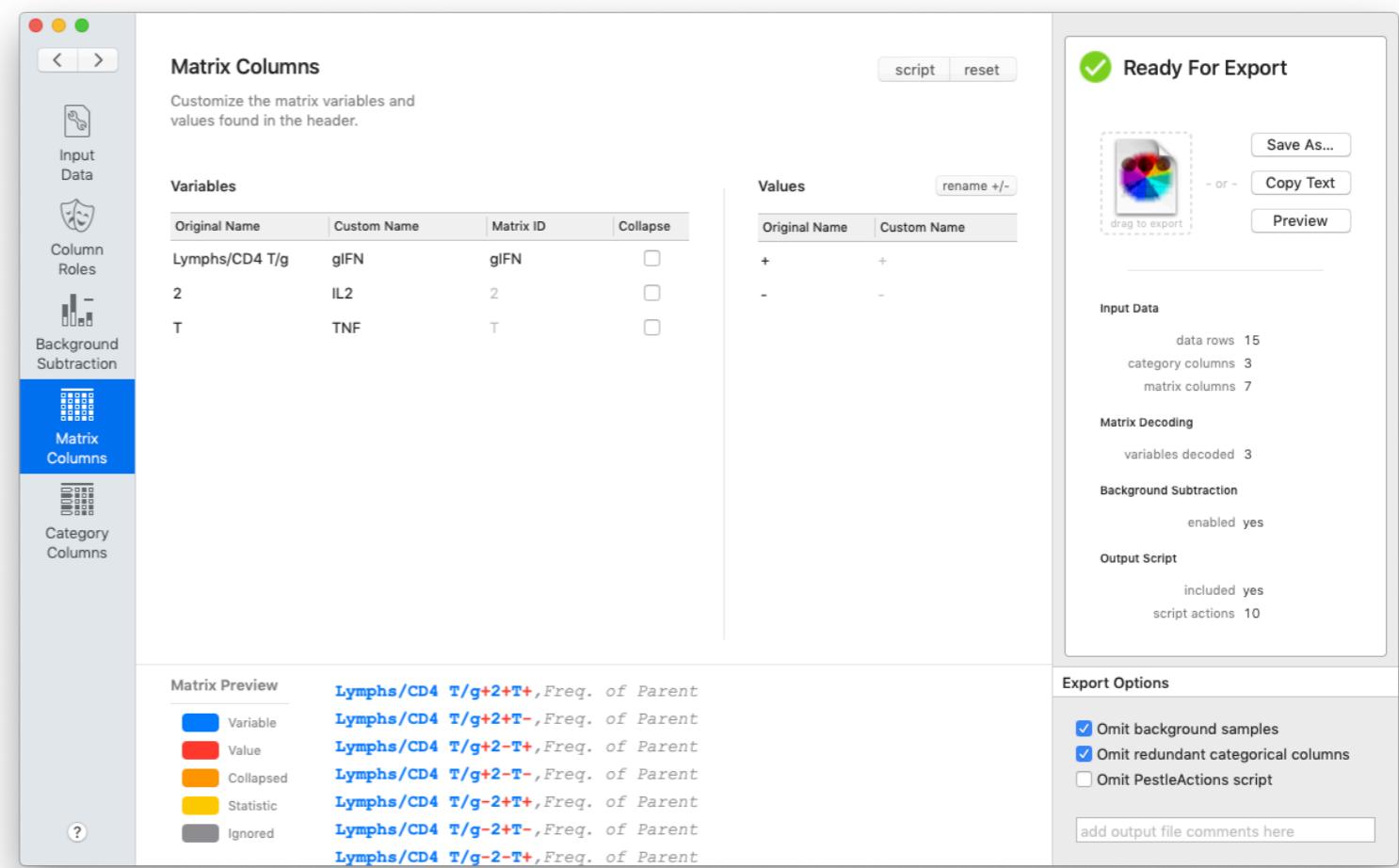
Figure 2.1.1g

Fine Tuning

The variable names as exported to SPICE won't be particularly helpful. Pestle can help with this using the Matrix Columns and Category Columns tabs. Let's clarify the automatically-decoded variable names.

Select the Matrix Columns tab. Here (**Figure 2.1.1h**) you are shown two sections in which you can edit variable names and values. The Variables section lets you rename variables that appear in the FlowJo Boolean matrix. Pestle has scanned the column names and identified the

possible variables (the differences between different matrix column names), the strings used to identify those variables, and the variable values. You can rename the variable Pestle has scanned using the Custom Name field. In this example, edit the variables to *gIFN*, *IL2*, and *TNF*, as shown. The possible variable values in this example are only + or -; you do not need to edit those; but if you wished to, you would do so in the Values section. After editing the Matrix Column names, the window should appear as shown in figure 2.1.1h.



Exporting

At this point, you have finished all the preparations. To export the data as a SPICE-compatible SPD file, use the controls at the top of the panel on the right edge of the window. You can either drag the file icon to a folder or your desktop, or click the Save As button and choose a location.

You can also choose to copy the text to the pasteboard for additional fine-tuning in a text editor, or simply preview the output before saving using these controls.

Figure 2.1.1h

The Export Options section at the bottom of this panel allows for additional settings as well as the addition of comments to the SPD file for your own reference.

The resulting SPD file is a plain text document you can open in any text editor or import into SPICE via the File > Open or by dropping the file onto the SPICE icon.

Scripting

Pestle stores all of the actions that you perform on your data set in the SPD file (unless you choose to omit them). You can choose to save these actions separately (or apply saved actions from another file to the current data in Pestle) using the script button at the top of the window. This simplifies future data preparation.

For an example of how this works, export the SPD file then close Pestle. Reopen Pestle and choose the *simpleFJ.txt* file again, as though this were a similar experiment. Click the script button, then choose Run Script from the popover. Select the SPD file that you just exported for this tutorial. The Run Script sheet will appear (**Figure 2.1.1i**), showing all the actions you took to create the SPD file. Click the Run button. Pestle will now automatically carry out all the same operations, resulting in the same SPD file. More importantly, using stored Pestle Actions scripts also ensures that you will have consistent naming and analysis for each experiment. You can verify that SPICE has correctly carried out the actions by navigating around the various tabs or exporting and comparing the SPD files.

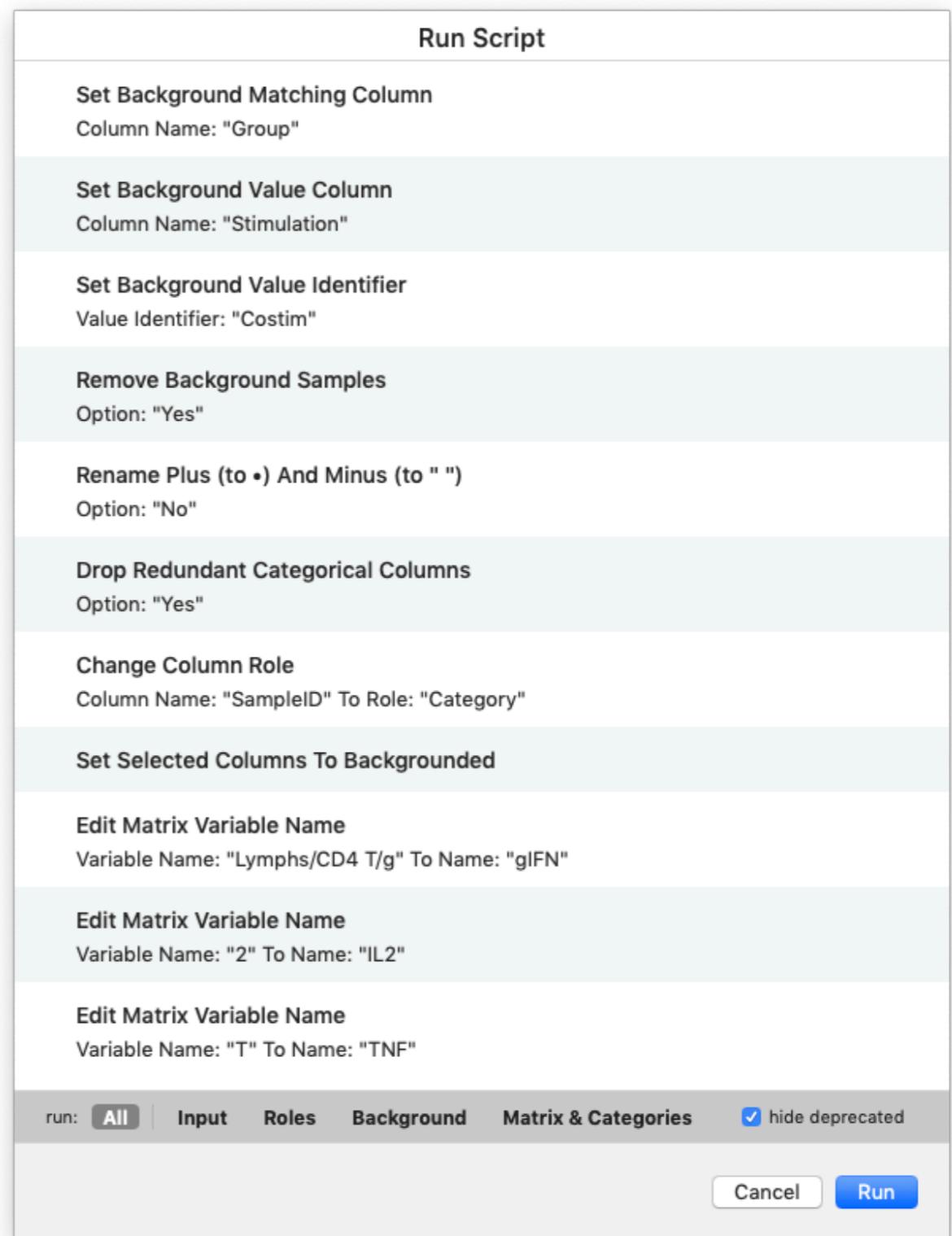


Figure 2.1.1i

2.1.2 Complex Example

This example illustrates all of the different operations Pestle can perform. It is based on several analyses for which the frequencies and cytokine mean fluorescence intensities (MFIs) were determined for a 5-functional analysis of CD4 and CD8 T cells from 8 individuals at several time points, using a variety of different stimuli to identify antigen-specific T cells.

Overview

To process this data for consumption in SPICE, we will do the following:

- Join two separate FlowJo output files.
- Merge demographic data from another tabular text file.
- Verify the appropriate column roles.
- Apply background subtraction to frequency statistic.
- Edit the output statistic names.

Input Files

Launch Pestle, then navigate to the file “CompSet1.txt” provided with the documentation and select it. At the top of the Data Files section, click the *append data* button and select “CompSet2.txt” from the same location. The second

file appears in the list along with a control beneath it labeled *behavior* (**Figure 2.2.2a**).

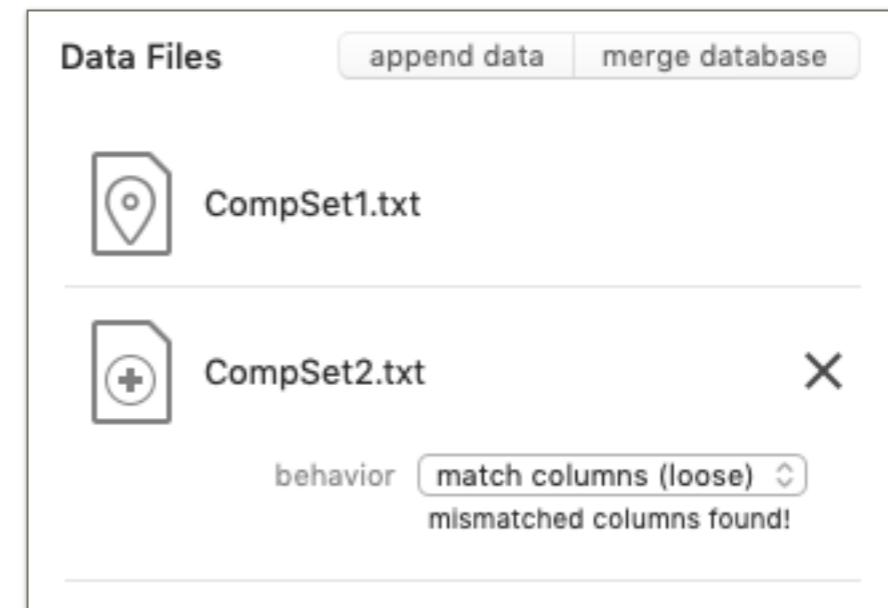


Figure 2.1.2a

Pestle is unable to match the column names using the default setting. Change this to *match columns (loose)* to resolve this error. This setting ignores case variations and differing orders of columns and looks only for any columns matching those of the input data file.

Note: The ignore column names setting matches columns only by order, whereas the strict setting requires all columns to match in name and order.

Now you will merge in a data set that has additional categorical information (for example, demographic data). In this case, there is information about the treatment status and sex of the subjects in the trial. It does not matter if the database file has more information than is present in the experimental analyses; Pestle will only extract the values which match those in the data files. Thus, you could merge demographic data from a complete database encompassing more patients and categorical information than what you need.

Table 2.1.2 shows how the demographic data has been formatted (in a text file). One of the columns (*Sample ID*) contains values that exactly match a column of data in the exported data file (also named *Sample ID* in this example). Pestle will insert new output columns into the resulting data file containing the other information in this file, matching the values based on when the two *Sample ID* columns have the same value.

In most cases, Pestle will automatically select the correct columns - as in **Figure 2.1.2a** - but you can choose the

Sample ID	Status	Sex
004-022	Treated	M
004-023	Treated	M
004-029	Control	M
004-030	Control	M
004-034	Treated	F
004-042	Control	F
004-047	Treated	F
004-050	Treated	F

Table 2.1.2

correct columns using the *database column* and *input data column* controls beneath the merged database file.

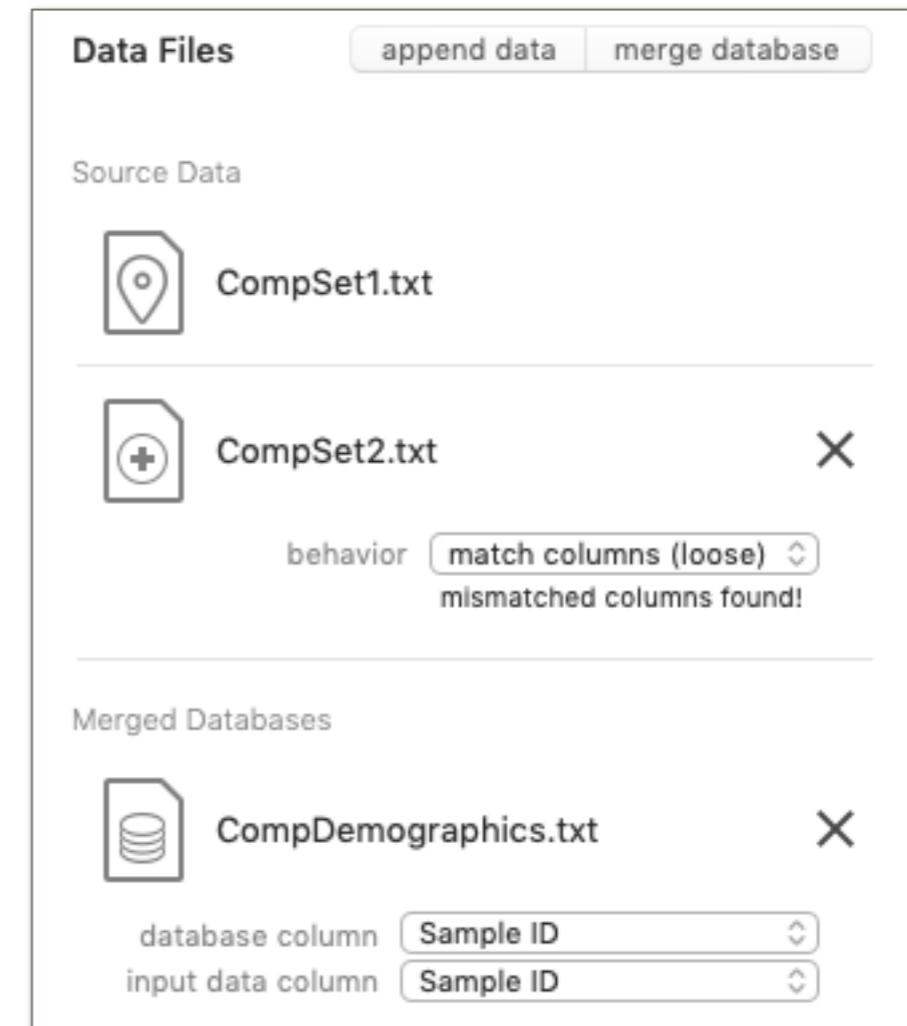


Figure 2.1.2a

Since Pestle has correctly identified the *Sample ID* column for both controls, no additional steps are needed here. This satisfies steps A and B.

Column Roles

Before continuing, select the Column Roles tab and review the roles Pestle has automatically assigned based on their content. In this example, not everything was correctly identified. That is, some of the columns representing matrix values have been identified as *category* instead of *value*.

To correct this, click the Find & Select Columns field at the top of the list, then type *Singlets* and press return. Pestle will select all column names containing *Singlets* (which happen to be all of the *value* columns). Click the *value* segment of any role control for any of the selected columns. This will set the role for all selected columns to *value*, satisfying step C.

Pestle will now update the data and show that it is ready for export. The summary beneath the export controls should show that there are now 54 data rows, 6 category columns, 372 matrix columns, and 8 matrix variables.

Background Subtraction

As with the simple example in section 2.1.1, we will apply background subtraction. Unlike that example, the steps will only be outlined here.

1. Select the Background Subtraction tab.
2. Click the *enable* switch to activate it.
3. For the positive samples columns, click *select* and choose *Sample ID* and *Timepoint* columns (hold the Command button to choose more than one), then click *choose*.
4. For the background sample column, choose *Peptide*; for the value, choose *28/49d*.
5. Click the Search Matrix Columns field, type *freq*, then press return. Pestle will select all *Freq. of Parent* columns.
6. Click the checkbox next to any of the selected columns to specify that all of the selected columns should be background-subtracted.

Pestle will update the data, this time applying background subtraction to the output. For larger data sets, this will marginally increase the time it takes to update. This satisfies step D.

Editing Variables

Select the Matrix Columns tab. Notice the output names for these are either unhelpfully simple or absurdly long. There is some cleanup we can do in addition to renaming them so that they are more helpful when imported into SPICE.

The first two entries refer to a difference in the subset for CD4 or CD8. This is because the gating tree for CD8 is *Singlets/Live/Lymphs/CD3+/CD4-/CD8+* and for CD4 it is *Singlets/Live/Lymphs/CD3+/CD8-/CD4+*. The differences are underlined in this text. You will note this is a redundancy; these two differences both identify the same distinction (CD4 or CD8).

Thus, you need to “collapse” the first difference so that SPICE will treat the two differences - scanning across these column names - as a single variable. Place a check in the *collapse* checkbox for the first entry in the Variables table.

This will result in a single column with the values 4 and 8 being output for the subsets, however the variable name for this new variable is now *-/CD*, which isn’t particularly helpful. Edit its Custom Name and Matrix ID fields both to be *Subset*.

You can rename the variables and values here to match

Figure 2.1.2b.

Variables				Values		rename +/-
Original Name	Custom Name	Matrix ID	Collapse	Original Name	Custom Name	
<i>Singlets/Live/-/CD</i>	<i>Singlets/Live/Subset</i>	<i>Singlets/Live/Subset</i>	<input checked="" type="checkbox"/>	+	+	
<i>+/7</i>	<i>CD107</i>	<i>CD_107</i>	<input type="checkbox"/>	-	-	
<i>G</i>	<i>IFNg</i>	<i>G</i>	<input type="checkbox"/>	4	4	
<i>2</i>	<i>IL2</i>	<i>2</i>	<input type="checkbox"/>	8	8	
<i>M</i>	<i>MIP1b</i>	<i>M</i>	<input type="checkbox"/>	Freq. of Parent %		
<i>T</i>	<i>TNF</i>	<i>T</i>	<input type="checkbox"/>	Median,<B515... iMFI IFN		
<i>Statistic</i>	<i>Statistic</i>	<i>Statistic</i>	<input type="checkbox"/>	Median,<G56... MIP mfi		
				Median,<G78... TNF mfi		
				Median,<R66... IL2 mfi		
				Median,<R710... CD107 mfi		

Figure 2.1.2b

Now select the Category Columns tab. The variable names here look reasonable, but on this tab you can also create new categorical columns based on existing ones. This does not create any new information; it simply creates new groups on which to subset the eventual data displays in SPICE. For example, here you could create a new variable that has the sum of the number of positive responses.

To do so, click *create sum variable* in the Sum Variables panel, select the categories to sum, then assign a name to the new category.

In this example, we've created two sum variables (**Figure 2.1.2c**). The first, named *5FunctionSum*, is composed of the *CD107*, *IFNg*, *IL2*, *MIP1b*, and *TNF* variables. The second, *3FunctionSum*, is composed of *IFNg*, *IL2*, and *TNF*. This completes step E.

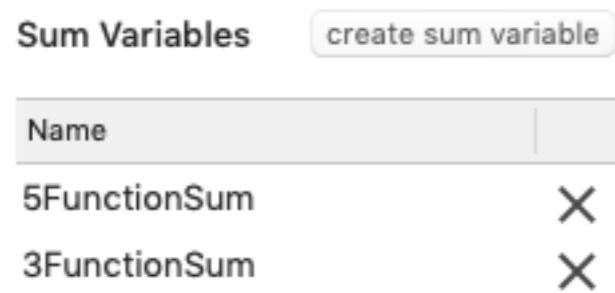


Figure 2.1.2c

At this point you are ready to export using the controls in the upper-right corner of the window.

This was a complex series of steps, but by going through this process you can ensure the data has been properly processed and edited for analysis in SPICE. As with the simple example in section 2.1.1, you can use the *script* button to save these actions and apply them to new analyses.

3

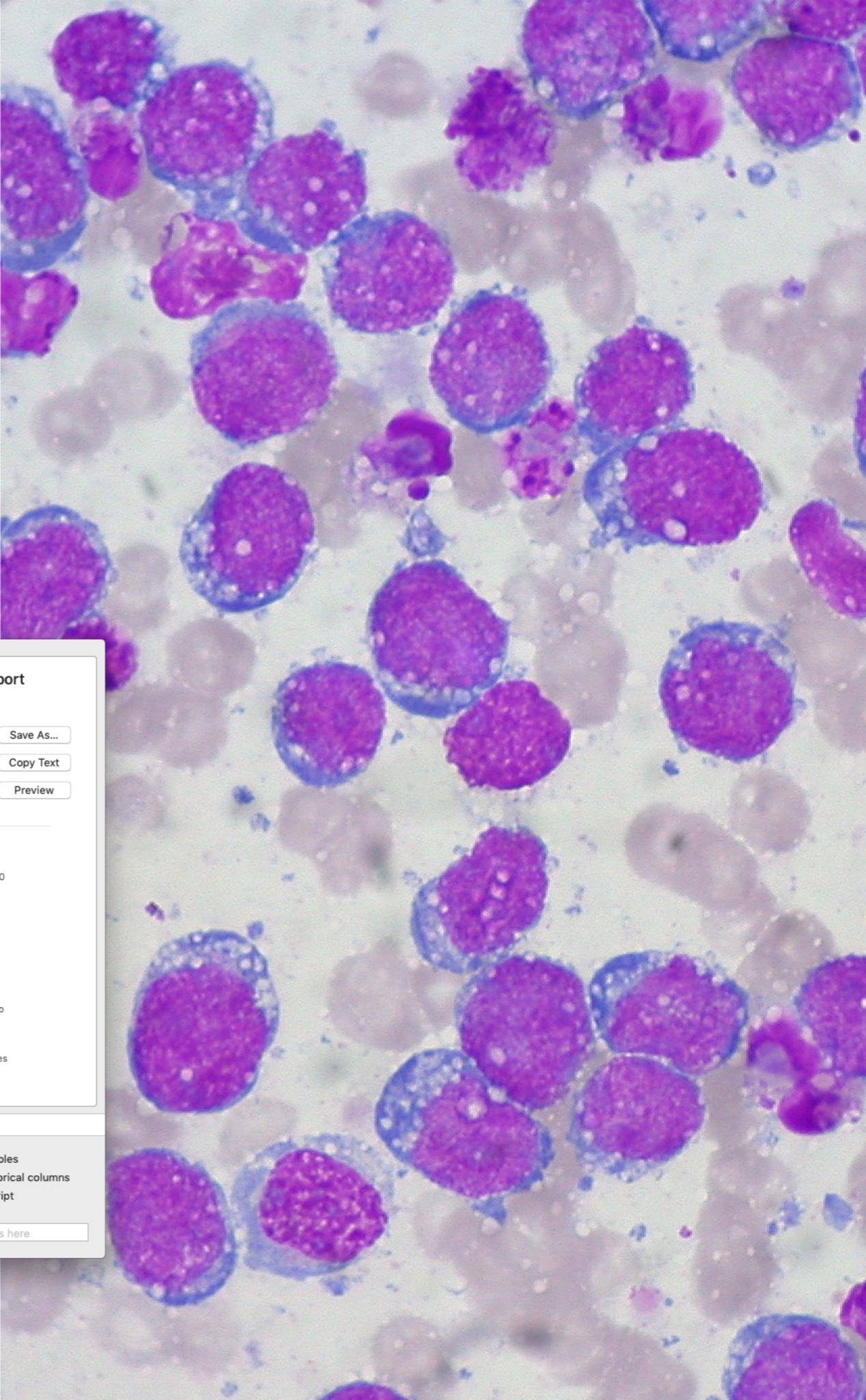
USER INTERFACE

This section covers all aspects of the Pestle user interface.

The topics begin with basic navigation and help, error management, and exporting the processed output. Then the use of each tab is explored in detail.

The screenshot shows the Pestle application window. On the left is a sidebar with icons for Input Data, Column Roles, Background Subtraction, Matrix Columns, and Category Columns. The main area has two tabs: 'Input Data' (selected) and 'Ready For Export'. The 'Input Data' tab shows a list of data files, with 'simpleFJ.txt' selected. Below it is a table of stimulation data. The 'Ready For Export' tab shows settings for export, including 'Input Data' (20 rows, 3 category columns, 7 matrix columns), 'Matrix Decoding' (3 variables decoded), 'Background Subtraction' (disabled), and 'Output Script' (included, 5 actions). It also includes an 'Export Options' section with checkboxes for omitting background samples, redundant categorical columns, and the PestleActions script, all of which are checked.

Sample	Stimulation	Group	SampleID	Lymphs/CD4 T/g+2+T-, Freq. of Parent	Lymphs/CD4 T/g-2+T+, Freq. of Parent
1:	Sample_001_Well_001_A05.fcs	Lymphs/CD4 T/g+2-T-, Freq. of Parent	Costim	Untreated	1 0.018 0.00439 0
2:	Sample_001_Well_002_A06.fcs	Costim + Peptide	Untreated	2 0.074 0	
3:	Sample_001_Well_003_A07.fcs	Costim + Peptide	Untreated	3 0.03 0	
4:	Sample_001_Well_004_A08.fcs	Costim + Peptide	Untreated	4 0.072 0	
5:	Sample_001_Well_005_B05.fcs	Costim A	5 0.026 0.00217 0		
6:	Sample_001_Well_006_B06.fcs	Costim + Peptide A	6 0.27 0.028 0		
7:	Sample_001_Well_007_B07.fcs	Costim + Peptide A	7 0.3 0.027 0		
8:	Sample_001_Well_008_B08.fcs	Costim + Peptide A	8 0.28 0.035 0		
9:	Sample_001_Well_009_C05.fcs	Costim B	9 0.00696 0.00232 0.00232 0		
10:	Sample_001_Well_010_C06.fcs	Costim + Peptide B	10 0.26 0.012 0		
11:	Sample_001_Well_011_C07.fcs	Costim + Peptide B	11 0.66 0.019 0		
12:	Sample_001_Well_012_C08.fcs	Costim + Peptide B	12 0.4 0.011 0		
13:	Sample_001_Well_013_D05.fcs	Costim C	13 0.015 0.00057 0.00014 0		



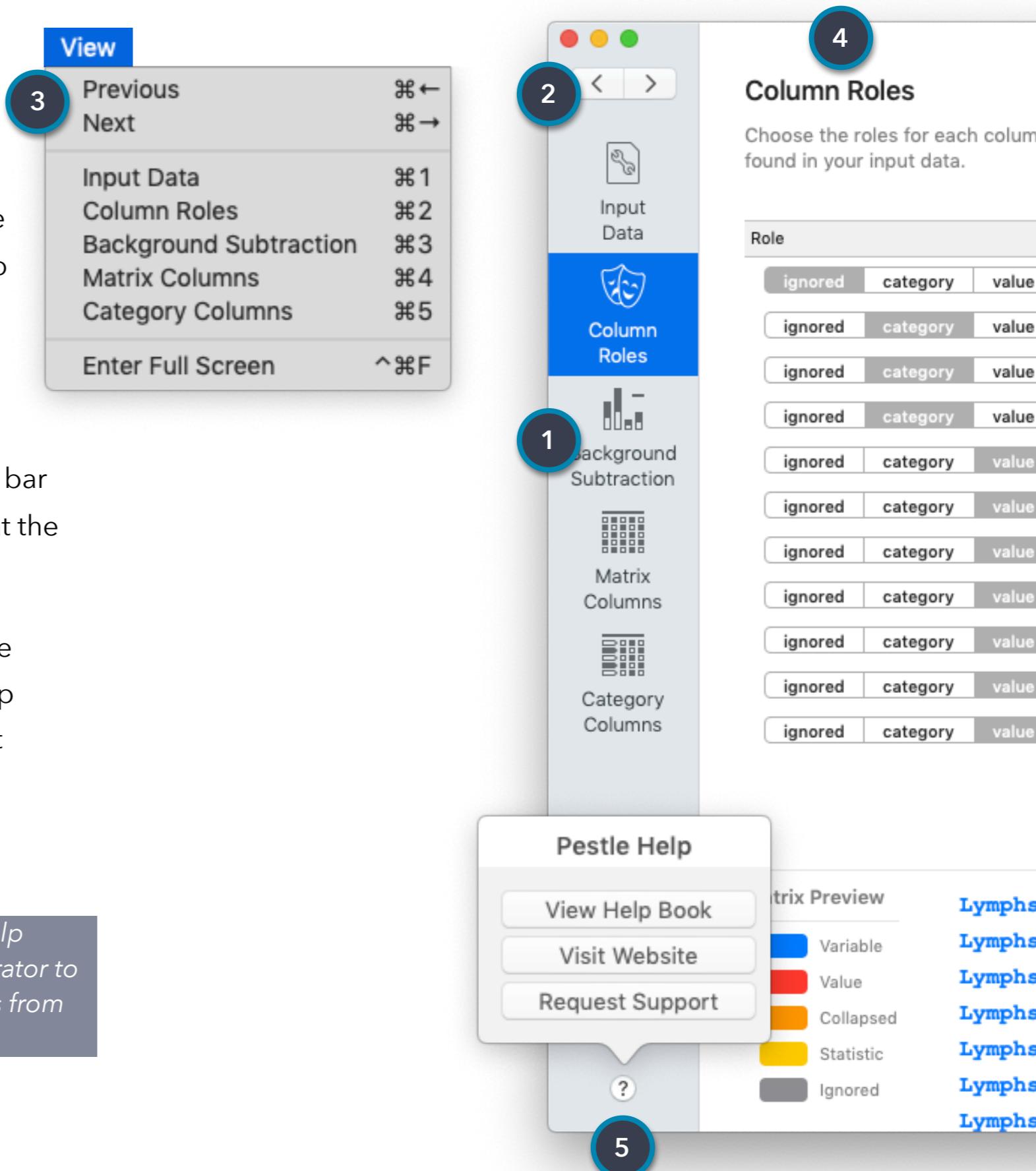
3.1 Navigation & Help

The images to the right contain several numbered areas of the primary navigation controls. Pestle's primary functions are easily navigated via tabs in the navigation bar ①, the back/forward button at the top of the navigation bar ②, the application's View menu ③, or by the keyboard shortcuts shown in the View menu.

The currently selected view is highlighted in the tab bar and the current view's title and description appear at the top-left of the view ④.

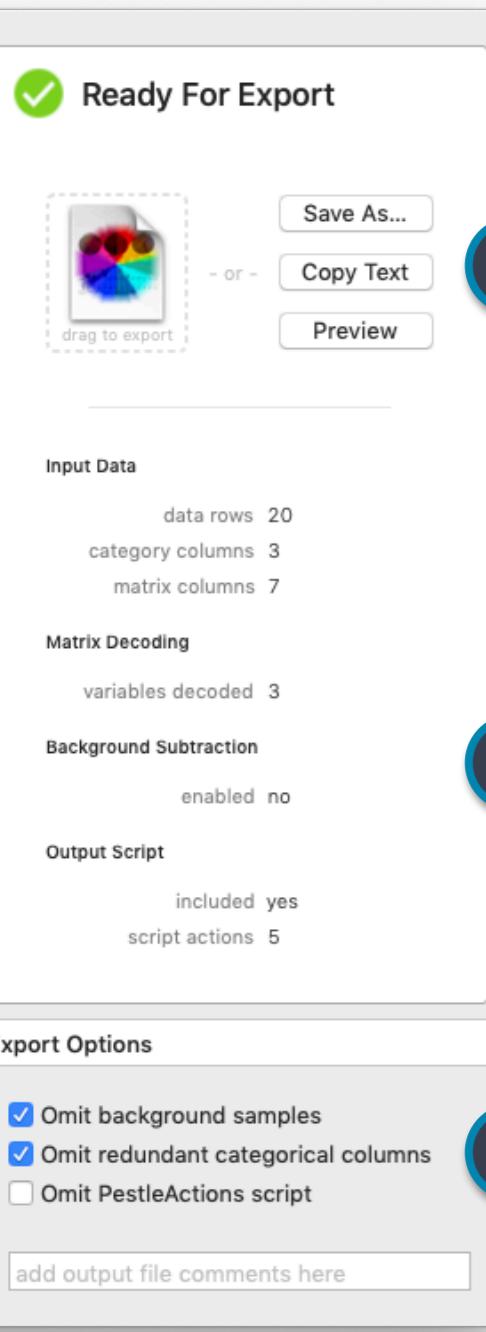
If you need help, you can click the Help button at the bottom of the navigation bar ⑤. From the Pestle Help menu, you can choose to view this user manual, visit Pestle's website, and submit a support request.

Note: An Internet connection is required to use the Help functions. You may need to ask your network administrator to allow Pestle https access if you receive error messages from Pestle or your Mac when using them.



3.2 Errors & Output

When Pestle is able to export its processed output as a valid SPD file, the “Ready For Export” view will appear in the right-hand panel.



The controls at the top ① allow you to drag the file to a folder or the desktop, choose a location and name with the standard Save As dialog, copy the output to the pasteboard, or preview the output without exporting it.

The summary section ② contains an overview of the processed output for your review.

The Export Options section ③ lets you further customize the output prior to export, including adding your own notes as comments in the SPD file.

When Pestle is unable to process the data and provide output, the right-hand panel will instead show “Export Unavailable” and list one or more relevant errors explaining why ④.

You can click the arrow buttons on each error ⑤ to navigate to the relevant tab to correct the error.

Any tabs with errors preventing output are highlighted with a red dot ⑥ next to the tab in the navigation bar.

As individual errors are resolved, they disappear from the error list. When all errors are resolved, Pestle once again displays the “Ready For Export” view.

Note: “No errors” does not necessarily mean “useful output”. It is possible and even easy to create an SPD file that will import successfully into SPICE yet be unhelpful or nonsensical. It’s up to you to Think Before You Export.

3.3 Input Data

Input data is the beginning of the Pestle workflow. In order to function, Pestle must have at least one input file. You may combine additional files in the form of appended data or merged databases. Pestle combines the input data for use in its later operations. You can also perform find & replace style text replacement on this combined input.

3.3.1 Opening an Initial Input File

When initially launching Pestle, it will prompt for an input file (**Figure 3.3.1a**). You must select an input file in order to proceed.

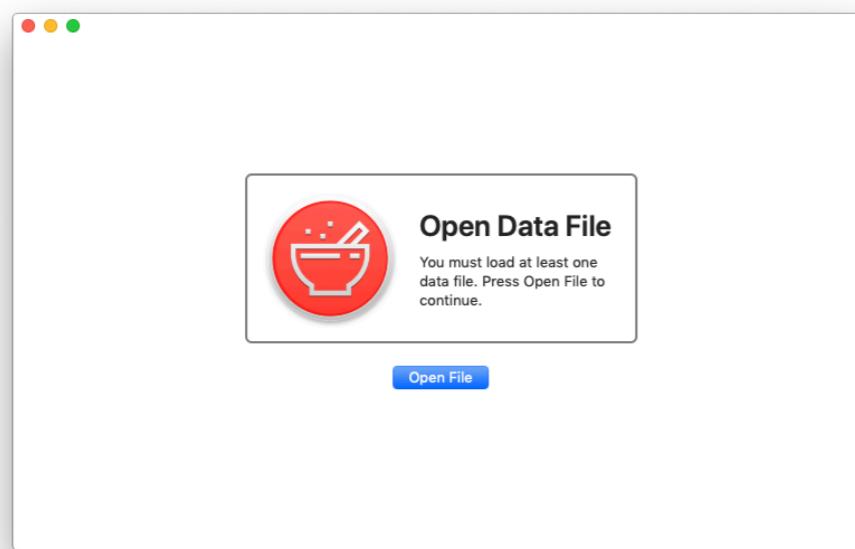


Figure 3.3.1a

Once you choose a file, the navigation controls appear and the Input Data tab is active (**Figure 3.3.1b**).

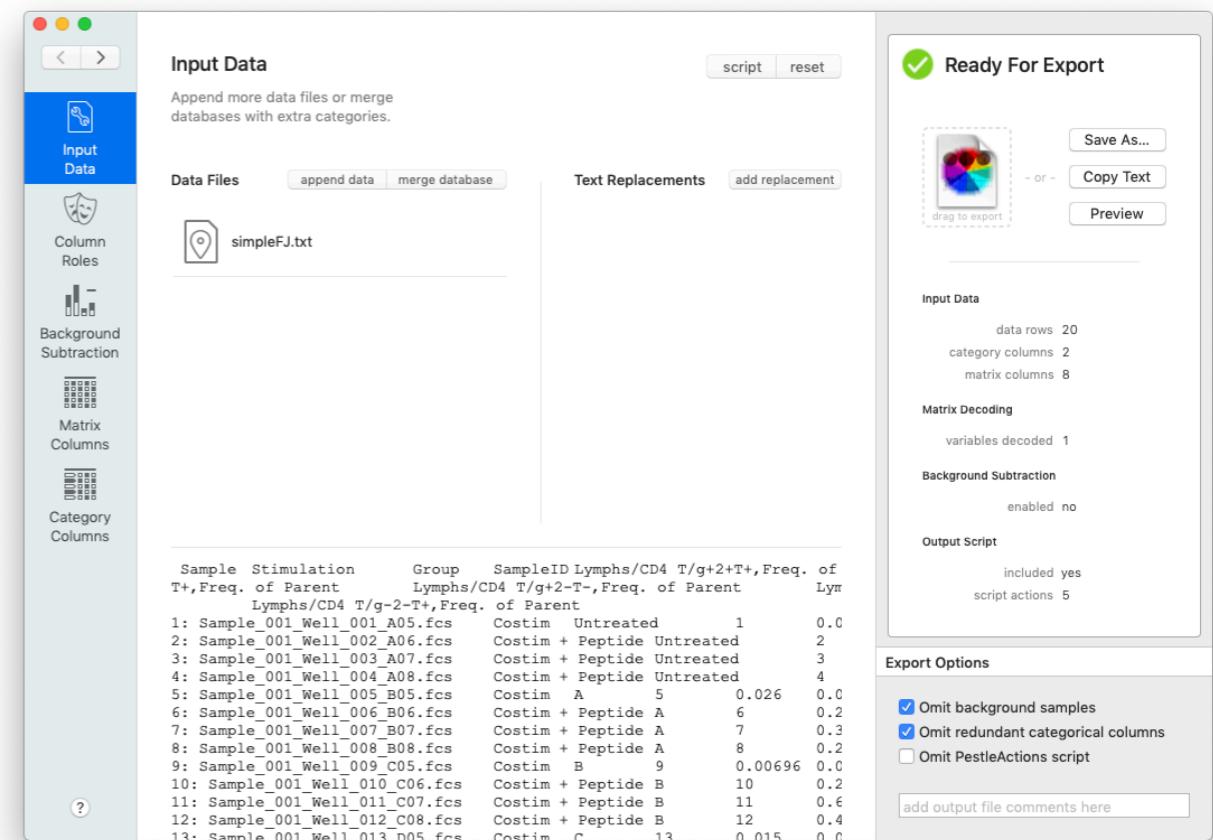


Figure 3.3.1b

Notice the preview pane at the bottom of the Input Data view. This shows the input data text after all data files have been combined and all text replacements have been applied. This is useful for verifying proper merging as well as figuring out unexpected errors, as it is the final input data Pestle will use in all downstream operations.

3.3.2 Appending Data

You can append a data file by clicking *append data* and choosing the file to append. It will appear in the Data Files list with an additional *behavior* control to allow you to select the merge behavior.



There are 3 merging options (*strict* is the default option):

strict, which requires the appended file to contain the same columns with the same names, in the same order as the existing files;

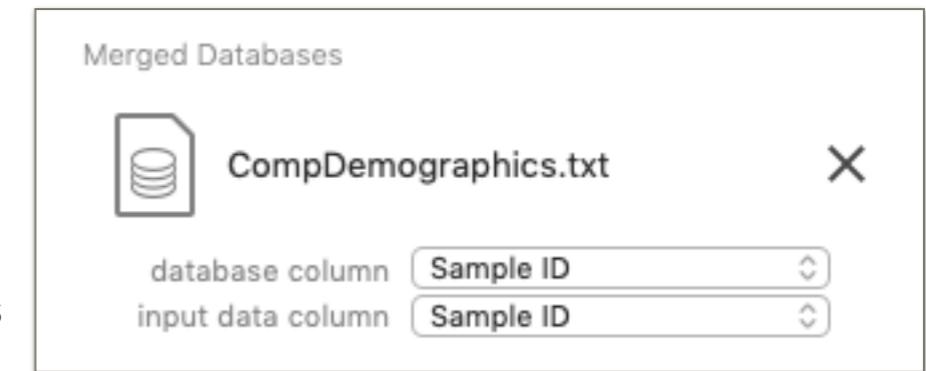
loose, which merely matches by column name in any order;

ignore, which attempts no matching and merely appends the columns in the order they're encountered.

3.3.3 Merging Databases

You can merge databases (such as categorical information such as demographics) by clicking *merge database* and selecting an appropriate file. The file will appear in the Data Files list (under the Merged Databases group).

Pestle will initially attempt to auto-detect the columns by which to merge the categorical information. This may or may not succeed.



See the complex example in section 2.1.2 for details. If you make your own selections in the controls beneath the database file, these selections will persist (Pestle will not attempt to auto-select them again, unless there is an error with the current selection).

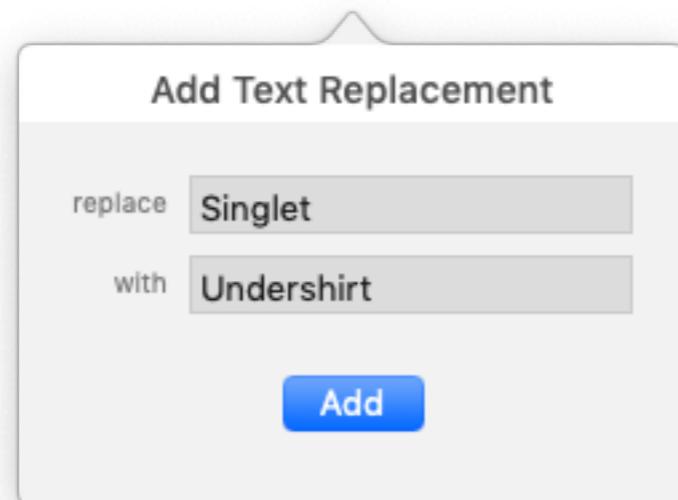
The *database column* and *input data column* controls designate the text columns (in the merged database file, and the combined data files, respectively) that will be used to match categories when merging.

3.3.4 Input Text Replacement

As mentioned, Pestle can perform text replacements on the combined input data. These replacements are performed prior to any operations on the remaining tabs.

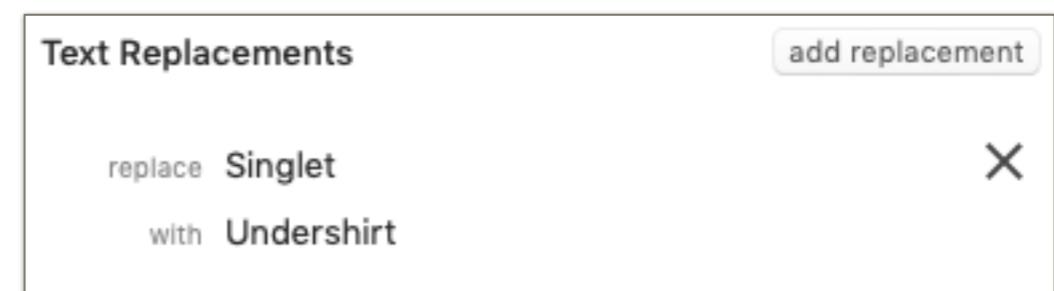
This can be handy for a number of reasons as the replacements are performed on all the combined input data (as opposed to each individual data file) and do not affect the content of your input data files. You can have as many replacements as you like and can drag to reorder them, which allows you to layer successive replacements together to perform more complex text transformations.

To add a text replacement, click *add replacement* above the Text Replacements column. A popover will appear, into which you can enter



the term to replace (*replace*), and an optional replacement term (*with*), which can be left blank to delete the *replace* term altogether. Click Add to add the new replacement.

The new text replacement will appear in the Text Replacements list. You can drag the replacements into any order by clicking and dragging anywhere on the replacement except the X, which will remove the replacement.



You can verify the results using the preview pane at the bottom of the Input Data view. There you can see our example above has caused all occurrences of "Singlet" to be replaced with "Undershirt" in the input data for the complex example in section 2.1.2.

Sample ID	Peptide	Trial	Timepoint	Undershirt
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M+T+,Median,<G				
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M+T+,Median,<R				
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M+T-,Median,<G				
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M+T-,Median,<R				
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M-T+,Median,<G				
Undershirts/Live/Lymphs/CD3+/CD4-/CD8+/7+G+2+M-T+,Median,<R				

3.4 Column Roles

In the Column Roles view, Pestle displays the text columns from the final input data (**Figure 3.4**). Each text column must be assigned one of three possible processing roles:

ignored – the column will not be used by pestle in any way and will be omitted from the final output;

category – the column is used to discriminate data values into subsets;

value – the column contains data values to be analyzed quantitatively by SPICE.

Pestle initially attempts to guess the appropriate roles for each column (and will only do so again if the input data changes significantly). Changing column roles will usually “break” downstream settings (those in the subsequent tabs).

3.4.1 Assigning Roles to Columns

Pestle makes it easy to assign roles to one or more columns. To change a single column’s role, simply click the desired role next to the column name in the list.

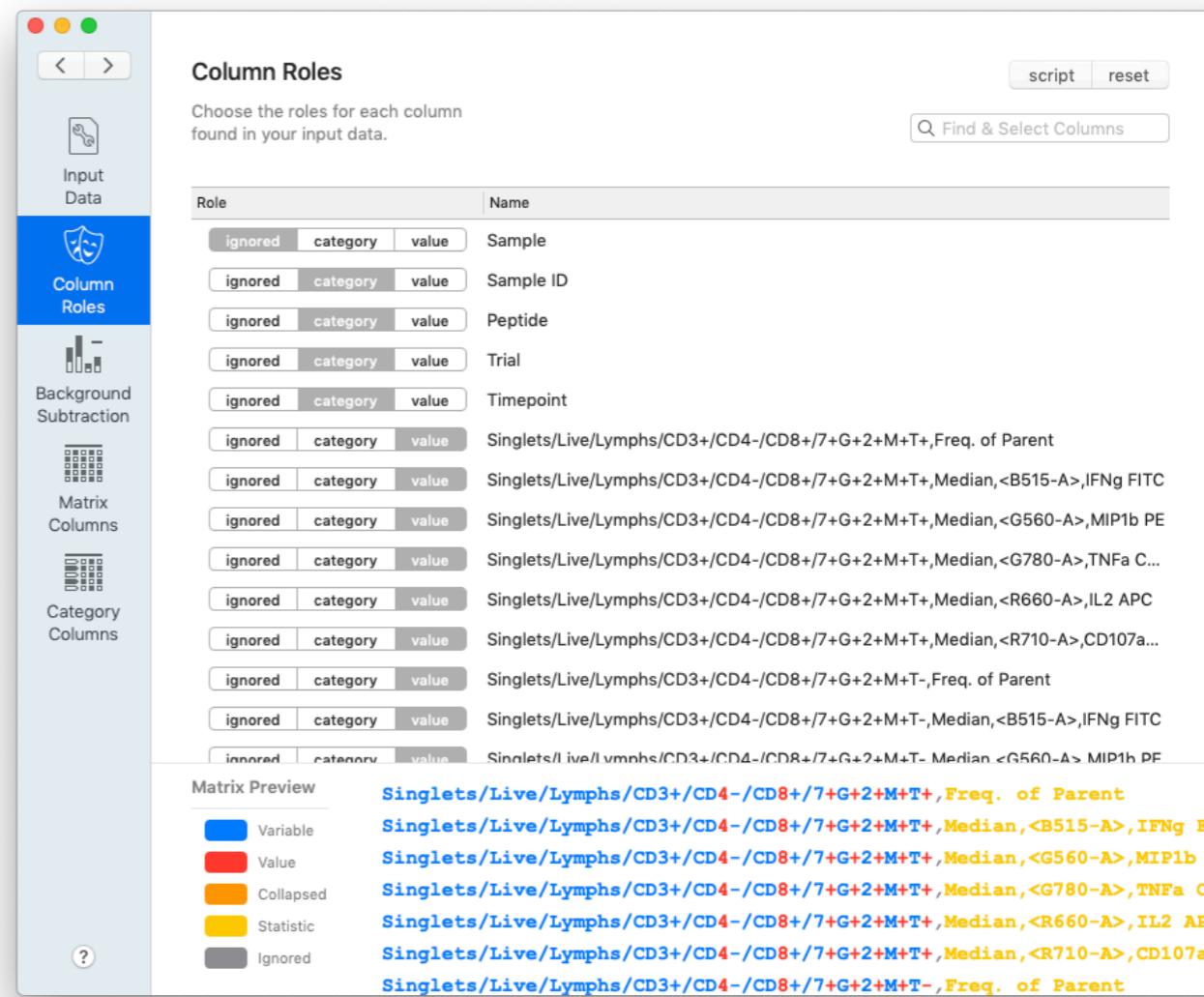


Figure 3.4

You can also select multiple columns with the mouse and keyboard, or by using the Find & Select Columns field at the top of the list. Changing the role of any selected column will change the roles of *all* selected columns.

3.4.2 Matrix Preview

Pestle runs through a heuristic routine to attempt to identify matrix variables and values based on the names of the columns with a role of *value*.

The Matrix Preview pane at the bottom of the Column Roles view in figure 3.4 is designed to help you understand how Pestle is interpreting the value column names, which often contain FlowJo gating paths. If something doesn't look right here, you should first check to make sure every column in the list has the correct role assigned.

In figure 3.4, Pestle is correctly determining the distinction between variables and values as identified by the gating path common to the *value* columns.

The color key on the left of the preview pane is used to read the color-coded list of *value* column names. In this example, there are 8 matrix variables (the 7 proper variables as well as a Statistic variable denoted by the trailing commas in each name).

If Pestle does not decode any variations of the post-comma portions of the names (or there are none), no Statistic variable is created.

Pestle prefers to look for similarities in the names of all *value* columns. Gating paths, for example, have ranges of characters in common (used as variables) and those that differ (used as values).

If, however, the *value* column names start with a difference, all subsequent differences and similarities are ignored and a single matrix variable called *Variable* is created using each *value* column name as a value of that single variable. This is also true if each *value* column name is unique.

In both of these cases, Pestle does not attempt to decode a Statistic variable and any "post-comma" text is assumed to be part of the value name.

3.5 Background Subtraction

Pestle allows you to perform background correction on certain values. In a common example, datasets contain frequencies of cytokine positive events in subsets after stimulation with antigen; the control sample (stimulation without antigen) value needs to be subtracted from each of these values. You can do this using the Background Subtraction tab (**Figure 3.5**).

For background subtraction, you need to specify which columns should be processed for background subtraction, and how Pestle is to associate the background sample with each positive sample.

Configuring Background Subtraction

Figure 3.5 shows a fully-configured background subtraction operation for the simple example in section 2.2.1 (the simpleFJ.txt file). For a walkthrough, please refer to that example.

As it's a multi-step process and modifies the values, you must first enable it with the "big old enable switch" ①. Doing so will initially draw complaints from Pestle in the form of errors about missing configuration.

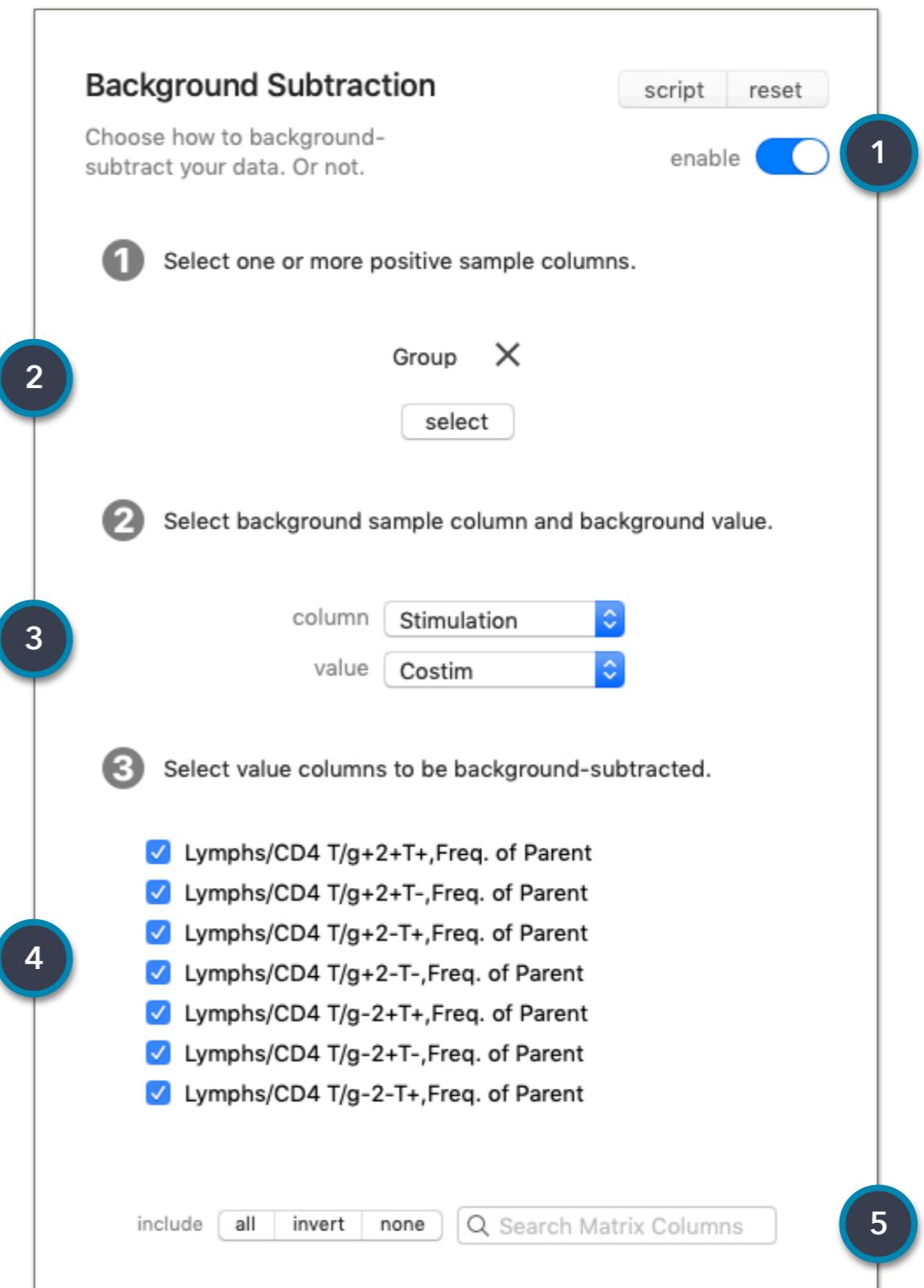


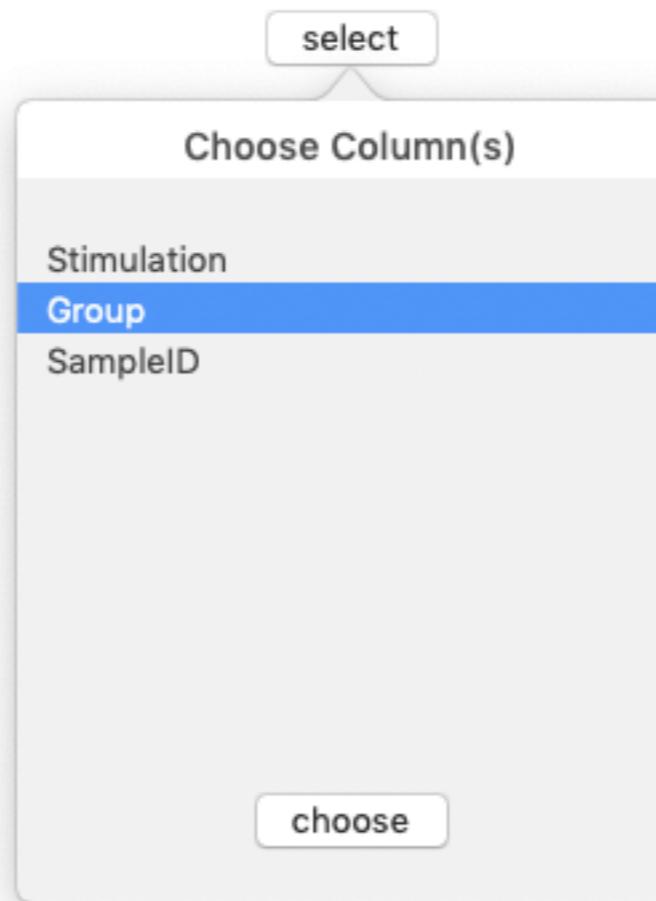
Figure 3.5

You'll also be prompted, one step at a time, to select one or more positive sample columns ②, the background sample column and value ③, and the value columns to be background-subtracted ④.

To satisfy step 1, click *select* and choose one or more columns in the popover. As with any list or table in macOS, you can hold the Command button while clicking to select multiple items. Click *choose* to commit your selection. Step 2 will be revealed.

Step 2 requires you to designate the column and its value used to identify background samples. Select these using the *column* and *value* controls to move on to step 3.

The third and final step requires you to designate the *value* columns to be background-subtracted. To do so, place a check next to the desired columns. You may select multiple columns and check (or uncheck) any member of the selection to apply that change to all selected columns.



You may also use the convenience controls at the bottom of step 3 ⑤ to manipulate the selection. The *include* control makes it easy to include all or none, or invert the selection you've made (ie, if it's easier to select a few you do *not* want to be included, do so, then *invert* that selection). The search field will select columns matching your search term. Just enter the search term and press return to select the matching columns, then check (or uncheck) any one of them to change the inclusion of all selected columns, as usual.

Once all the steps are satisfied, Pestle will update and (assuming there are no errors in other tabs) be ready to export. In the Export Options panel, you could choose *not* to omit the background samples from the output (they are omitted by default).

You can also easily toggle background subtraction once it's configured by flipping the *enable* switch. Provided no input data or column role changes have occurred, your configuration is remembered if you reenable this function.

3.6 Matrix Columns

The Matrix Columns tab is used to perform the final identification of variables extracted from the names of *value* columns (for example, as encoded by the Boolean gate combination names), as well as to edit and reformat variable and value names for best presentation in SPICE. The idea is to annotate the data sets to provide the easiest path to analysis of the data.

Matrix Variables

The Variables list ① shows all of the variables Pestle decoded from the *value* column names. The settings here affect not only the appearance in SPICE but how the matrix is encoded and compressed in the SPD file. It isn't necessary to make any changes here but it's often desired.

You may specify a custom name for any variable by editing the Custom Name field. This will override the original name and affects the variable's name as displayed in SPICE.

The Matrix ID field is used to override

the string used to identify the associated variable in the exported matrix columns. This can be used to simplify (and shrink) the matrix header for readability. More importantly, it can be used to resolve "name collisions", which may cause SPICE to decode the matrix incorrectly or not at all. Pestle tries to prevent the most obvious collisions (duplicate matrix IDs or those that are contained in a value or variable's matrix ID).

To remove a custom name or matrix ID, simply delete it.

The Collapse field is used to treat a variable's values as belonging to the *next* variable after it in the list. See the complex example in section 2.1.2 for an example of when this might be useful.

Variables		Values			
Original Name	Custom Name	Matrix ID	Collapse	Original Name	Custom Name
Lymphs/CD4 T/g	gIFN	gIFN	<input type="checkbox"/>	+	+
2	IL2	2	<input type="checkbox"/>	-	-
T	TNF	T	<input type="checkbox"/>		

Matrix Values

The Values list ② lists the unique values of all variables. In the case of simpleFJ.txt, all the decoded variables have only a + or - value, so these are only shown once. As with variables, values can have custom names. This affects how the values appear when displayed in SPICE.

You could, for example, rename + and - to *Positive* and *Negative* – or *Bob* and *Not-Bob* if you like – by editing the custom name field.

You can also use the *rename +/-* button to apply a one-time change to rename + to • (a mid-dot) and - to a space (since values cannot be blank, a space is used).

Again, this is a one-time operation for convenience, since it is a common preference.

To remove custom names, just delete them from the Custom Name field and the original name will be used.

Original Name	Custom Name	Matrix ID	Collapse
Lymphs/CD4 T/g	gIFN	gIFN	<input type="checkbox"/>
2	IL2	2	<input type="checkbox"/>
T	TNF	T	<input type="checkbox"/>

Original Name	Custom Name
+	+
-	-

Matrix Preview

- Variable
- Value
- Collapsed
- Statistic
- Ignored

```

Lymphs/CD4 T/g+2+T+,Freq. of Parent
Lymphs/CD4 T/g+2+T-,Freq. of Parent
Lymphs/CD4 T/g+2-T+,Freq. of Parent
Lymphs/CD4 T/g+2-T-,Freq. of Parent
Lymphs/CD4 T/g-2+T+,Freq. of Parent
Lymphs/CD4 T/g-2+T-,Freq. of Parent
Lymphs/CD4 T/g-2-T+,Freq. of Parent
Lymphs/CD4 T/g-2-T-,Freq. of Parent

```

3.7 Category Columns

The Category Columns tab, like the Matrix Columns tab, lets you customize the names of the categorical variables (category columns become category variables when exported to SPICE) in the exported SPD file. It also lets you create new categorical variables for convenience.

Category Variables

The Category Variables list ① displays all of the categorical variables that will be exported to the SPD file. Here you can override variables' original names using the Custom Name field. This affects how these variables appear when viewed in SPICE in the same way as custom names for matrix variables.

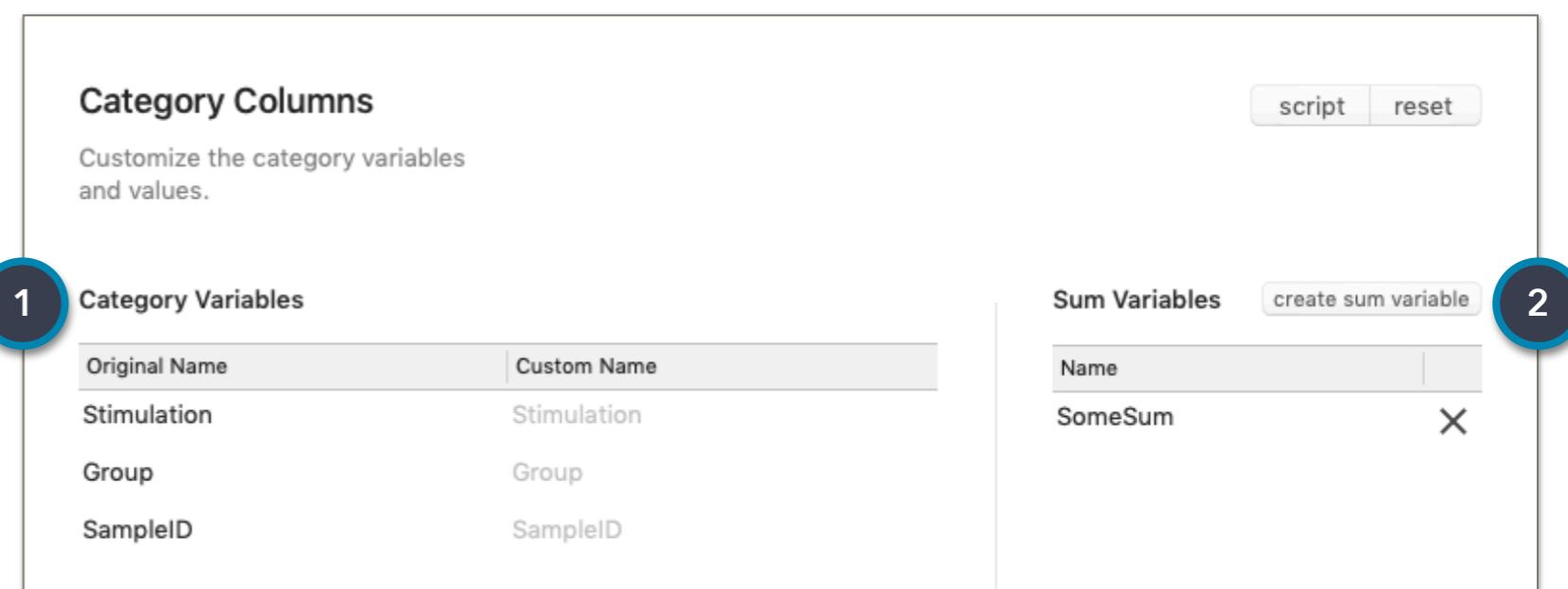
If you'd like to remove these customizations, simply delete the custom name to resume using the original name.

Redundant Columns

Redundant columns are categorical columns with only a single value. That is, every data row that is exported has the

same value for a given categorical column. This would produce a rather useless variable in SPICE. The Export Options pane has a helpful checkbox called *Omit redundant categorical columns*, which is enabled by default.

This prevents Pestle from including such columns in the SPD file, thus putting it out of SPICE's misery. You may of course choose to disable this, but note that it could result in "duplicate categories" warnings in SPICE, which means that only the first measurement decoded for a given category is actually imported and the others are discarded.



Sum Variables

The Sum Variables list ② allows you to create additional categorical variables for use as additional descriptors of the data.

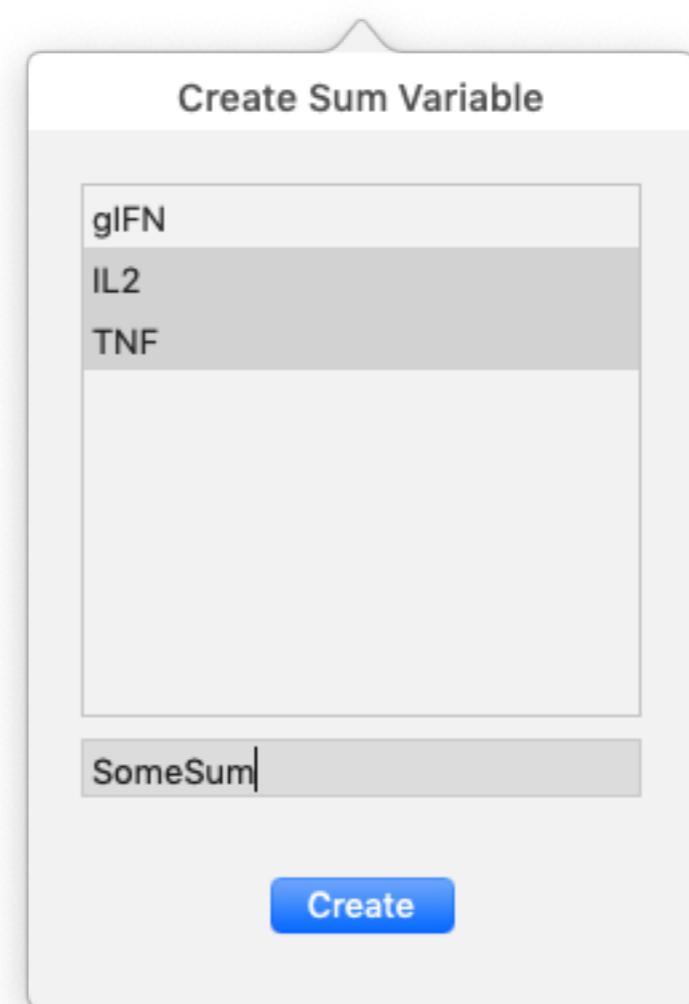
For example, in multifunctional studies of T cell subsets, in which several functions were simultaneously measured, you might wish to have a category which defines how many functions are expressed simultaneously. Thus, an *IFN+IL2+TNF+* would be a 3+ category; *IFN+IL2+TNF-*, *IFN+IL2-TNF+*, and *IFN-IL2+TNF+* would all be 2+, and so forth.

This would allow you to group or overlay by functional state. In this case, a + is considered as having a value of 1, and a - is considered as 0.

To create a sum variable, click the *create sum variable* button at the top of the Sum Variables list. A popover will appear, showing all of the available matrix variables. You will need to select at least two or more matrix variables. You can multi-select by holding down the Command key while clicking.

Once you've made your selection, you'll be prompted to provide a name for the new sum variable. This name must be unique.

Enter the name and click Create or press return to create the new variable.



The new variable will appear in the Sum Variables list. To delete it, click the X to the right of the variable.

4

SCRIPTING

Pestle keeps track of your actions as you manipulate your data. This allows you to make sure you have consistent and complete manipulations performed on every similar data set.

These Pestle Actions can be saved or loaded and applied to a new data set. Scripts are also embedded by default in the SPD files it generates unless you disable this in the options panel prior to exporting.

Whether stored in the SPD file or alone in a separate file, Pestle Action scripts are lists of plain-text commands that can be viewed and edited in any text editor.

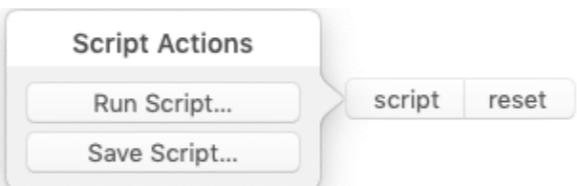
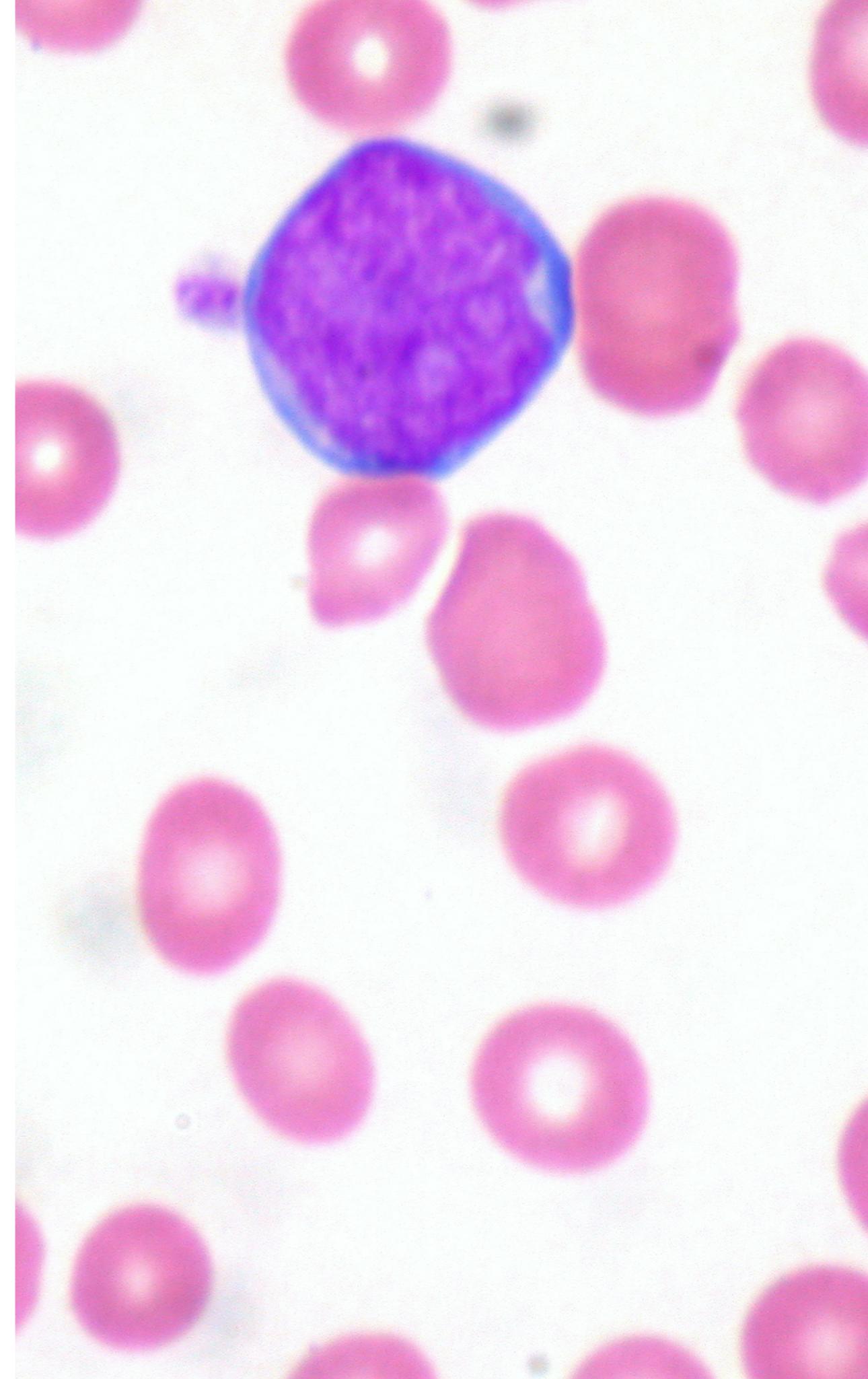


Figure 4a

You can access Pestle's scripting capabilities via the Script controls (**Figure 4a**) or the Script menu.



4.1 Running Scripts

As mentioned in the beginning of this section and demonstrated at the end of section 2.1.1, you can run a script to apply (or “replay”) actions taken from a Pestle-generated SPD file or standalone text file using the Run Script sheet. This is accessible from the *script* button seen in figure 4a, or the Script menu.

To do so, choose Run Script from the *script* button (or press Command+L), select the text or SPD file containing the script, then click Open. Pestle will interpret any script actions it finds and you will be presented with the Run Script sheet seen in **Figure 4.1a**. If Pestle is unable to find and successfully interpret at least one valid Pestle Action script command, Pestle will inform you and the Run Script sheet will not appear.

In this sheet, each successfully-interpreted action is displayed in the list. The controls at the bottom allow you to filter for one or more categories of actions so that only those actions will be applied when the script runs.

Note: You can also choose to show or hide actions that have been deprecated since previous versions. Deprecated actions will not run whether they are shown or not.

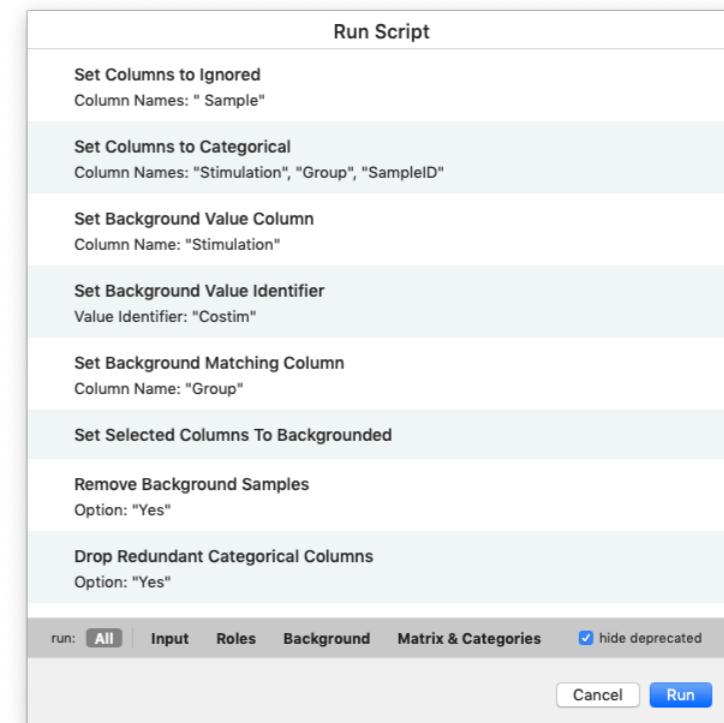


Figure 4.1a

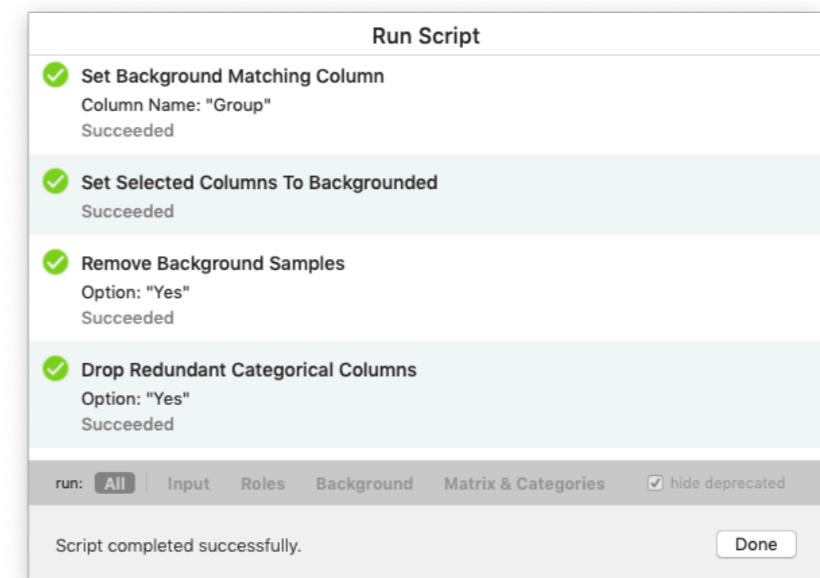


Figure 4.1b

You can of course cancel the sheet without running the script or choose Run to apply the actions to your data set.

Figure 4.1b shows the script from figure 4.1a as having run successfully. Note the green checkmarks next to each successfully-completed action. Failed actions will instead show a red X and the script will halt at that point. In such cases, rather than seeing “Succeeded” beneath the failed action, you will see error details. You can click Done to dismiss the Run Script sheet. You can then also run additional scripts as needed.

4.2 Saving Scripts

Saving scripts is easy to do from any tab in the Pestle interface. Click the *script* button, choose Save Script, then choose the folder and file name for the new script and click Save. This can also be done from the Script menu.

4.3 Command Reference

Following is a complete reference of commands Pestle recognizes. You can edit them manually (inTextEdit, for example) if you want to change them.

Each Pestle action is a single line in the file. Some actions require a parameter, and a few require a value as well; these are on the same line, separated from each other by a space.

Action names must be preceded by an asterisk (*); the presence of the asterisk is ignored by Pestle. Any line that does not contain a recognizable Action is ignored by Pestle. An Action name is all one word (no spaces).

Action parameters and values must be enclosed in double-quotes; if a parameter or value contains a double-quote, then that double-quote must be quote-escaped by placing two double-quotes where the single double-quote should be (“value ““with quote”” inside”) in order to identify it as part of the parameter or value, and not as a quoted field. This is similar to comma- and tab-separated data files.

The command reference list follows on the next page.

Column Roles

Action Name	Parameter	Value	Explanation
ChangeColumnRole	Column Name	"Ignored" "Category" "Variable"	<i>Set specified column to specified role</i>
SetIgnoredColumns	Text-stream-encoded list of column names		<i>Set specified columns to ignored role</i>
SetCategoricalColumns	Text-stream-encoded list of column names		<i>Set specified columns to category role</i>
SetValueColumns	Text-stream-encoded list of column names		<i>Set specified columns to value role</i>

Background Subtraction

Action Name	Parameter	Value	Explanation
BackgroundMatchColumn	Column Name		<i>Set background matching column</i>
BackgroundValueColumn	Column Name		<i>Set background value column</i>
BackgroundValueIdentifier	Value Name		<i>Set background value identifier</i>
RemoveBackgroundSamples	"Yes" "No"		<i>Remove background samples from output</i>
SelectAllBackgroundColumns			<i>Select all background-subtractable columns</i>
SelectBackgroundColumnsByString	Search String		<i>Select background-subtractable columns by searching for the specified term parameter</i>
SetSelectedColumnsForBackground			<i>Set the selected columns to be background subtracted</i>
SetSelectedColumnsNoBackground			<i>Set the selected columns to not be background subtracted</i>
ChangeBackgroundStatus			<i>Toggle the background subtraction status of the selected columns</i>

Variables & Values

Action Name	Parameter	Value	Explanation
CollapseVariable	Name		<i>Collapse specified matrix variable</i>
EditMatrixVariableName	Name	New Name	<i>Rename specified matrix variable to new name</i>
EditColumnName	Name	New Name	<i>Rename specified categorical variable to new name</i>
EditValueName	Name	New Name	<i>Rename specified matrix variable value to new name</i>
DropRedundantCategoricalColumns	"Yes" "No"		<i>Omit redundant categorical columns from output</i>
RenamePlusAndMinus	"Yes" "No"		<i>Change "+" values to "•" and "-" values to a space</i>
ReorderVariable	Name	Index (an integer)	<i>Move the specified variable to the specified index</i>
CreateCategoricalMatrixCol	New Name	Formula	<i>Create a sum category with the specified name, using the specified formula, which is a list of category column names separated by "+"</i>

Input Data Search & Replace

Action Name	Parameter	Value	Explanation
DataFileSearchReplace	Text-stream-encoded list of search and replace values		<i>Perform a search and replace of the combined Input Data text with the specified text-stream-encoded table of search and replace values</i>

Deprecated Commands

Action Name	Parameter	Value	Explanation
<i>Input Data - Deprecated in v2; Pestle Actions now only work once the Input Data has been configured</i>			
ReadDataFile			<i>Deprecated</i>
AppendDataFile			<i>Deprecated</i>
MatchAppendColumnNames			<i>Deprecated</i>
LoadDatabaseFile			<i>Deprecated</i>
SetDatabaseMatchColumn			<i>Deprecated</i>
DatabaseMergeKeyColumn			<i>Deprecated</i>
ExcludeDatabaseRow			<i>Deprecated</i>
<i>Multiplication - Deprecated in v2; Pestle no longer supports the premultiplication functionality of v1</i>			
UseFormula			<i>Deprecated</i>
FormulaColumn1			<i>Deprecated</i>
FormulaColumn2			<i>Deprecated</i>
FormulaColumnName			<i>Deprecated</i>
DoNotIncludeFormulaInputs			<i>Deprecated</i>

4.4 Example Script

Following is a Pestle Action script created using the simple example in section 2.1.1.

```
*PestleActions
*SetIgnoredColumns "0,0, Sample"
*SetCategoricalColumns "0,0,Stimulation,1,0,Group,
2,0,SampleID"
*SetValueColumns "0,0,Lymphs/CD4 T/g+2+T+,Freq. of Parent,
1,0,Lymphs/CD4 T/g+2+T-,Freq. of Parent,2,0,Lymphs/CD4 T/g+2-
T+,Freq. of Parent,3,0,Lymphs/CD4 T/g+2-T-,Freq. of Parent,
4,0,Lymphs/CD4 T/g-2+T+,Freq. of Parent,5,0,Lymphs/CD4 T/
g-2+T-,Freq. of Parent,6,0,Lymphs/CD4 T/g-2-T+,Freq. of
Parent"
*BackgroundValueColumn "Stimulation"
*BackgroundValueIdentifier "Costim"
*BackgroundMatchColumn "Group"
*SetSelectedColumnsForBackground
*RemoveBackgroundSamples "Yes"
*DropRedundantCategoricalColumns "Yes"
```

[end of file]

