FACScan 4 and 5 Color Upgrade
Rainbow
Users Guide
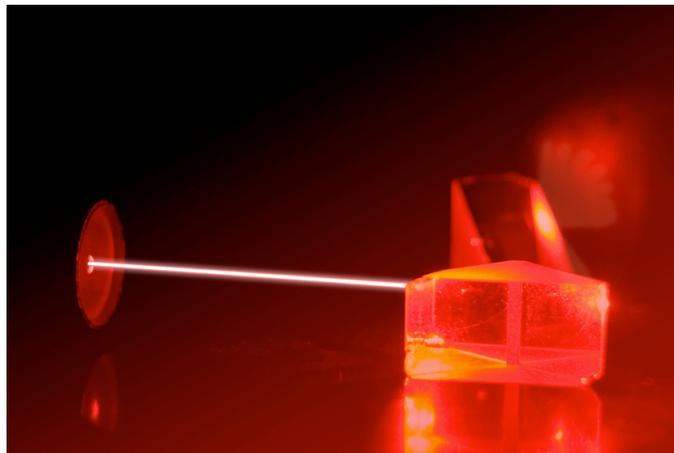
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Overview

This upgrade provides for one or two additional fluorescence parameters, using red laser excitation. The additional parameter(s) are controlled from the Macintosh computer using the Rainbow software.

Hardware Specifications

A 25mW red diode laser or a 40mW violet solid state laser is focused approximately 130 microns above the existing blue laser. This intercept is imaged into a 2mm diameter fiber, and then re-imaged onto one or two red sensitive PMTs.

For the violet laser, the fourth color uses a 475/15 or 430/30 filter for Pacific Blue, and for the fifth color, a 545/30 filter for Pacific Orange. For the red laser, the fourth color uses a 666/27 filter for use with APC, and the fifth color uses a LP740 filter for use with APC-Cy7.

Rainbow Software

When the Rainbow software application is launched, the following window appears (the labels on the controls will vary depending on the laser configuration chosen in the preferences window):

Rainbow Settings

Status: Ready

Compensation

FL3 – 12.9 % FL4

FL4 – 21.2 % FL3

FL4 – 30.5 % FL5

FL5 – 22.8 % FL4

Detectors/Amps

Parameter 7: ⊙ FL4
○ Width

FL4 PMT Voltage: 673 Volts

FL4 Amp Mode: ○ Linear
⊙ Log

Parameter 6: ⊙ FL5
○ Area

FL5 PMT Voltage: 652 Volts

FL5 Amp Mode: ⊙ Linear
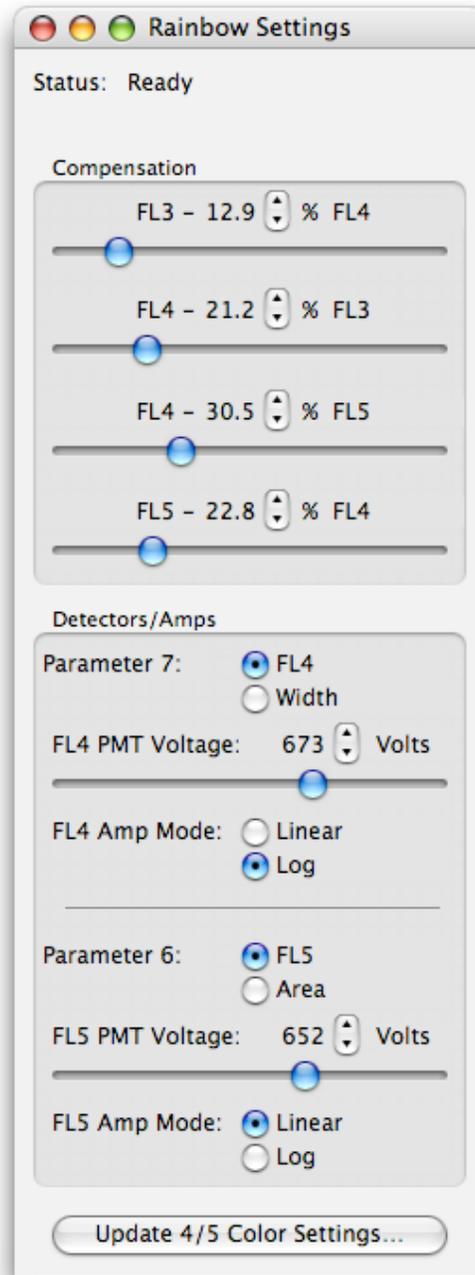○ Log

Update 4/5 Color Settings…

Figure 1

From this Rainbow Settings window, the compensation, detectors and amplifiers settings can be adjusted.
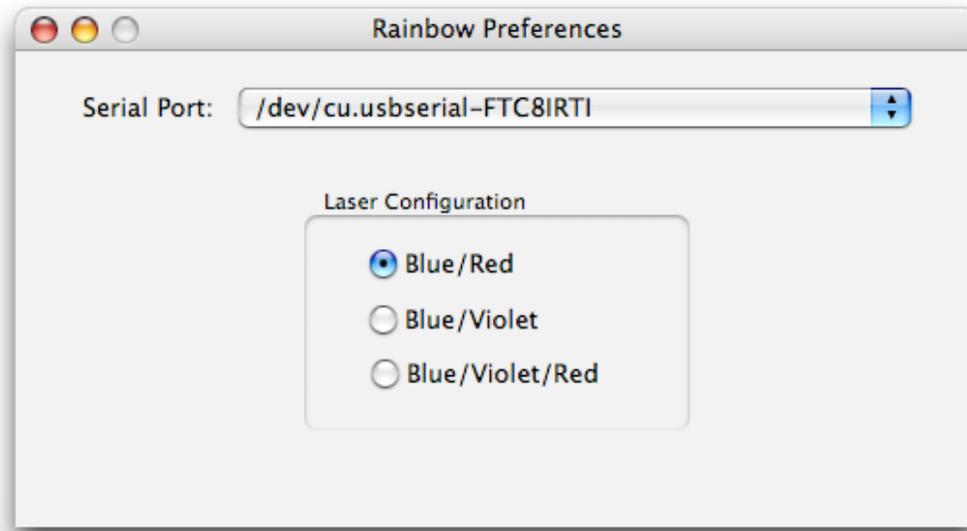
Rainbow Preferences Window

Figure 2

The Rainbow Preferences window (Figure 2) can be opened by selecting *Preferences...* from the *Rainbow* menu in Mac OS X, or from the *Edit* menu in Mac OS 9. From this window the serial port that is used to communicate with the hardware can be selected, as well as the laser configuration of the hardware. If the laser configuration changes, the labels in the main Rainbow settings window will change to reflect the lasers in use.

Please Note: *The preferences set in the Rainbow Preferences window (as well as the settings from the Rainbow Settings window) are unique for each User on Mac OS X. If you create a new User in Mac OS X, the first time you launch Rainbow when logged in as that User there will be no saved preferences or settings for Rainbow to use, so it will open with default values. You will have to adjust the settings and preferences, including possibly the serial port selection. The settings and preferences are saved for the currently logged in User when you quit Rainbow.*

Setting up the 1st, 2nd and 3rd Color Parameters

These parameters are set up as usual, using an unstained, FITC, PE, and PerCP or PE-Cy5 beads. Set the PMT voltages to place the unstained beads in the lower left quadrant on the FL1/2 and FL2/3 dot plots. Adjust the four compensation levels to place the stained beads in their respective quadrants.

Setting up the 4th and 5th Color Parameters

CellQuest Settings

Please Note: ***Be sure to specify that DDM is present in the Amplifier window, and be sure to save all 8 parameters in the acquisition and storage menu.***

Open up a CellQuest document as usual. Under *Acquire*, in the *Edit Reagent List* window, add "FL4-H" for P7, and "FL5-H" for P6. Under *Acquire* in the *Parameter Description* window, enter FL4-H for P7 and FL5-H for P6. Under *Plots* in the *Log Data Units* window, select 0-1023, so the FL4 and FL5 plots look the same as the other plots.

Set up a FSC/SSC, FL3/FL4, and FL4/FL5 dot plot. Under *Amplifier and Gains*, be sure to click DDM,**.**

Rainbow Settings

Open the Rainbow software. Note that switching between CellQuest and Rainbow is automatic when a window is clicked in either application. However, the first click is required to switch the application to the foreground, while a second click is required to actually change the desired parameter.

Make sure the appropriate serial port is specified for serial communication and that the correct laser configuration is chosen in the Rainbow Preferences window. The instructions below reference the labels that are shown if the "Blue/Red" laser configuration is chosen.

Set the FL4 and FL5 Amp Modes to "Log".

Select FL4 and FL5 parameters. If "Width" is selected instead of "FL4", the width parameter specified in CellQuest will be acquired rather than the FL4 parameter. Similarly, if "Area" is selected instead of "FL5", the area parameter specified in CellQuest will be acquired rather than the FL5 parameter. In the Rainbow Settings window, if "Area" or "Width" is selected, the corresponding Amp Mode is forced to "Linear", and the PMT Voltage controls are disabled.

Using a mix of unstained, PerCP, APC, and APC-Cy7 beads, set the FL3 PMT voltage in CellQuest, and the FL4 and FL5 PMT voltages in Rainbow to place the unstained beads around channel 128 on their respective axes.

Next adjust the FL3-%FL4, FL4-%FL3, FL4-%FL5, and FL5-%FL4 compensation levels to place the stained beads in their respective quadrants. For PerCP, APC, and APC-Cy7 typical values for the compensation are:

> FL3- %FL4 <1%
> FL4- %FL3 <25%
> FL4- %FL5 <50% (Note: If the APC-Cy7 tandem has been exposed
> to light, this value can exceed 80%)
> FL5- %FL4 <25%

If there is overlap of the FL3 reagent with the FL5 channel, it is necessary to use an off line compensation application such as Flo-Jo to perform the FL5-%FL3 compensation.

Running Samples

Set up gates, data file names, and data acquisition termination criteria as usual. Note that it is possible to gate on FL4 and FL5 parameters if desired. Data files will be saved with the same axis labels used to acquire the data (e.g. FL4-H or a width parameter).

It is necessary to use the "Linear" Amp Mode setting in FL4 and FL5 to obtain valid CVs. Other stats, such as means and percent positives, are valid in log or linear modes.

Using Rainbow to Save 4/5 Color Information to Data Files

Since Rainbow and CellQuest are run as separate applications, certain FCS data file header information will not be set correctly unless you either set up Rainbow to automatically update the file with the correct 5 color header information as the files are acquired, or use Rainbow to manually update the header information after the files are acquired.

Auto Scan

To allow Rainbow to automatically save your data files with the correct five color keywords please follow these steps:

1. Click on the *Update 4/5 Color Settings...* button at the bottom of the Rainbow Settings window. The following window will open:
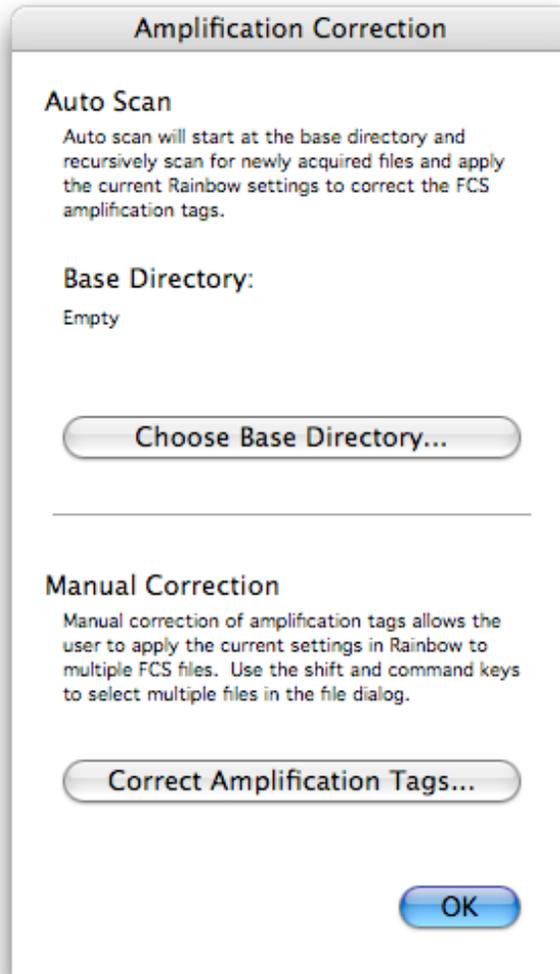
Figure 3

2. Click on the *Choose Base Directory...* button and select the directory where CellQuest will be saving the FCS data files. By choosing a directory to "Auto Scan", Rainbow will automatically update your CellQuest data files with the correct 5 color header information as they are saved by CellQuest into that directory.

Manual Correction

To use Rainbow to add or modify the five color header information in previously acquired FCS files so the information matches the current Rainbow settings, please follow these steps:

Please Note: Manually correcting the files will update the data files with the *current* Rainbow settings, which may be different from the Rainbow settings at the time of acquisition.

1. If the file is currently in use by CellQuest, you must quit CellQuest or CellQuest Pro in order for the file to be properly converted.
2. Click on the *Correct Amplification Tags...* button, and from the resulting file selection dialog select the files you wish to modify.
3. For each file that already contains five color header information, a warning dialog will appear that shows the information that is already in the file so that it may be compared with the current Rainbow settings before modifying the file. An example of this dialog appears below:
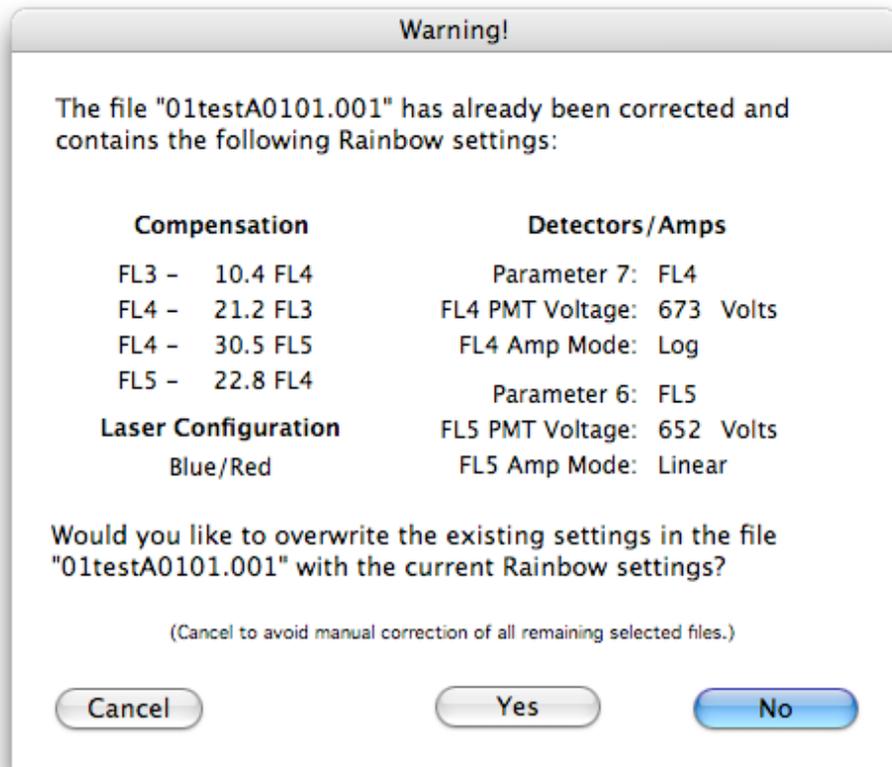

Figure 4

4. Clicking on the *Yes* button will modify the file. Clicking on the *No* button will skip the modification of that file, leave the file untouched. Clicking on the *Cancel* button will also leave the file untouched, and will abort the modification of any remaining files that were selected.

5. After all the selected files have been modified (or skipped), the *Amplification Correction* dialog will show the number of files that were corrected, as shown below:
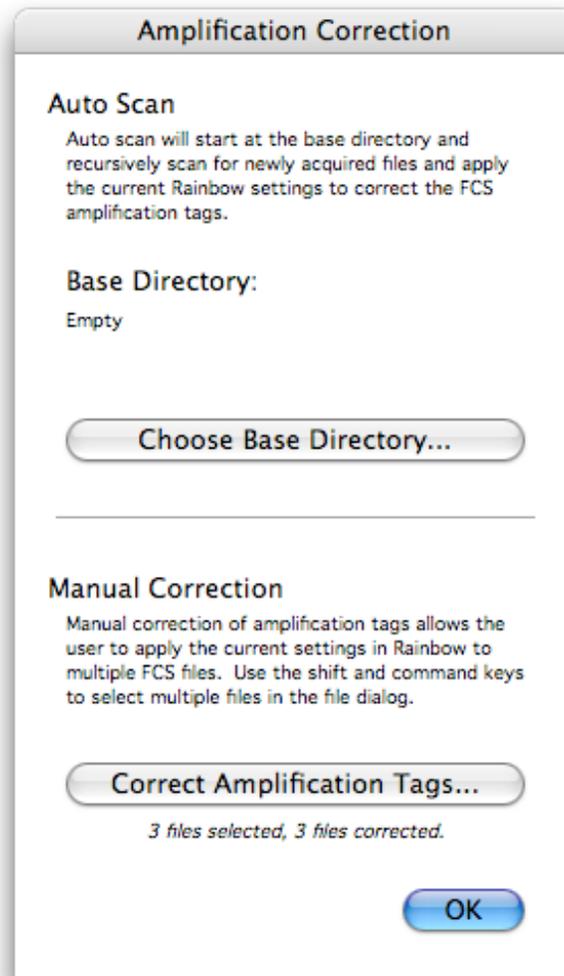

Amplification Correction

Auto Scan

Auto scan will start at the base directory and recursively scan for newly acquired files and apply the current Rainbow settings to correct the FCS amplification tags.

Base Directory:

Empty

Choose Base Directory...

Manual Correction

Manual correction of amplification tags allows the user to apply the current settings in Rainbow to multiple FCS files. Use the shift and command keys to select multiple files in the file dialog.

Correct Amplification Tags...

3 files selected, 3 files corrected.

OK

Figure 5

6. If you are finished with the manual correction of data files, click on *OK* to close the *Amplification Correction* dialog.

Importing Rainbow Settings

The five color instrument settings can be imported into the Rainbow software from any CellQuest data file that has previously been modified by Rainbow, either automatically or manually. This can be done by selecting the *Import Settings*... menu item in the File menu, or by pressing the "command (apple)–O" keys to open the data file containing the Rainbow settings you wish to import. After you choose the data file, the Rainbow Settings window will change to reflect the imported instrument settings.

Warning: If you import a file that was created on a system with a different laser configuration, the laser configuration in the preferences window will change as well. You will need to open the preferences window and ensure that the laser configuration is correct for your hardware.

Data Analysis

Flo-Jo v6.0b8 can be used to edit the 5 color data files so they correspond to log gains used for acquisition. The procedure is:

1. Open a new Flow-Jo workspace
2. Drag all files to be converted to the workspace
3. Change workspace preferences to "show all keywords"
4. Edit workspace columns by adding $P6N, $P6E, $P7N, and $P7E as columns
5. In the first file in the list, double click on $P6N (you will get a warning, click OK) and change to FL5-H and hit "option E". This will change all subsequent files to the change you made to the first file. Then double click $P6E and type "4,0", then "option E", and again all subsequent files will change. This changes the FL5 gain to log.
6. Repeat for $P7N, making it "FL4-H", and $P7E, making it "4,0" for log gain.
7. Highlight all the modified data files, then export as FCS files into a new folder.

All files are now ready to be analyzed by CellQuest or any other data analysis program. FL4 and FL5 plots will reflect the log axes, correct stats, and parameter names.