FlowJo Collectors' Edition Software User Guide

For Research Use Only

Not for use in diagnostic procedures

Cytek
FLOW CYTOMETRY PRODUCTS

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Glossary provides definitions of technical terms that appear in the guide.

1.1 Who Should Use It

This guide is intended for Flow Cytometer users with different degrees of knowledge and experience with Becton Dickinson FACScan™, FACSort™, and FACSCalibur™ flow cytometers. This guide assumes that you have some knowledge of basic flow cytometer theory, how to use the aforementioned flow cytometer models, and the Windows™ computer operating system.

For more information, see respective Becton Dickinson flow cytometer user guides, the Windows XP user guide (Windows XP User Guide), FlowJo Analysis on-line help (http://www.flowjo.com/home/manual.html) or the appropriate documentation.

1.2 Typographical Conventions

This document uses the following typographical conventions:

Command and option names appear in **bold type** in definitions and examples. The names of directories, files, machines, partitions, and volumes also appear in **bold**.

Variable information appears in *italic type*. This includes user-supplied information on command lines.

Screen output and code samples appear in `monospace` type.

In addition, the following symbols appear in command syntax definitions.

Square brackets [ ] surround optional items.

Angle brackets < > surround user-supplied values.

2 Introduction

2.1 Purpose

The purpose of this User Guide is to explain the acquisition module of FlowJo 7.5 (FlowJo Collectors' Edition).

- FlowJo Collectors' Edition (FJCE) is Cytek-branded acquisition software blending Tree Star FlowJo workspace features and data acquisition for Cytek modified cytometers. FJCE data acquisition software can be used with all Cytek cytometer upgrades and any Cytek configuration of lasers and detectors, including analog xP or digital DxP and the AMS automated collection system.

2.2 Scope

This manual will cover how to setup Cytek-modified cytometers using FJCE. The purpose of this software training is to highlight the features of FlowJo Collectors' Edition and demonstrate an example sample acquisition workflow.

The manual assumes the reader has a basic understanding of flow cytometric data acquisition and is familiar with its terminology. It is not intended to teach flow cytometrists how prepare flow cytometry-based experiments; nor does it cover biological and safety precautions associated with running flow cytometry samples.

2.3 System Organization

- Abbreviation(s)

    1. FJCE= FlowJo Collectors' Edition
    2. xP= extra parameters
    3. DxP= Digital extra parameters

- Analog Cytometers Supported

  xP3   3 Color FACScan or FACSCalibur w/o Pulse Processing

  xP3+   3 Color FACScan or FACSCalibur with Pulse Processing

  xP5   2-laser, 5 Color FACScan or FACSCalibur with Pulse Processing

- Digital Cytometers Supported

  DxP6   2-laser, 6 Color FACScan or FACSCalibur with Digital Electronics

  DxP8   3-laser, 8 Color FACScan or FACSCalibur with Digital Electronics

  DxP8Y   3-laser, 8 Color (yellow 3rd laser) FACScan or FACSCalibur with D. E.

  DxP9   3-laser, 9 Color FACScan or FACSCalibur with Digital Electronics

  DxP10   3, 4 or 5-laser, 10 Color FACScan or FACSCalibur with Digital Electronics

  DxP11   4-laser, 11 Color FACSort or FACSCalibur with Digital Electronics

  DxP12   4-laser, 12 Color FACSort or FACSCalibur with Digital Electronics

  DxP13   5-laser, 13 Color FACSort or FACSCalibur with Digital Electronics

  Custom configurations based on the above systems.

- Version Number(s)

  This user guide was written for FJCE 7.5.107.3 or later.

- Compatible Automation

  Automatic Micro-Sampling System (AMS)

  (96-well sample acquisition automation)

3 System Overview

FlowJo Collectors' Edition is part of a cytometer upgrade system manufactured by Cytek. This system consists of hardware and software that add functionality to existing cytometers by adding lasers, detectors, digital electronics and new acquisition software. Cytek upgrades may have 2-5 lasers and up to 13 colors.

3.1 Key Features

3.1.1 FlowJo Collectors' Edition has three main components:

1. The Workspace: Used to create a sample list and to apply attributes (e.g., antibody/fluorochrome labels) to the samples listed
2. The Cytometer Window: Its uses include
    i.   Control of the cytometer
    ii. Set up of file name and saving location preferences
    iii. Set up of acquisition preferences
    iv. Save and recall of cytometer settings
    v.   Control of software-based compensation for data viewing
3. The Data Scope: Used to view live data, and setup a collection gate while previewing and recording data.

3.1.2 Inventory

FJCE software is intended to be used for collecting data from a flow cytometer upgraded by Cytek Development.

The software uses the Windows XP operating system and has a similar look and feel to FlowJo analysis.

There are approximately 20 files and folders kept in the main program folder, which is illustrated in figure 3.1.2. By default the FlowJo CE folder is located at C:\Program Files\FlowJo CE, and it is recommended this location does not change.

For each Windows user there will be a separate CytekFJCE.prefs file located in their respective "My Documents" folder. This Preference file is read each time FlowJo is launched. There is also a USB driver file (drvxusb.drv) located in C:\Windows\System32\Drivers.
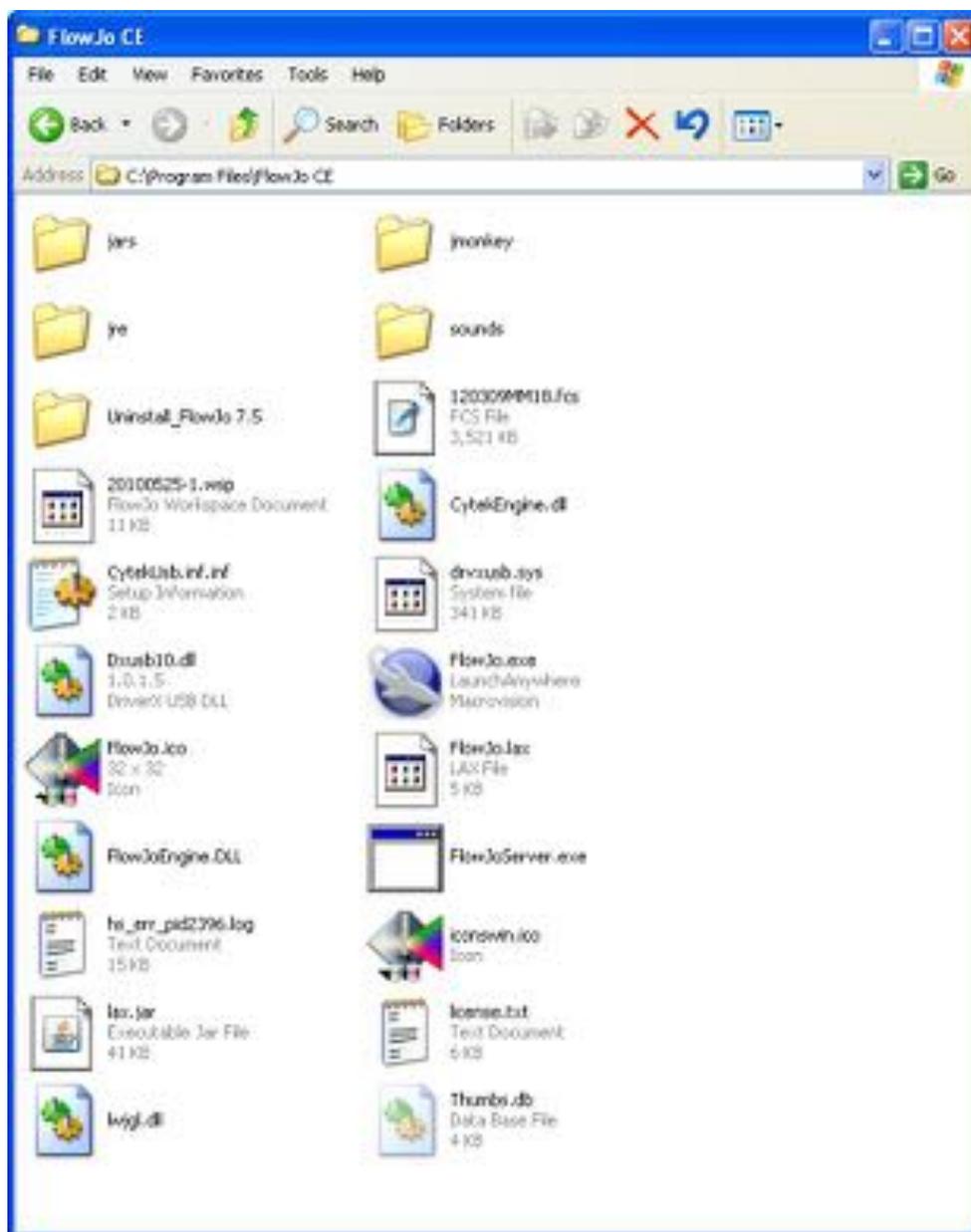

Figure 3.1.2

3.2 Environment

FJCE will operate in a standard lab environment that meets the environmental conditions for the Flow Cytometer.

3.3 Minimum Computer System Requirements

CPU 2.5 GHz or higher, 1.87 GB RAM, Windows XP, 4800+ AMD Processor,

USB port

High speed Internet connection recommended

Cytek xP upgrades require Doublet Discriminator (DDM) option installed

Does not support BD 4-color upgrade and sorting options on FACSCalibur™

or FACSort™ cytometers

Installing the FlowJo Collectors' Edition (FJCE):

The latest version of FJCE can be found on the Cytek development website in the products - FlowJo Collectors' Edition section: FJCE downloads.

A software license is required. Either a serial number based on the computer's MAC (Media Access Control) hardware address, or a USB dongle is required to run FJCE.

4 Installing, Starting, and Stopping the System

4.1 First-time Users

The cytometer must be turned on first before launching FJCE. It is **not** necessary to reboot the computer if the cytometer is turned off. Just quit FJCE, turn the cytometer back on, and restart FJCE. However, if you must reboot the computer for any reason, reboot with the cytometer OFF (or USB cable unplugged).

4.2 Access Control

- If necessary, obtain a Windows password from your IT administrator to run FJCE. The software can be run as a Limited User or Administrator.

- To change your password as a Limited User, go to User Accounts in the Control Panel, and follow the instructions to change passwords.

  Note: Data files and workspaces stored in My Documents will not be accessible to other Limited Users (but will be available to Administrator accounts).

4.3 Steps to install FlowJo Collectors' Edition

Note: You may skip steps 1-6 in the following section if FJCE was pre-installed on your computer by Cytek and you wish to update to a later version of the software.

INSTALLATION OF FLOWJO COLLECTORS' EDITION FROM CYTEKDEV.COM WEB SITE

1. To download the current version of FJCE, visit our website.
   http://www.cytekdev.com/pages/Software-Downloads.html
2. Click on the most current FJCE version to download.
3. Once complete, locate the downloaded application file. It will be named fjce_setup followed by the version number, for example, fjce_setup.7.5.107.3.exe, and will be found in your default save location (usually a download folder or on the desktop).
4. To install FlowJo Collectors' Edition simply double-click the fjce_setup file. You may receive a warning (Figure 4.3.1). Select Run and proceed with installation wizard.

Figure 4.3.1

5. Once installed, FJCE can be launched via the Program menu from the FlowJo CE folder or from the Desktop FlowJo CE shortcut. If you are using a dongle to verify your software license, this should be plugged into the USB port before launching the program.

6. Once opened, if you have do not have a valid FJCE dongle plugged in, FJCE will request a license serial number. Enter the serial number in the indicated space, hit enter, and the FJCE workspace window will open up.

    Contact Cytek at kle@cytekdev.com to obtain a serial number if one was not shipped to you.

If FlowJo Collectors' Edition software has been installed on your system previously, the above instructions will work to install the latest version, but you may also run an "updater" program to install a newer version over your current version, if a newer version exists:

    Open the Program menu and launch the FJUpdater application from C:\Program Files\FlowJo Cytek\Updates and click "Check for updates". If a newer version of FJCE is available, the version and description of changes will appear. The most recent version will be listed first, and it is recommended that the most recent version be installed.

4.4 Starting up FlowJo Collectors' Edition (FJCE)

1. Turn on the computer, wait for the operating system to boot, and then login if necessary.
2. Turn on the cytometer, and make sure the USB cable is connected.
3. Launch FJCE by clicking on its Icon in the start menu:

Fig. 4.4.1

Or double-clicking its shortcut on the desktop:

Or by selecting it in the programs menu:

Once you have launched FJCE, a blank Workspace will displayed as shown in figure 4.4.2.

Fig. 4.4.2

4. To connect to the cytometer and enter acquisition mode, press the acquire button. . Note: If the acquire button doesn't appear in the tool bar, click on **Edit menu**, then select the **Edit Buttons** command. A window will appear with additional buttons. Drag the acquire button from this window and drop it into the tool bar.

5. When acquisition launches, the Cytometer Window opens. Additionally, if the Data Scope window was in use when last closing the application, it will open along with the Cytometer Window. If the Data Scope is not visible, select **Menu** within the Cytometer Window, then choose **Show Data Scope**.

4.5 Exiting FJCE

To Exit FJCE you may click on the close button (red X) in the upper right of the Workspace window, or use the Workspace menu and click on **File,** then **Exit**, or use the CTRL+Q hotkey (Fig. 4.4.3).
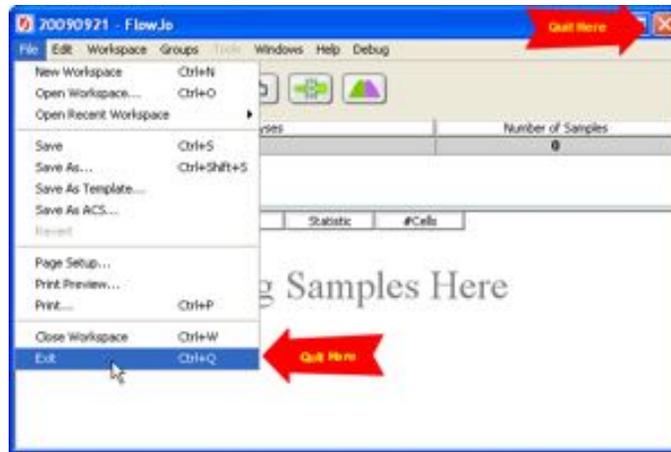
Fig. 4.4.3

Only one user can use FJCE at a time. When setting up to manage multiple Windows users (e.g., in a core lab), turn OFF the "*Use fast user switching*" checkbox in User Accounts located in Control Panels. This ensures that each user exits FJCE after their session, a necessary condition for logging each user's individual machine time. Core lab managers should contact Cytek Development regarding the use of the user-logging feature.
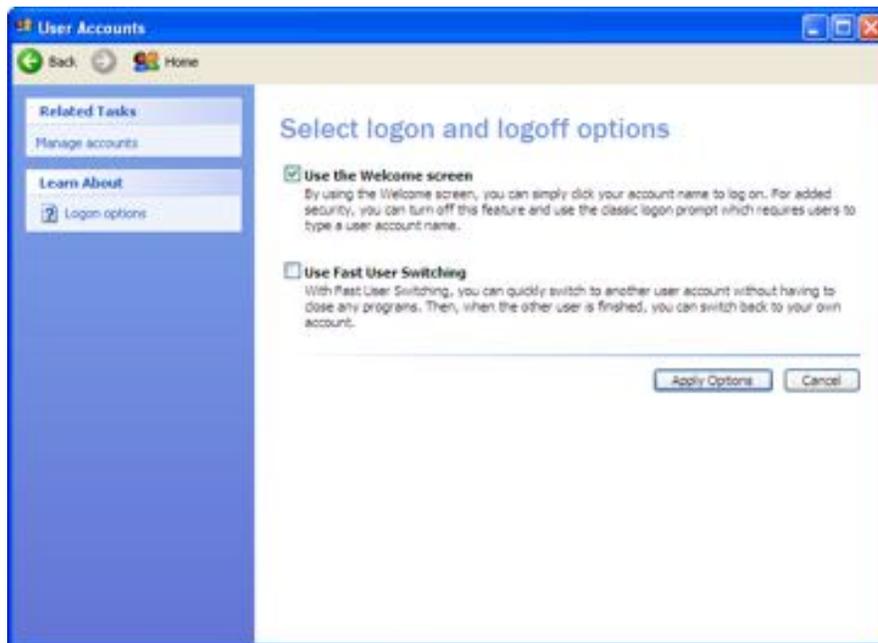
Fig. 4.4.4

5 The Workspace

5.1 The Workspace Window provides these capabilities:

1. Allows for selection of acquisition mode, which opens the Cytometer and Data Scope windows
2. Allows viewing a list of samples that have been acquired
3. Indicates which sample is being previewed/collected
4. Shows list of empty samples that have been added to the workspace
5. Allows for pre-acquisition addition of sample attributes (sample ID, reagent or fluorochrome labels, etc.)
6. Indicates whether a compensation matrix has been applied to a sample
7. Allows for analysis of acquired samples

Note: FJCE supports only limited analysis intended primarily to allow users to setup a compensation matrix. This feature is covered in <u>section 8.3.4.</u>

5.2 The Workspace Window and its Icons
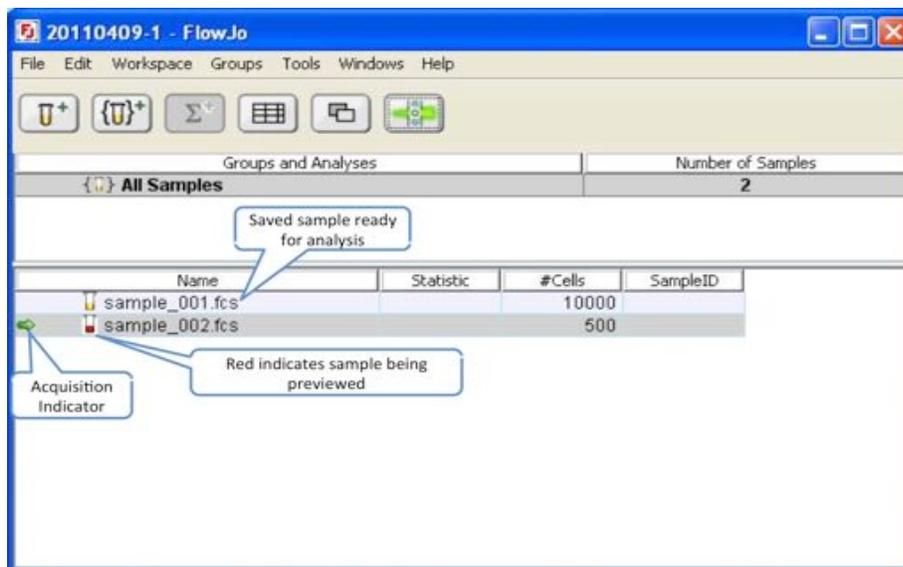
Figure 5.2.1 FJCE Workspace

There are typically 6 buttons in the default workspace as pictured in Figure 5.2.1. Most of these buttons are used during FlowJo analysis and are not needed for acquisition.

For acquisition, only the Acquire Samples button is used:

Acquire Samples: Pressing this button will connect FJCE to the cytometer, and launch the Cytometer and Data Scope Windows. If the cytometer is not connected, you will be asked to run in simulation mode.

> Note: When multiple Workspaces are open, Acquisition may only be associated with only one Workspace. To change the Acquisition to a different workspace, close the Cytometer Window, then re-launch Acquisition from the desired workspace window.

Buttons used for analysis only are:

Add Samples: Adds *already acquired* sample(s) to the workspace. See FJ Analysis for detailed explanation. (Same as Add Samples in Workspace Menu)

Note: this button is NOT used to added new (not already acquired) samples from the cytometer – this is done using the Cytometer window (see figure 6.2.1 for instructions).

New Group: Creates a new sample group for analysis. See FJ Analysis for detailed explanation. (Same as Add Group in Group Menu)

Add Statistic: Chooses statistics to add to parameters. See FJ Analysis for detailed explanation.

Table Editor: Allows you to edit tables of data statistics. See FJ Analysis for detailed explanation.

Layout Editor: Opens layout editor, which is used for graphically representing data. See FJ Analysis for detailed explanation.

5.3 The Workspace Symbols and Highlight Colors

⬚ Saved data file: A data file that has been recorded to disk using either FJCE or another data acquisition software. In the workspace these files are represented by a yellow test tube ⬚, with a light blue highlight.

⇨ Preview sample: The current sample the user has ready to be recorded (acquired). In the workspace these files are represented by a green arrow ⇨ and red test tube ⬚.

⇨ Current Analysis sample: Blue arrow indicates which sample is (optionally) being displayed in the selected FlowJo Graph window.

⬚ Recording sample (or sample being acquired): Indicated by a red test tube ⬚ and a green highlight with an increasing **#Cells** column, this is the sample currently being recorded to disk.

⬚ Paused sample: When recording of a sample is paused, the sample that was being recorded will have a green test tube ⬚ and a static **#Cells** column next to it.

⬚ Empty Sample: A sample that is awaiting preview and recording is represented by an empty test tube ⬚, and a pink highlight.

Different highlight colors on the rows illustrate the state of sample(s) in the workspace and indicate which sample is selected. In figure 5.2.1:

Figure 5.2.1

◼ Blue Highlight: Sample selected using single click

■ Yellow Highlight: Sample being previewed or acquired

Note: a selected preview sample will have a gray highlight rather than a yellow highlight.

■ Pink Highlight: Empty sample

An acquired sample that is not selected will have light pink color (figure 5.2.1 IFNalpha2.fcs).

5.4 Adding Keywords to the Workspace

Keywords may be added to the Workspace using the **Add Keyword** and **Edit columns** commands located in the **Workspace** pulldown menu.
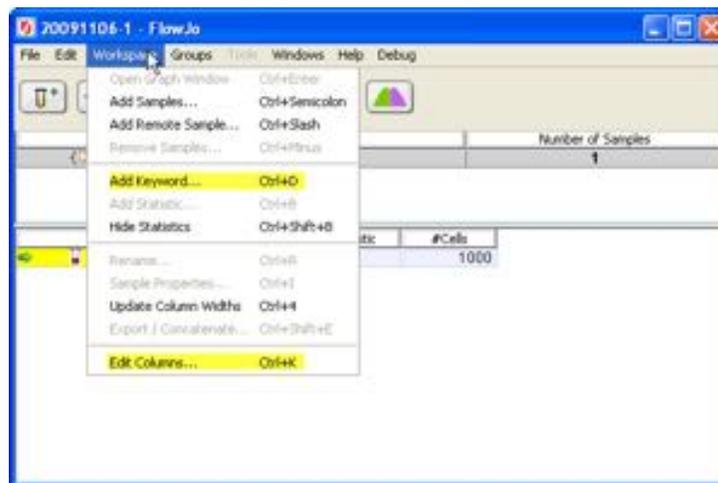
- The **Add Keyword** command allows a user defined keyword to be added to the workspace.
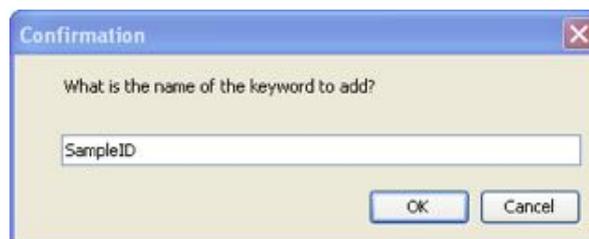
- The **Edit Columns** command allows the user to manage predefined keywords in the workspace. With this command the user may add or remove keywords in the edit columns list, one at a time or in multiples.
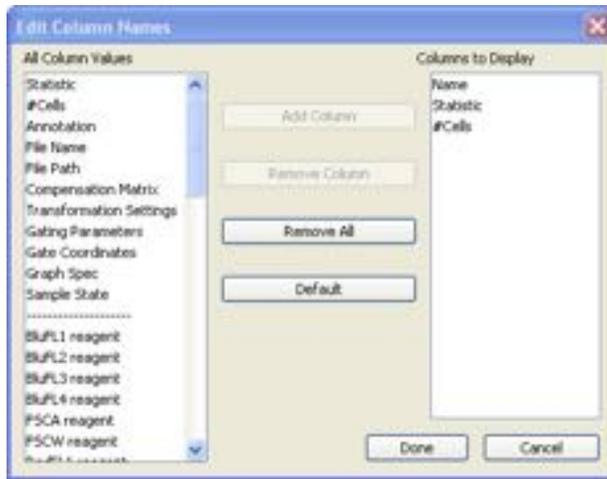
Note: For detailed instructions on how to edit columns in the Workspace see section 8.3.3

6 The Cytometer Window

6.1 Cytometer Window Capabilities:

The Cytometer Window allows the user to:

1. Define the file name(s) and folder where the data to be acquired will be saved
2. Determine stop criteria, such as number of events to record or time duration for data to be acquired.
3. Monitor the status of the cytometer
4. Control the PMT voltage and amplifier settings
5. Turn lasers on and off
6. Define and apply a spillover compensation matrix (digital instruments)
7. Set up hardware compensation (analog instruments)
8. Define threshold parameter(s)
9. Setup the area and width parameter
10. Record a sample, pause recording, erase & re-record a sample, queue-up next sample to be recorded

Illustration 6.1.1 shows the Cytometer Window in a minimized view.

In the top window bar the cytometer configuration and Cytometer name are shown. You may assign each cytometer a nickname (Menu / Show Cytometer Info). The cytometer illustrated is nicknamed "Shemp". If you are operating more than one of the same Cytek instrument model, naming them is essential to tracking each machine's use history in the log.
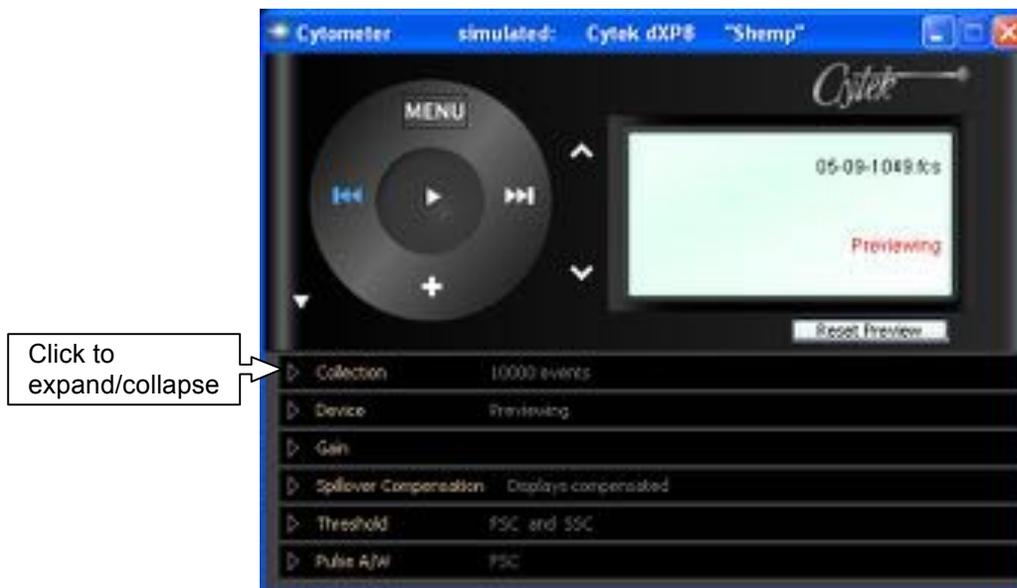
Figure 6.1.1

Each section can be expanded or minimized by selecting the triangle located on the left.

6.2 The Cytometer Menu

In the Cytometer window Menu (Fig 6.2.1) the user may:

1. Add one empty sample (without copying any keywords/attribute labels)
2. Add multiple empty samples (without copying any keywords/attribute labels)
3. Define sample storage and naming
4. Define acquisition preferences
5. Open instrument settings
6. Save instrument settings
7. Display the Data Scope
8. Set the Time parameter scale
9. Show Cytometer Info.
10. Display help
11. Enter the test menu

Fig 6.2.1

6.2.1 Add One Empty Sample

Clicking on **Add One Empty Sample** will add one empty sample to the workspace. This empty sample will use the file naming convention defined in the **Sample Storage and Naming…** dialogue. Using this command will NOT copy any keywords/labels to the new samples. To automatically have keywords from the current Acquisition Sample (green arrow) copied into the new empty sample, use the **'+'** at the bottom of the Cytometer Window control wheel.

6.2.2 Add Empty Samples…

To add multiple empty samples to the workspace, click on the **Add Empty Samples…** command in the Cytometer Window menu. The window in figure 6.2.2.1 will appear.
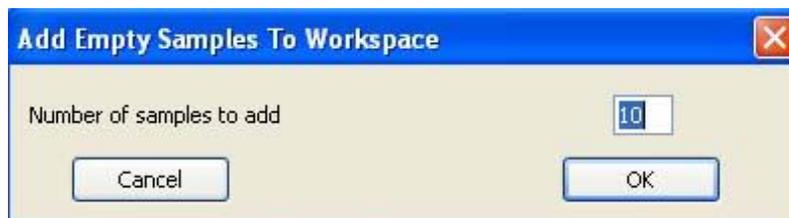
Figure 6.2.2.1

- **Number of samples to add**: defines the number of empty samples to add.

6.2.3 Sample Storage and Naming…

Clicking on the **Sample Storage and Naming…** command will bring up the window shown in figure 6.2.3.1. Use this window to choose a folder to store acquired files in, and to input keyword(s) to append to the file name (File Name Stem).
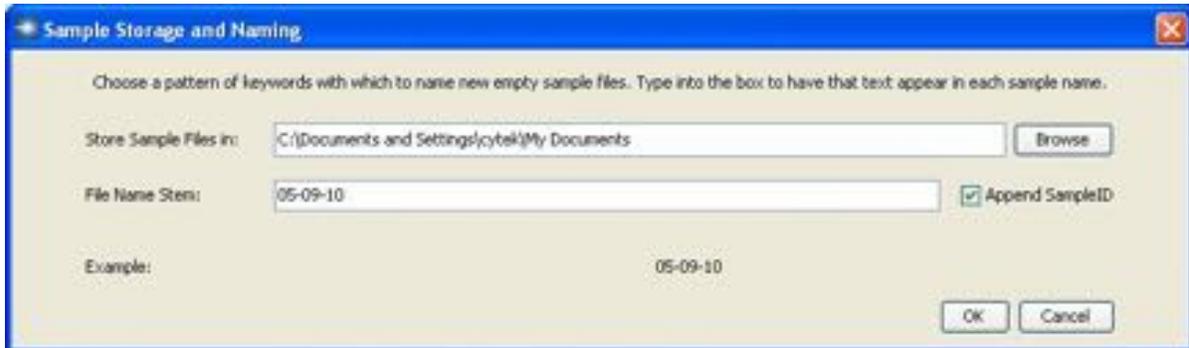
Figure 6.2.3.1

1. **Store Sample Files in**: clicking the **Browse** button opens the folder selection dialogue box (shown in figure 6.2.3.2). Here the user may select an existing folder or create a new folder where data files will be saved.
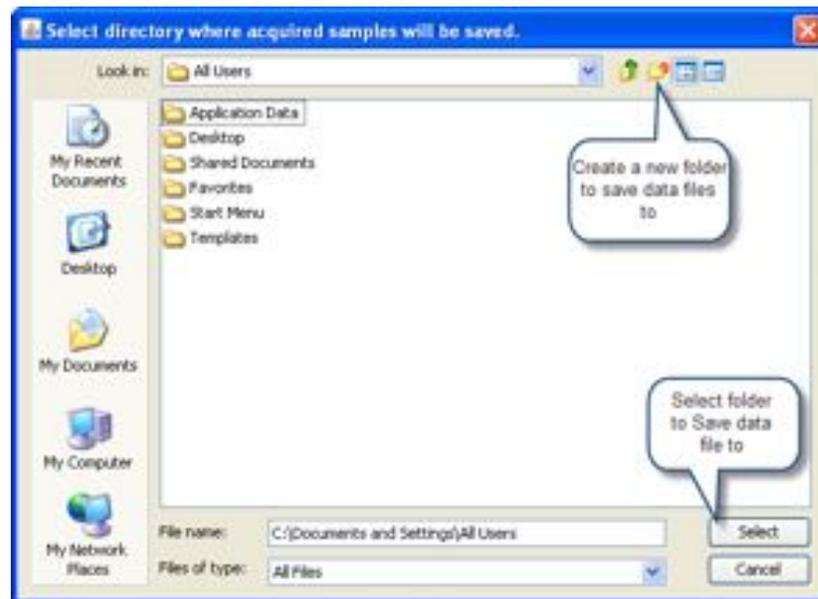
Figure 6.2.3.2

2. **File Name Stem**, defines a prefix to be used within the file name for all new samples. For example, the date and/or the experiment number could be entered here.

3. **Append SampleID checkbox**. Checking this box will add the SampleID as a filename "suffix" (concatenated to the file name stem). A text entry field in the Cytometer Collection Panel is used to enter the SampleID.

6.2.4 Define Acquisition Preferences

The **Acquisition Preferences** command located in the Cytometer Window menu allows the user to change preferences specific to acquisition. Figure 6.2.4.1 illustrates the Acquisition Preferences window

Figure 6.2.4.1

- **Refresh graphs every:** Defines the refresh interval for Data Scope plots.

Note: For large datafiles (>1,000,000 events), keeping the Data Scope open will slow down FJCE performance. For mega-recordings, closing the Data Scope is recommended.

- **Population colors**: The palette of population colors in the order they will be assigned.

  - **# colors**: The program automatically chooses colors based on the number of populations you plan to create (up to 16). When increasing the number of colors, the default color is white. Press the "Auto-color" button to have the program assign colors from the palette. You may change any individual color by clicking on it, and selecting a color on the pop-up palette window.

- **Name Preface:** The default stem name for all new populations is Pop_ with a number automatically appended to the end. In this field, the user can change this default stem name. Note: Population names for individual gates can be manually changed from the default by double-clicking on the population name within the Data Scope Population panel and typing in the new name.

- **Automatically advance to next sample after auto-stop**:

  - Box checked: Once the collection for the current sample is complete, the acquisition pointer will advance to the next available (empty) sample in the

workspace. If no empty samples exist, a new empty sample is automatically added and acquisition advances to that sample for previewing.

- o Box not checked**:** Acquisition does not advance to the next sample. This allows inspection of the final statistics for a recorded sample in the Data Scope. When done inspecting statistics, manually advance to the next sample. Use either the ">>|" button on the wheel, or use the "down" arrow control to the right of the wheel. The green arrow shows the sample queued-up for recording. This is the same sample whose statistics are currently displayed in the Data Scope.

- **Generate Sample log file:** When this box is checked a text file of samples (acquisitionUserLogFile) is generated in the My Documents folder, which contains a log of each file name and time of acquisition.

- **Simulation Mode:** FJCE may be run in Simulation mode. This mode is used predominantly by Cytek personnel for training and demo purposes, and is not a supported mode for users. When running in simulation mode, FJCE uses a previously saved FCS data file as the data source and repetitively "streams" the datafile to simulate data coming from the cytometer.

  - o **Simulation source FCS data file:** select a simulation source FCS data file. Note: Compensation is not pre-applied to a digitally-acquired source datafile, since a digital cytometer sends uncompensated linear data.

  - o **Simulation event rate:** sets the data event rate of the simulated data file (must be > 0).

- **OK:** saves changes to acquisition preferences

- **Cancel:** aborts changes made to acquisition preferences and reverts to the previous settings.

6.2.5 Open Settings….

The acquisition settings file (.acqt) bundles together all instrument settings (e.g., PMTs, threshold), software-applied Spillover Compensation matrix, the full set of Data Scope plots, population gates, the population hierarchy, statistics layout, as well as recording stoppage preferences.   Saved settings files are loaded using the **Open Settings….** command located in the Cytometer Window menu. Clicking on **Open Settings…** will allow the user to import previously saved acquisition settings. Settings files will have an .acqt extension.
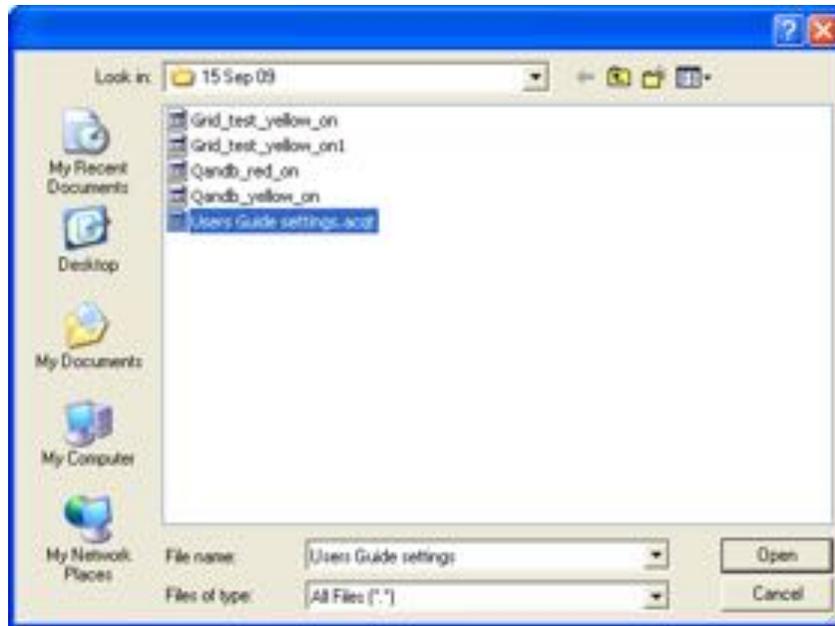
Figure 6.2.6.1

6.2.6 Save Settings…

Left clicking on the **Save Settings…** command in the Cytometer Window menu will open a Windows dialogue box similar to figure 6.2.6.1 where the user may save an instruments setting file. This instrument settings .acqt file will contain the settings described in section 6.2.5 at their current values. Note that instrument settings at shutdown time are automatically saved in each user's preferences, and will be recalled for that user when the program is relaunched.

6.2.7 Show Data Scope

Left clicking on this command will display the Data Scope if not presently open. When launching acquisition in FJCE, the Data Scope will load in the same plot configuration as it was when FJCE was last quit.

6.2.8 Time Parameter Scale…

For experiments, such as those measuring calcium ion flux, plotting Time as a parameter is useful. This dialogue defines the scale for plotting the Time parameter. When changing this upper limit for plotting time in the Data Scope, FJCE will prompt the user with the option to also set the collection time limit to this same value. During Previewing, a plot of the Time parameter "flies back" like an oscilloscope. During recording, the Time parameter advances monotonically from 0. (Figure 6.2.8.1)
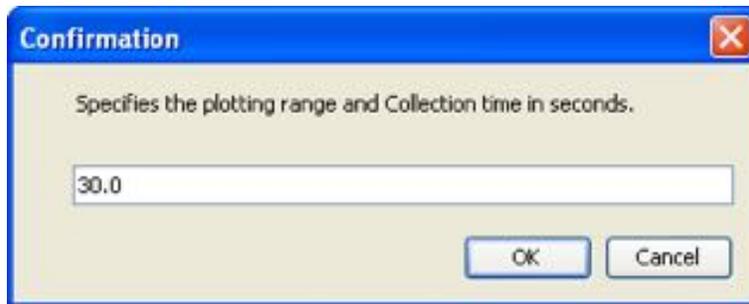
Figure 6.2.8.1

6.2.9 Show Cytometer Info

Left Clicking on the **Show Cytometer Info** command in the Cytometer Window **Menu** opens the window illustrated in figure 6.2.9.1

Figure 6.2.9.1

- **Cytek dxP8**: cytometer model detected during launch of acquisition. The cytometer model is set in the hardware at the factory or by a Field Service Engineer
- **USB FW**: Firmware version of the USB program running in the hardware (U47 Status Control Board).
- **Cytek FW:** Firmware version of U31 on Status Control Board, and U57 SSG Board.
- **Cytometer Nickname**: Enter the moniker you wish to give the cytometer. The nickname is imprinted in FCS data files, and positively IDs the instrument's usage in the Experimenter's Log file.

6.2.10 Test…

These features are generally used for diagnostics only.

• Test Pulses ALL turns on all test pulses

• Test Pulses FSC only turns on FSC test pulses. This is useful for checking noise.

• The two log file items, debug frame window, and parameter dump are used for diagnostic purposes.

6.3 The Cytometer Window Action Wheel

▶ The Record button: Pressing this starts recording. By default the program is always previewing live data from the cytometer. The recording of data to an FCS file will begin when the record button is pressed. During recording this button will change to a pause "||" button which can be clicked to pause recording. When paused, the button changes back to a record symbol that can be clicked to resume recording.

◀◀ Erase and Re-Record: Problem sample? Use this control to erase and re-record a sample. To protect against inadvertent data loss, the user will be presented with the following warning:

Confirmation

Erase and re-record?
C:\Documents and Settings\sample1.fcs

Yes   No
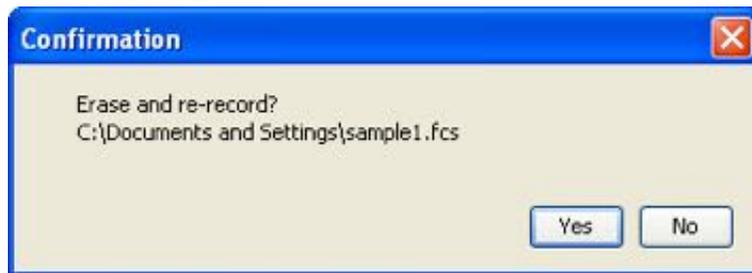
Figure 6.3.1.1

Note: If you elect to erase and re-record , the sample reverts to the empty Previewing state. Previously recorded data in the file is removed. This allows settings to be adjusted, or the sample restirred, before restarting recording. Samples imported from other acquisition programs (non-FJCE) can not be recorded over using this feature.

▶▶| Next Acquirable sample: Advances the acquire pointer to the next available empty sample ready for acquisition. The order of samples is the same order in which empty samples were created. Use the "up" and "down" arrows to the right of the wheel to manually select the next sample to be acquired.

+ Add One Empty sample to the Workspace with automatic keyword copying: This button provides a quick way to add one empty sample to the workspace. New files are named using the stem name previously setup in the "Sample Storage and Naming" dialogue from the Cytometer Window Menu. Unlike the **Add One Empty Sample** menu option, using this button copies any keyword values from the Current Acquisition Sample (pointed to by the green arrow).

∧ ∨ On-deck sample navigation arrows: These controls are used to navigate to the next sample in the workspace list that you want to record, with the current sample indicated by the green arrow.

6.4 The Acquisition Information Window

- o Displays the name of the current sample.
- o Displays the status of the current acquisition: Previewing or Recording

Figure 6.4.1

- • **Reset Preview**: Clears the Data Scope's current event memory, allowing fresh events (and gating decisions) to be displayed. Also clears Data Scope statistics to begin afresh.

6.5 Sample Collection Control

Figure 6.5.1

- **# of events**: Checking this box will terminate recording when the number of events in the numeric field has been counted for the population selected. In the example in Figure 6.5.1, recording will cease when 10,000 cells belonging to Pop_1 have been counted.

- **# of Seconds**: Checking this box will stop recording at the total number of seconds defined in the numeric field to the right (pausing suspends the stoppage timer).
  - o Note: When multiple termination criteria are defined, the first satisfied condition terminates acquisition.

- **SampleID**:   You may enter a unique ID here to be appended to the current acquisition sample's file stem name (the checkbox in Menu / Sample Storage and Naming must be ON to append the SampleID). Whether an ID is entered here or not, FJCE will auto-number the samples with a three character numeric suffix to keep their names unique, in the same order as new empty samples are created.

- **Record to FCS File** [Population]: The default is to record All Events, but you have the option to record only a specific population (including all its sub-populations), for

instance only T-Lymphocytes. A warning is given to remind you that not all events are being recorded.

6.6 Device Control

Figure 6.6.1

- Device Status: Displays the status of the cytometer
  - Previewing
  - Acquiring to file
  - Standby: May indicate this when STBY mode selected, or no sample pressure
  - Not Ready: May be due to 5 minute warm-up, sheath empty, waste full, or no 488 laser power.
- Diff. Pressure: Measures the differential pressure between the sheath and sample. Should be approximately 0.5 PSI for low, 0.75, and 1.0 PSI for high
- Fluid Tanks: Bright orange indication when a tank needs routine service.
- Laser Excitation: Colored sections depict each laser intersect the cell passes through (specific to the Cytek model connected). Coaxial lasers occupy the same laser intersect, and the laser color bar shown is the color addition of lasers currently turned ON (red + violet

= magenta). When a 488 *solid-state* laser is installed, the power is preset and not monitored. The power on all other solid state and diode lasers are preset and not monitored.

- Lasers may be turned on and off using the check boxes to the left. On DxP10, the operator must manually switch the green and yellow lasers inside the instrument compartment.

6.7 Gain Control
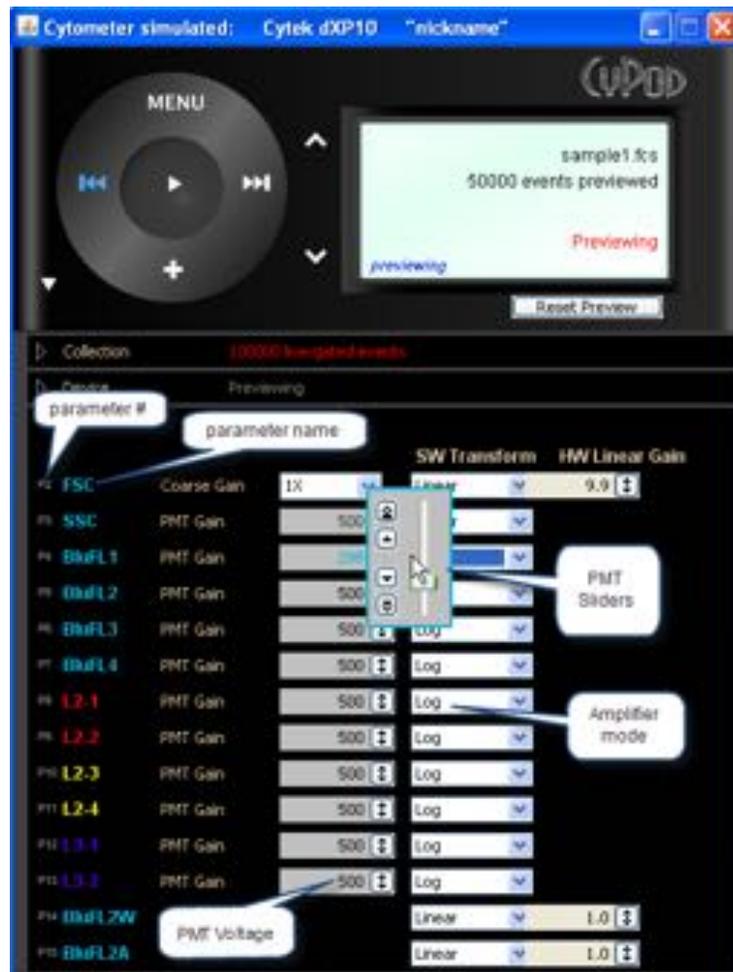
Figure 6.7.1

- Parameter #: The parameter number as will be used in the FCS file keywords. Parameter numbers start at two because parameter number one is time.

Note: Figure 6.7.1 illustrates a 4 laser 10-color system. Different Cytek instrument models will vary from this appearance.

- Parameter name: Name of the parameter.
    - Laser 1 is occupied by the 488nm laser and is the first laser intercept position. It excites the scatter and Blue parameters.
    - Laser 2 can be shared by three lasers and is the second laser position. In figure 6.7.1, L2-1, and L2-2 are excited by the red laser and color-coded as such. L2-3 and L2-4 are excited by the 561 yellow laser and color-coded as such.
    - Laser 3 is occupied by the 407 violet laser and is the third laser position.
- PMT Voltage: Shows the voltage of the PMT. Ranges from 1 to 999 volts.
- Amplifier (or digital software transform) Mode: Select LOG or LINEAR. When log is selected, scaling in the Data Scope will show 4 decades for analog instrument and 5 decades for digital instruments
- PMT Sliders: Adjust PMT voltage and, for FSC, the amplifier gain.
    - ⬆ Adjusts the PMT Voltage up by 1 volt
    - ⬇ Adjusts the PMT down by 1 volt
    - ⬆ Adjusts the PMT up by 10 volts
    - ⬇ Adjusts the PMT down by 10 volts

6.8 Spillover Compensation (DxP digital instruments)

6.8.1 Spillover Compensation Panel

On digital installations, all FCS data is recorded as uncompensated linear data; however, the user may find it useful to view compensated data during collection. FJCE allows the user to define and apply a software compensation matrix that is then used for display in the Data Scope during sample acquisition.   This software compensation is defined and applied using the Spillover Compensation panel and the ACQUISITION Spillover Matrix, which opens in a separate Compensation Editor Window. The Cytometer Window Spillover Compensation control is shown in figure 6.8.1.1.

Figure 6.8.1.1

To set up a compensation matrix, press **Spillover Matrix,** which opens this dialogue box:
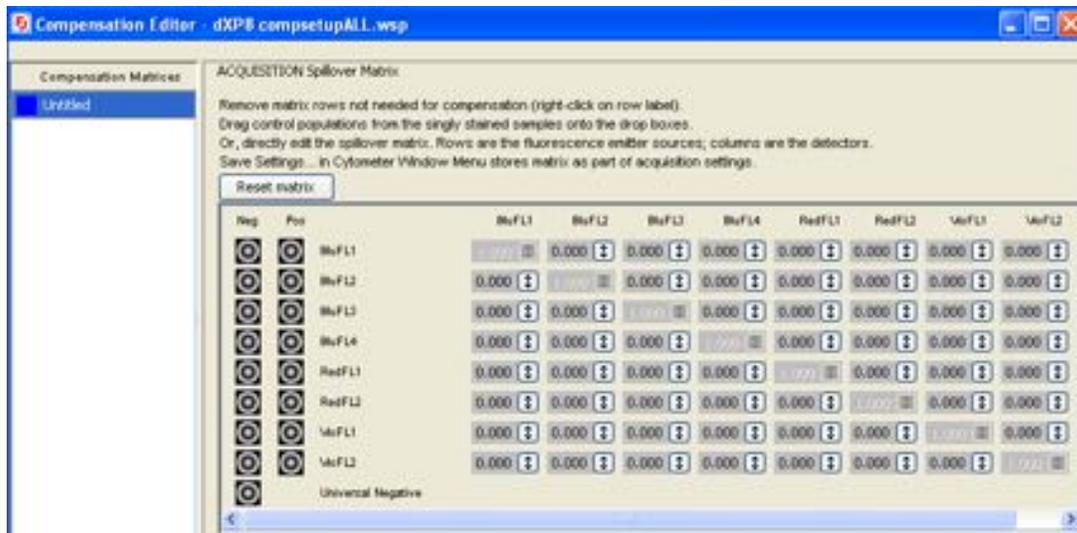
Figure 6.8.1.2

Each row in the matrix represents the amount of light a fluorochrome "spills over" unintentionally into each detector (columns). Compensation values can either be added manually using the sliders, or can be automatically calculated by dropping single-stained populations into the appropriate boxes on the left. Auto-compensation instructions are outlined in the box above the

matrix with an overview below. Additional details can be found in the Auto-Compensation Procedure Section 8.3.4.

Once spillover matrix values are loaded into the compensation editor window, the Compensation panel indicates "Defined", and you may display compensation on the live Data Scope plots by checking the "**Apply Compensation"** button.
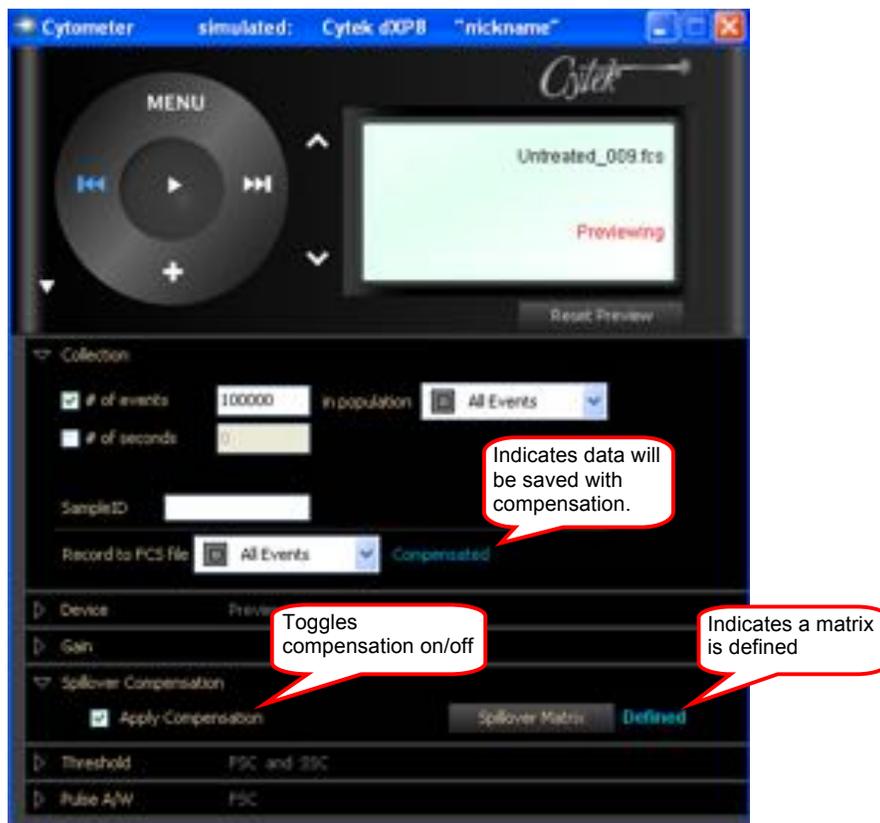
Figure 6.8.1.3

The Matrix is saved to the recorded data file and is included in saved acquisition settings. Toggling the "**Apply Compensation"** on will also save the matrix with any data files recorded while this option is checked. Collection of compensated data is indicated in the collection panel as shown in Figure 6.8.1.3.

6.8.2 Creating the Spillover Matrix

You may create a Spillover Matrix manually or automatically.

Manual:

1. Click Spillover Matrix to open the Compensation Editor window and then click **Reset matrix**. This resets all compensation values to 0.
2. Set PMTs to correct values.

3. The sliders next to each spillover value may be used to change the spillover value.

Automatic:

1. Click Spillover Matrix to open the Compensation Editor window and then click **Reset matrix**. This resets the matrix for uncompensated acquisition (a requirement for acquiring single-color controls).

2. Set PMTs to correct values for all parameters, acquire single color controls, and negative control, and create appropriate gates if necessary.

3. Click **Apply Compensation** so that the compensation matrix can be viewed in the Data Scope, then click on the Spillover Matrix button.
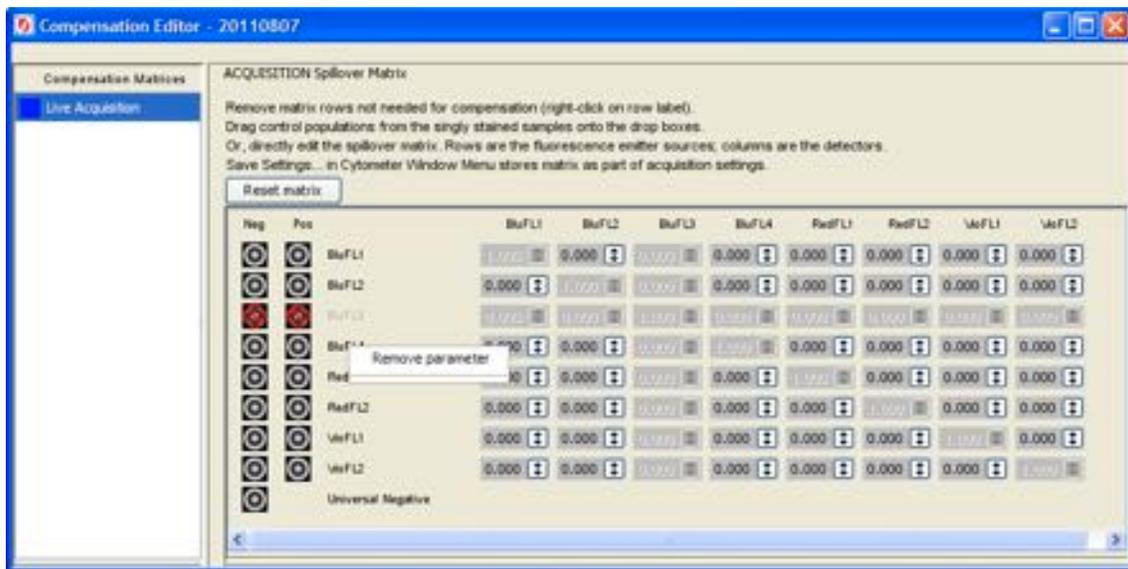
Figure 6.8.2.1

4. Within the Spillover Matrix window, remove any parameters that will not be used for the experiment. For example, in Figure 6.8.2.1, BluFL3 does not have a fluorochrome assigned in this experiment and was removed. This is done by right clicking on the name of that parameter and selecting **Remove parameter**.

5. Drag the negative control sample from the Workspace to the Universal Negative drop box

6. Drag the single color controls to the appropriate positive drop box
   a. NOTE: If positive control samples also contain negative populations, you must gate on the positive subpopulation and drag only this subset to the positive drop box. See section 8.3.4 for more detail.

7. After the last positive control has been added to the matrix, spillover values will be automatically calculated.

NOTE: <u>Collecting uncompensated samples</u>. Some samples do not make sense to collect with compensation applied, such as single-color control samples. Before acquiring such samples, make sure the "Apply Compensation" checkbox is unchecked in the Spillover Compensation panel of the Cytometer window. The FCS files collected will NOT be associated with a SPILL matrix, and the vertical bar will not appear in the Workspace list, all indicating that compensation is not specified for such samples. Alternately, open the Spillover Matrix Window and use the "Reset matrix" to clear the spillover values.   The blue "defined" indicator in the Cytometer panel changes to "undefined", and the "Apply Compensation" checkbox will be unchecked.

6.8.3 Saving the Spillover Matrix

You may save your spillover matrix settings in several ways. For an experiment you plan to repeat, Saved Settings should be your primary choice, as it saves everything associated with acquisition setup into a single convenient file. This data includes the spillover matrix, plus other essential cytometer settings such as PMT voltages.

1. <u>Saved Settings file</u>: Save an instrument setting file using the **Save Settings**… command located in Cytometer window menu. This .acqt file will contain PMT and amplifier settings as well as the spillover matrix, populations and statistics setup.

2. <u>The FJCE Preferences File</u>: When exiting FJCE, the current Spillover Matrix will be saved to the preference file located in the *My Documents* folder. It will automatically be recalled when restarting FJCE.

3. <u>FCS data file keyword</u>: Whenever you acquire a FCS file from a digital cytometer (DxPn), and the Spillover Matrix is defined and the "Apply Compensation" checkbox is checked in the Spillover Compensation panel of the Cytometer window, the Spillover Matrix will be saved to FCS data file header under the SPILL keyword. This matrix can be viewed using FlowJo analysis software or other third party flow cytometry data analysis programs.

6.9 Hardware Compensation (xP Analog Instruments)

To apply hardware compensation to your data during preview and record, use the controls shown in figure 6.9.1.

Figure 6.9.1

Hardware compensation for the analog instruments can only be performed on adjacent fluorescent parameters.

Use the slider control to manually set the compensation value between 0-99%.

⬆   Adjust compensation up in 1% increments

⬇   Adjust compensation down in 1% increments

🔼   Adjust compensation up in 0.1% increments

🔽   Adjust compensation down in 0.1% increments

For a detailed tutorial on compensation principles see http://www.drmr.com/compensation/

6.10 Threshold

The cytometer threshold sets the minimum signal level required to have the cytometer process an event. When an event goes through the detection area and does not meet the threshold level it is ignored; therefore, it does not contribute to the event or collection count. DATA BELOW THE THRESHOLD IS NOT SAVED. An event that exceeds the threshold amount is processed by the cytometer. Figure 6.10.1 illustrates the threshold controls for DxP instruments.

Figure 6.10.1

☑ **Primary Threshold**: sets the first threshold parameter. Can be set on any primary laser parameter, and is the first criterion the event must meet in order to be detected.

- ⊟ **Secondary Threshold**: sets the second threshold parameter. Can be set to **None** or any primary laser parameter not already used as the primary threshold.

- ⊟ **AND**: sets the primary and secondary threshold logic to AND. This means the event must first exceed the primary threshold value and then meet the secondary threshold parameter before the event will be detected.

- ⊟ **OR**: sets the primary and secondary threshold logic to OR. This means the event will be detected if either threshold value is met.

To adjust the threshold, use the slider control shown above.

- ⏶ Adjust threshold up in increments of 1

- ⏷ Adjust threshold down in increments of 1

- ⏶ Adjust threshold up in increments of 0.1

- ⏷ Adjust threshold down in increments of 0.1

    Use the slider control to manually set threshold from 0 to 100%.

Adjusting the threshold on analog (xP) cytometers is the same as for digital except that only one threshold parameter is available.

6.11 Area and Width parameters (DxP Digital Instruments)

On DxP cytometers, Pulse Area and Width may be extracted on any ONE available parameter (except Time). Figure 6.11.1 illustrates the Pulse Area/Width controls.

Figure 6.11.1

Source parameter: Selects the parameter for which area and width will be calculated.

Width Offset: The width offset is used to subtract the width of the laser beam, to get a 2:1 width ratio on doublets/singlets. For calibration, run a sample (e.g., CEN from Biosure) that includes singlets, doublets, triplets. Increase the offset so doublets/singlets=2, (this moves everything downscale), then use the width gain in the Gain menu to bring singlets and doublets back upscale.

To adjust the Width Offset, use the slider control shown above.

⬆ Adjust Width Offset up in increments of 0.1 (tenths)

⬇ Adjust Width Offset down in increments of 0.1 (tenths)

▲ Adjust Width Offset up in increments of 0.01 (hundredths)

▼ Adjust Width Offset down in increments of 0.01 (hundredths)

Use the slider control to manually set Width Offset from 0 to 1.

6.12 Area and Width parameters (xP Analog Instruments)

When using an analog cytometer, pulse Area and Width may be selected on the primary laser's FL1, FL2 or FL3 parameters. On xP5 upgraded cytometers Width and FL4 Red share parameter

7 (P7), and Area / FL5 share P8; therefore, only Width OR FL4 can be used, likewise only Area or FL5 can be chosen using the radio button controls as shown in Figure 6.12.1.

Figure 6.12.1

Source Parameter: Choose FL1, FL2, or FL3

Use the left radio button pair to choose FL4 vs. Width. Use the right pair to choose FL5 vs. Area.

7 The Data Scope

The Data Scope is the section of the acquisition module that can be used to:

1. View live data.
2. Apply viewing options, such as compensation, log transformation, gridlines and background color.
3. Draw gates to create populations.
4. Inspect population and parameter statistics.

The Data Scope window has plots arranged as a grid. Each of these plots can be viewed as either a Histogram or dot plot. As the Data Scope window is resized, it will adjust to show the maximum number of full size plots.

7.1 Title Bar and Plot Controls

Toggle background

7.1.1 Title Bar

7.1.2 Plot Controls

Figure 7.1.1

7.1.1 Title Bar

The title bar of the Data Scope displays event counts and rates. From left to right:

7.1.1.1 Last *N* Events
Shows the total number of Preview Events in each Data Scope plot. This number is set in the Preview Events dropdown box in the Data Scope window immediately below.

7.1.1.2 All Events/sec:
Displays total event data rate in events per second.

7.1.1.3 Pop_1 events/sec:
Displays the Data Scope gated event rate in events per second. The name of the gated population will vary according to how you setup data collection (see section 6.5).

7.1.2 Plot Controls

7.1.2.1 Preview Events

This menu controls the number n of events displayed in the Data Scope.

NOTE: Using the largest choices for Preview Events may degrade acquisition performance, especially in conjunction with a complex population hierarchy. For best results, use the *smallest* value of Preview Events adequate to visualize populations.

7.1.2.2 Gridlines

Check the gridline selection box in the header section of the Data Scope to add gridlines as a quick visual guide for your data. Gridlines on dot plots and histograms are shown below.
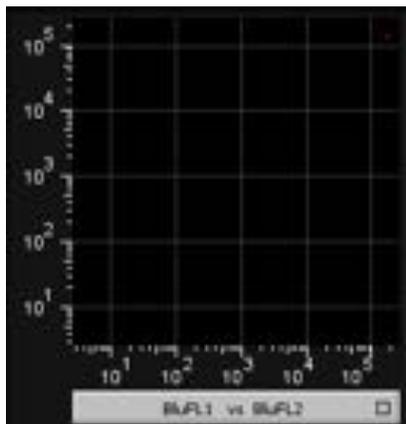
| Dot plot with gridlines | Histogram with gridlines |
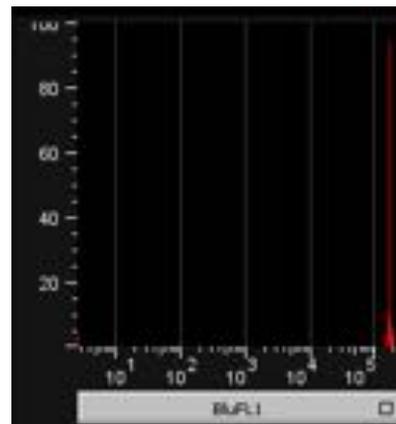
Figure 7.1.2

7.1.2.3 Plot background color selection

Toggling the Data Scope background color selection switch will toggle the background color of the plots from black to white. When the plot background is white, the ungated data will appear black (shown in figure 7.1.3). When the plot background is black, the ungated data will appear gray.
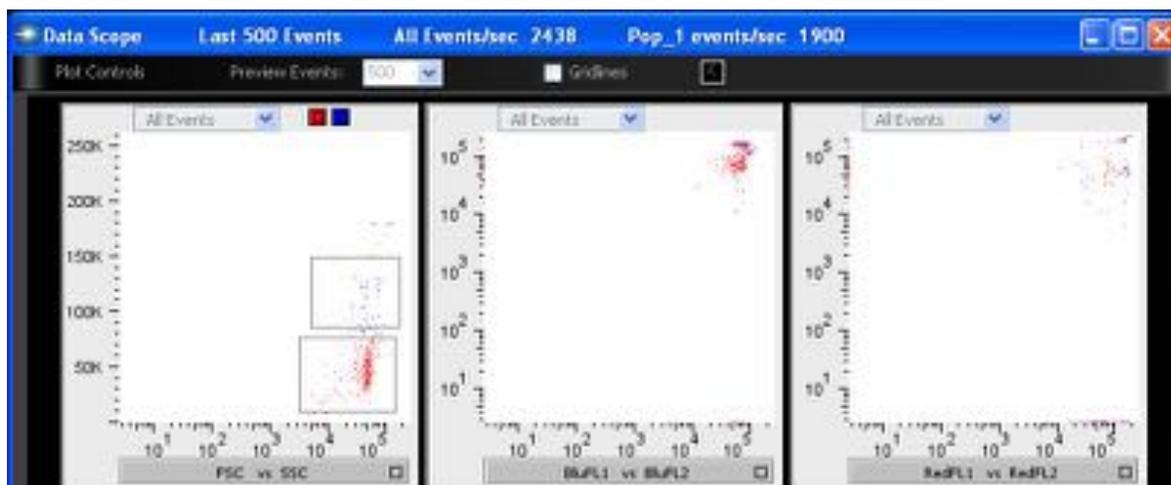
Figure 7.1.3

7.2 Viewing Live Data

In the Data Scope, the flow cytometer user may view both Histograms and Dot Plots as shown below (figure 7.2.1) Each plot has the following parts:

7.2.1 Population Chooser

This drop down allows you to limit the events displayed in a plot to a certain population (including its children).

7.2.2 Gate Reminder

This section displays small icons reminding you what gates have been defined using this plot.

7.2.3 Parameter Chooser

This allows you to choose the axis (for a histogram) or axes (for a dotplot).

Population Chooser

Gate Reminder
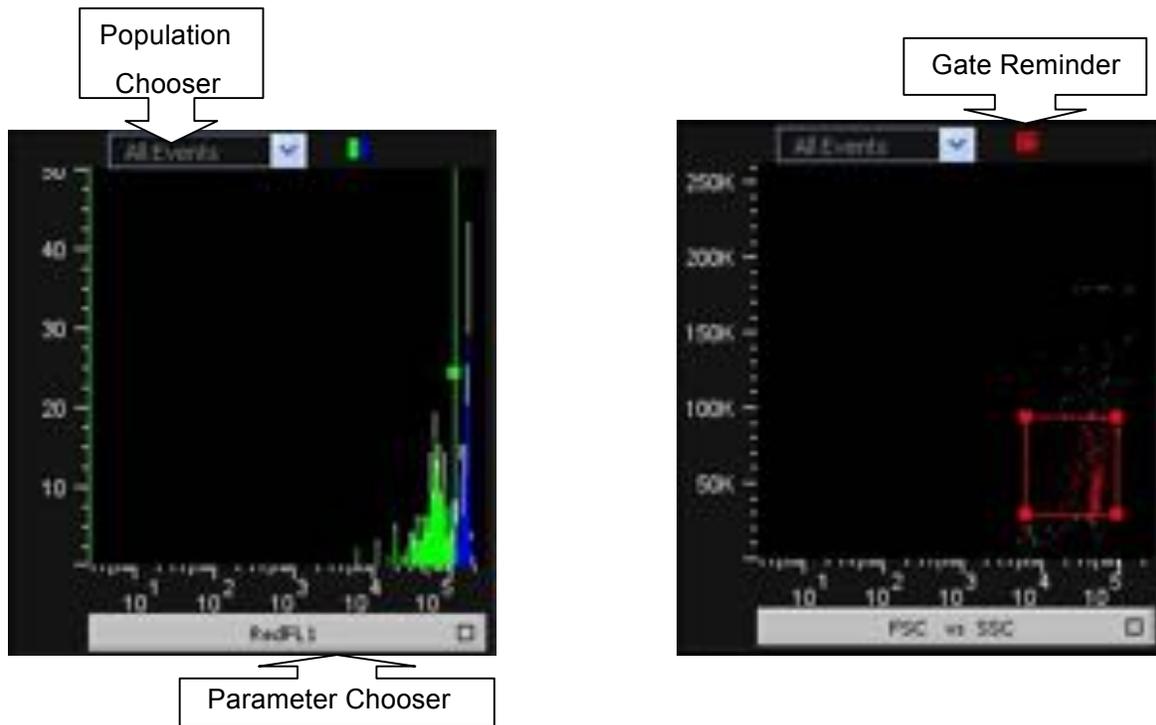
Parameter Chooser

Data Scope Histogram   Data Scope Dot Plot

Figure 7.2.1

7.2.1 To make a Histogram (figure 7.2.2):

1. Click on the parameter chooser at the bottom of the plot
2. Choose the X-Axis parameter
3. Move to the right (follow the little arrow) and then choose Histogram
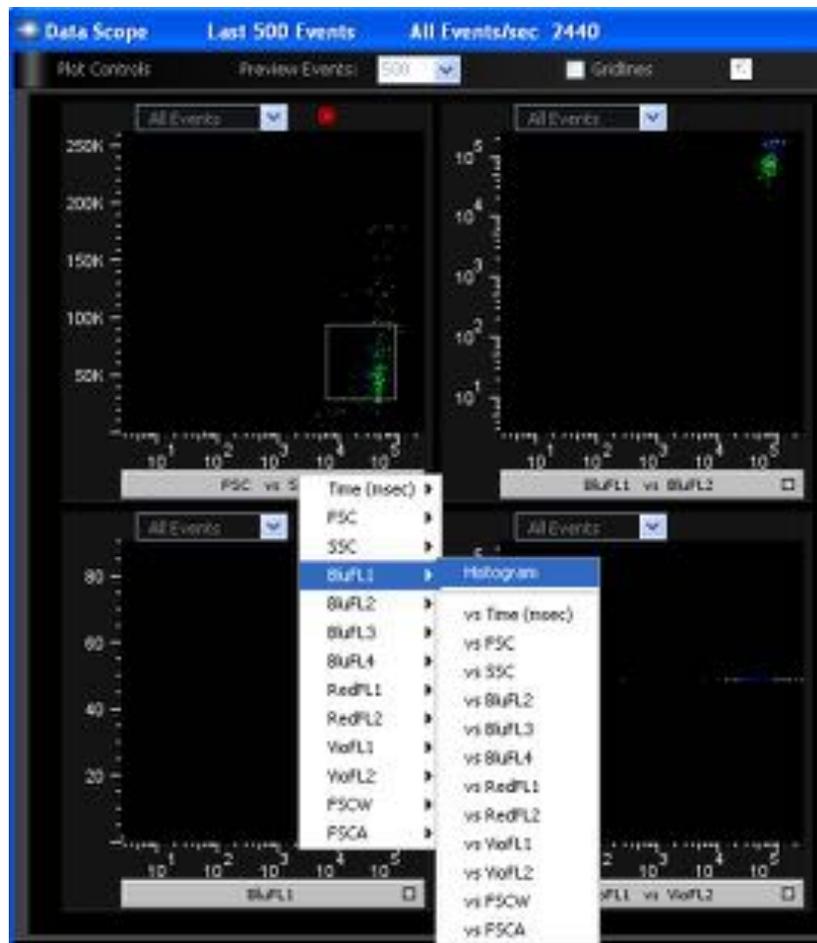
Figure 7.2.2

7.2.2 To Make a Dot Plot

1. Click on the parameter chooser at the bottom of the plot
2. Choose the X-Axis parameter.
3. Move to the right (follow the little arrow) and choose the Y-Axis parameter.

Note: The parameters displayed in the parameter selection bar are listed X axis first and Y axis second. E.g. BluFL1 vs. BluFL2: BluFL1 = X axis parameter, and BluFL2= Y axis parameter.

7.2.3 Displaying Reagent labels on Data Scope plots

Data Scope plot labels will display reagent, names as shown below, when these have been added to the Workspace as keyword columns (see section 5.4).

Name ▲	Statistic	#Cells	BluFL1 reagent	BluFL2 reagent
➡ 📕 sample2.fcs		50000	cd4 fitc	CD3 PE
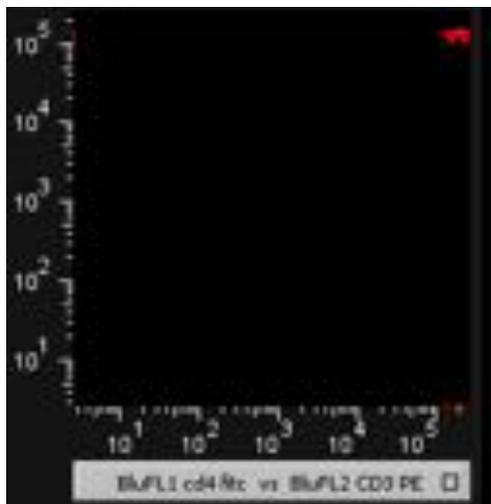
Figure 7.2.3.1

7.3 Introduction to Data Scope Populations and Gates

The Data Scope offers functionality for population analysis (not as extensive as the FlowJo Analysis package). The Data Scope can be used for these functions:

1. During setup and adjustment of PMT Gains, to position a bead or reference cell at a desired location.

2. To verify expected population percentages and means.

3. To create and name a population to use for determining the collection recording stop point based on a count of a specific population (as opposed to All Events).

4. To specify a subset (population) of All Events to be recorded in the FCS file.

5. To inspect the final statistics drawn on all the events and populations classified during FCS recording.

7.3.1 Population and Statistics Section

Create populations using the lower section of the Data Scope; the Populations and Statistics section. Resize the section vertically by clicking and dragging in the top divider bar, and then resize the two sections horizontally by dragging the central divider bar.
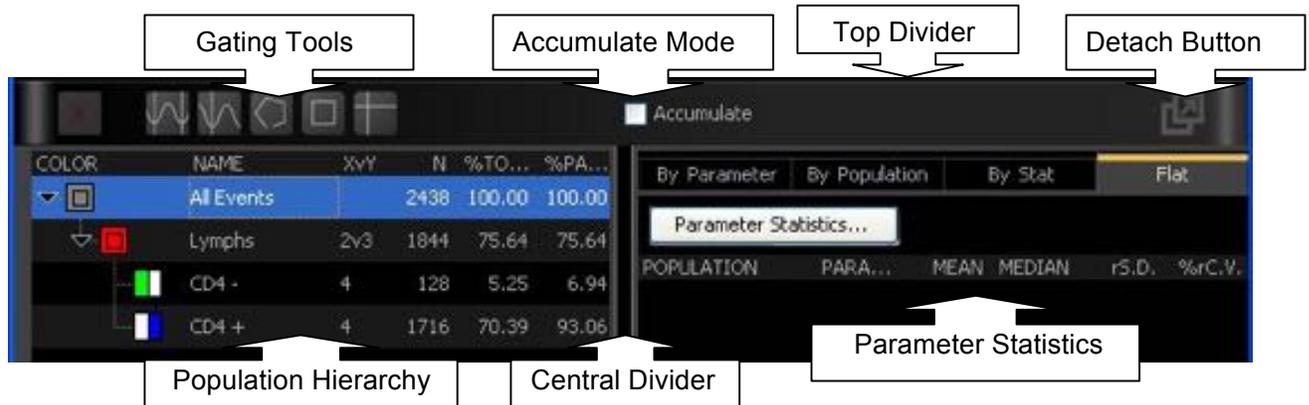
Figure 7.3.1.1

7.3.1.1 Gating Tools

There are multiple gate drawing tools available to the user. From left to right, there are icons for delete, unimodal histogram gate, bimodal histogram gate, and the three possible dotplot gates (polygon, rectangular and quadrant).

7.3.1.2 Detach Button

The various tables and statistics can get large, and the optimal shape for viewing may not match that of the data displays. To view this section in a separate window, click the detach button. You may resize this window, move it within your display, or "minimize" to hide it and concentrate on the data-plots. Closing the window will reattach it to the Data Scope.

7.3.1.3 Population Hierarchy

This table shows all the populations hierarchically. Every population has a name and a color automatically assigned. Click on the triangle to the left of each population icon to reveal/hide child populations. The table has the following columns:

COLOR   The color and icon for the population. To change the color, double-click on the icon and select a new color.

NAME   To change the population name, double-click the default name and type. Population names must be unique.

XvY   The parameters used in creating the population to classify events.

N   The number of events in the population.

%TOTAL   The percentage of events in the population relative to the total (i.e. All Events)

%PARENT   The percentage of events in this population relative to its parent.

Note: To change the default population stem name and colors for all *future* populations, use the Acquisition Preferences dialogue available under the Cytometer Window menu. (see 6.2.4.1).

7.3.1.4   "All Events" Population

By default, a population called "All Events" is pre-defined. Every event belongs to this population, and you consult the counts and statistics for All Events to track the behavior of the entire data distribution.

7.3.1.5 Accumulate Mode, Parameter Statistics

This checkbox and the parameter statistics tables are explained in more detail in section 7.5.

7.4 Creating Hierarchical Populations

In the FJCE hierarchical population model, every population has a *parent* population that must be selected as the first step in creating a new population. The All Events population serves as the default parent, but once a population is created under it, *that* population may be selected as the parent for creating additional population subsets. The next section explains how to use the gating tools to create populations.

7.4.1 General Workflow

1. Select one parent population row in the Population Hierarchy. Figure 7.4.1.1 shows a sample gate drawn in red on a FSC vs. SSC population named 'Single cell'. To draw a new gate on CD4+ cells (BluFL2+) within this Single Cell gate, click on the parent population (Single cell) in the gating panel.
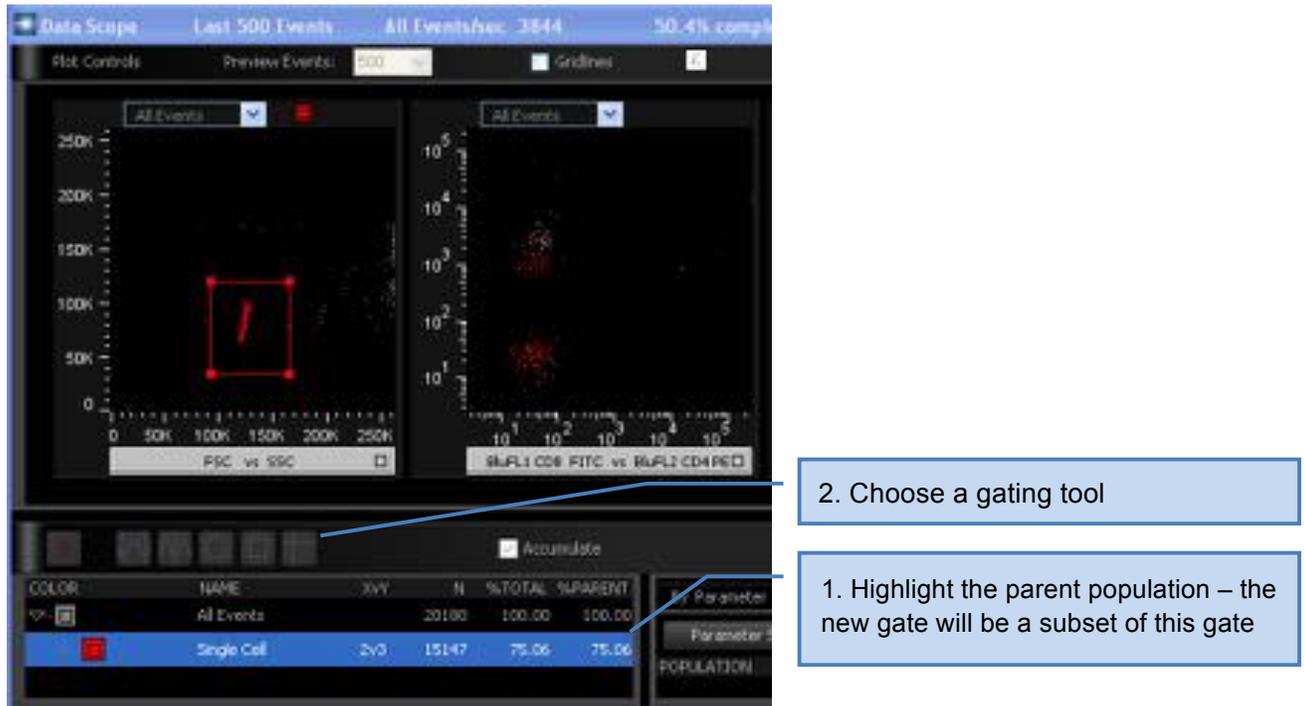
Figure 7.4.1.1

2. Decide whether you want to form the population(s) in a Histogram or Dot plot and click on a gating tool appropriate for your plot choice (7.3.1.1)
3. With the parent highlighted, draw the desired gate on the plot. In this example (Figure 7.4.1.2), a rectangle gate on the CD4+ cells (BluFL2+) is shown on the left and the same population gated on a histogram is shown on the right.
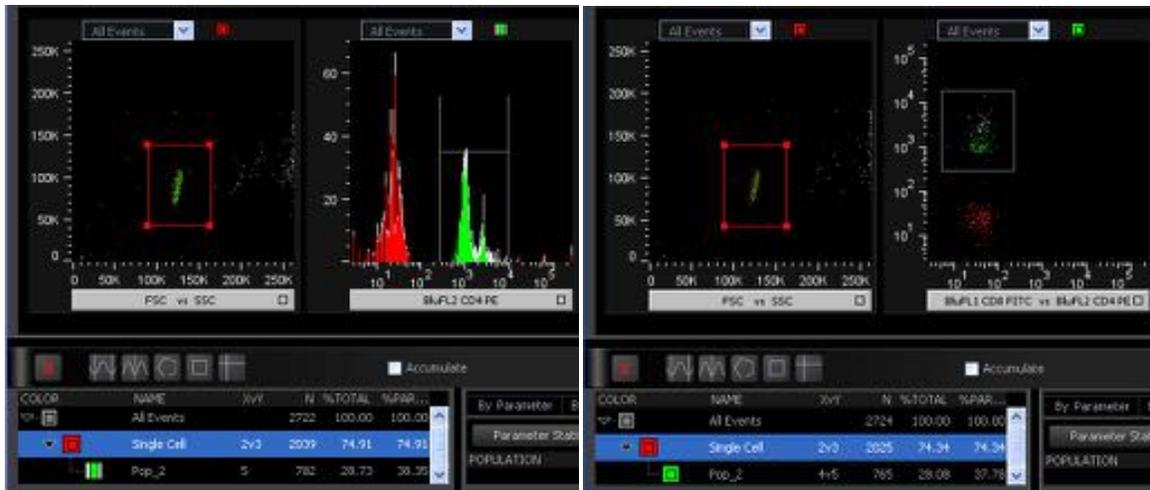
Figure 7.4.1.2

    a. For drawing most gates, you simply put the mouse down in the plot, click and drag. The polygon gate is an exception: click once for each vertex, and click inside the initial vertex to complete the polygon. To cancel, click somewhere outside of any dot plot.

4. Observe the new population(s) in the Population Hierarchy.

5. If desired, adjust the gate, or change the population's name and color.

7.4.2 Gating Tools

Gates have "handles", which are small boxes that show up when the gate is selected, or when the mouse is nearby. They are used to adjust the gate size/shape. You can also move the *entire* gate by dragging on a section of edge which does not contain a handle, or by selecting the gate and using the keyboard's arrow keys.

7.4.2.1 Unimodal gate (goal-post)

This gate selects a single region in a histogram. To move just one side of the gate, drag on the handle.

7.4.2.2 Bi-modal separator

This gate divides a histogram into a positive and a negative region. To adjust the gate, drag either the line or the handle.

7.4.2.3 Polygon gate

This gate classifies dot plot events inside the polygon. To adjust a vertex, drag a handle.

7.4.2.4 Rectangular gate

This gate classifies dot plot events inside the rectangle. To move a vertex, drag a handle.

7.4.2.5 Quadrant separator tool

This gate divides a dot plot into four quadrant populations.

7.4.3 Deleting a population

To delete a population, select it, either on the graph or in the table, then, either click on the delete button (see 7.3.1.1) or hit delete on your keyboard. A warning dialogue will list all the populations that will be deleted. Deleting a parent will also delete all of its descendants. Deleting any population that is based upon a bi-modal or quadrant separator will delete all related "sibling" populations.

7.4.4 Working in a constrained plotting space

Let's say you are working on a laptop with limited screen space. It is possible to redefine a plot's display parameters *after* a gate has been created in the plot. The gate will disappear, but its icon will remain in the gate reminder area at the top-right of the plot, indicating that a gate was drawn using this plot. Hovering the mouse over the icon reveals more information about that gate. If you click on one of these icons, the plot will revert to the parameter choice(s) for that gate, and it will reappear.

7.4.5 Event coloring notes

An event is plotted using the color of the MOST-SPECIFIC population it can be classified into.

For statistics, events can be classified in multiple geometrically overlapping gates. However graphically, only one color can be attached to the event. The color associated with events that fall within multiple gates is dictated by the order in which the populations are painted to the plot.

7.5 Statistics

7.5.1   Population summary statistics

As part of the Population Hierarchy, summary statistics are provided for every population. Since the populations are defined hierarchically, a single event may contribute to the statistics of *multiple* populations. For instance, an activated CD4 T-cell will be counted in the statistics of All Events, Lymphocytes, T-cells, CD4+ T-cells, and activated+ CD4+ T-cells.

7.5.2 Parameter (univariate) statistics

The section of the Data Scope to the right of the Population Hierarchy is where you may view Parameter Statistics. The statistics available are:

Mean: arithmetic mean of the parameter's univariate data distribution.

Median: the parameter value signifying the 50th percentile of the data distribution.

Robust Standard Deviation (rS.D.):   Cluster width, as measured by the range of parameter values bracketing the central 68% (1-sigma) of the data distribution. rS.D. is obtained by histogram-binning the values, and then reading out the 15.87th percentile, and the 84.13th percentile. Outlier values at the tails of the distribution never enter the SD calculation, giving it a robust quality.

%rC.V.:   rS.D. / Median (as a percentage).

There are four tabs, each providing a different option to view Parameter Statistics.

7.5.2.1 Parameter

On this table, you select exactly one parameter, *e.g.* FSC. The values of that parameter, for all populations and statistics, will be displayed.

7.5.2.2   Population

On this table, you select exactly one population, *e.g.* All Events. The values of that population, for all parameters and statistics, will be displayed,

7.5.2.3   Stat

On this table, you select exactly one statistic, *e.g.* Median. The values of that statistic, for all populations and all parameters, will be displayed.

7.5.2.4 Flat

This table is unlike the others in that you have complete flexibility as to which populations and parameters will be displayed. Click on the **Parameter Statistics…** button to bring up a table where you may select them. For each population-parameter pair selected, all statistics will be displayed.

7.5.3 Accumulating Statistics (vs. fast statistics)

Statistics may be produced in two different modes:

> Fast mode - statistics reset once per second, which is appropriate for a non-stationary data distribution (e.g., while you are dragging a PMT voltage).
>
> Accumulate mode - statistics build up continuously until the user changes a setting, changes sample, or does a Reset Preview.

Use the **Accumulate** checkbox located above the Population Hierarchy to control the mode. There is no limit to how long statistics can accumulate, however; the software will automatically reset statistics when the user takes any action that disrupts the stability of the data distribution.

Note: Statistics are linearized for an xPn cytometer producing a log-transformed parameter. In order to produce parameter statistics corresponding to linear expression of a fluorochrome, parameters log-transformed by means of analog electronics are anti-logged by the FJCE software prior to inputting values to statistics.

7.5.4 Statistics during recording / Inspecting final statistics

When the **start record** button is hit, all statistics are reset, and begin accumulating on the events being recorded. At the end of recording the sample, if you wish to inspect the final statistics, the Acquisition Pointer (green arrow) must be pointing at this sample. This requires that the **Automatically advance to next sample after auto-stop** checkbox in Menu / Acquisition Preferences be turned OFF. After inspecting the statistics, manually advance to the next sample to be recorded.

8 Step by Step Acquisition Workflow Instructions

In this chapter we will be describing how to use the FJCE acquisition module. Using a general workflow overview, as illustrated in figure 8.1, this chapter will step you through these processes, and describe the features associated with each step.

Figure 8.1

8.1 Start-up Cytometer

1. Boot-up the computer first, and log into Windows. Turn on the cytometer power and wait 5 minutes for the cytometer to warm-up. During the warm-up time check the Cytek LCD display and ensure you can see cytometer Status displays Ready, and the Sheath and Waste display 'OK' (figure 8.1.1)

Figure 8.1.1 Cytek LCD Display

8.1.1 Launch FJCE and connect to the cytometer

8.1.1.1 Launch FJCE in one of the following ways:
1) through the start menu

Figure 8.1.1.1

2) OR by double clicking on a desktop shortcut.

1. Once FJCE has been launched left click on the **Acquire Samples** button.
   The Cytometer and Data Scope windows (see note) should open.

Note: If the Data Scope does not open it may be opened in the Cytometer Window **Menu.**

8.2 Check Cytometer Performance

Cytek recommends running the Q&B validation procedure for checking your cytometer performance.

An overview of the instruction is as follows:

1. Dilute one drop of Cytek Q&B particles in 1 ml of sheath fluid.
   (http://www.cytekdev.com/products.php?product=Q%26b-Validation-Particles)
2. Load test tube onto cytometer and set flow rate to low.
3. In the Gain Panel, set the scatter parameters to Linear and the Fluorescent parameters to Log
4. Set a scatter gate around the single bead (non-doublets) population
5. While viewing live fluorescent histograms, set the blank bead population within the first decade for analog instruments and to the second decade for digital instruments.
6. Acquire 10,000 gated events to a data file.

7. Refer to the detailed instructions on the Cytek website on how to analyze the data and generate a Q&B report.
(https://www.cytekdev.com/pages.php?pageid=20&from=qb.php%3Faction%3D Start#NewSetup)

8.3 Set-Up Experiment

8.3.1 Select a folder where the data file will be saved and choose a Name Stem for the samples.

1. In the Cytometer Window Menu select **Sample Storage and Naming…** the following window appears:
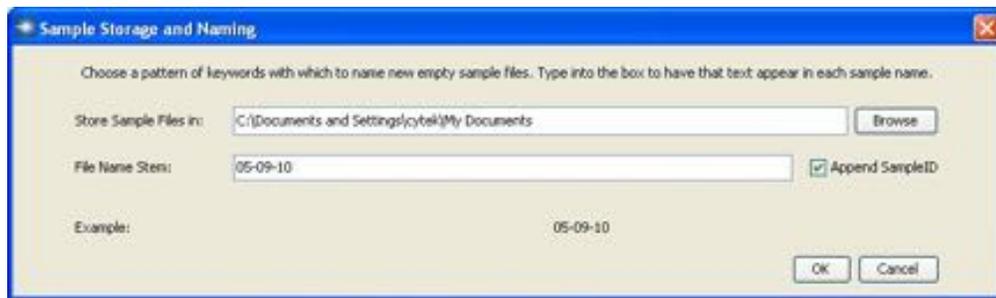
Figure 8.3.1.1

2. Left Click on the **Browse** button to select or setup a folder in which to save FCS data.

3. Type the file name "stem" (name prefix) which you wish to use. Spaces and special characters are not allowed.

4. Click OK to save.

8.3.2 Add Empty Samples to the Workspace

1. Add an empty sample (without keyword copying) by selecting **Add One Empty Sample** in the **Cytometer Menu,** or with keyword copying from the current Acquisition Sample by left clicking the on the Cytometer Window wheel.

2. You may add multiple samples (without keyword copying) by selecting **Add Empty Samples…** in the **Cytometer Menu**
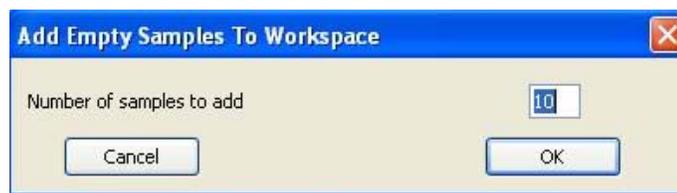
Figure 8.3.2.1

In the example figure above, 10 empty samples will be added to the workspace.

The figure below shows the workspace after the OK button is clicked with the 10 empty samples added to the workspace.
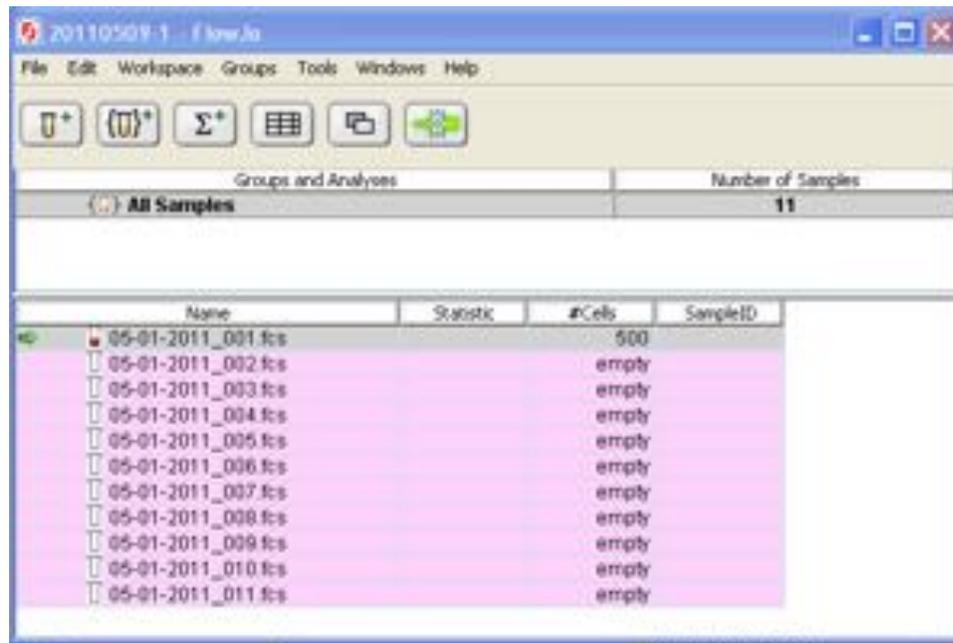
Figure 8.3.2.2

Note: In the above figure the date 05-01-2011_ was used as the stem name. File 05-01-2011_001.fcs was added when connecting to the cytometer and the remaining files were added using the **Add Empty Samples To Workspace** function.

8.3.3 Setting up a Workspace of keyword-coded Empty Data files

An experiment that you expect to repeat should only have to be setup once. The Workspace document populated with empty samples provides a convenient template for an entire experiment that may be saved and reused with minimal data entry.

Sample information can be placed into Keywords and displayed in Workspace columns. Keywords can be defined for any experimental variable you want to code, so long as the values can be entered as text.

As an example, we will add a column for the Patient ID for each sample. In the workspace menu bar click on Workspace and then choose **Add Keyword** from the drop-down menu.
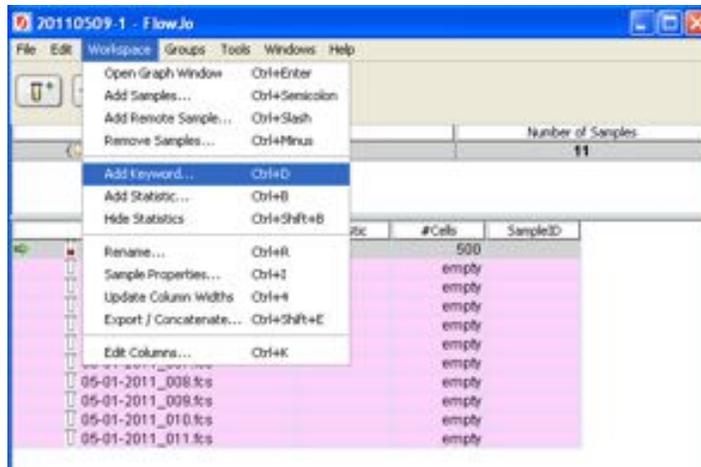
Figure 8.3.3.1

This will open up the a Keyword dialogue box where you can enter the column name:
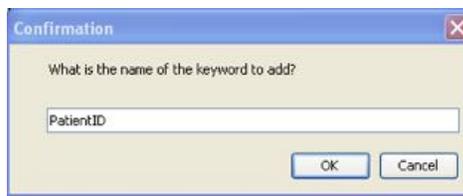
Figure 8.3.3.2

Type in PatientID, click OK, the new column is now displayed in the workspace.
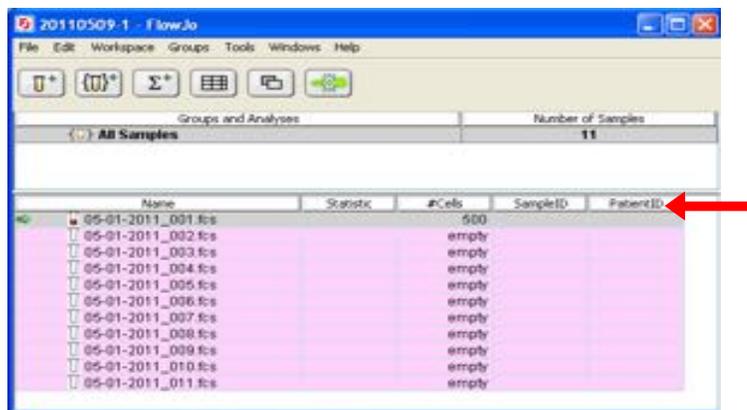
Figure 8.3.3.3

To add columns for tracking the reagents used to stain samples, you will find predefined keywords that may be added using **Workspace / Edit Columns…**. The keywords available

for display as columns in the workspace are listed in the left column. Keyword columns that will be displayed in the workspace are listed in the right column.

Note: For a complete list of FCS keyword definition see
 http://www.isac-net.org/index.php?option=com_content&task=view&id=101&Itemid=46

In this example, columns to name the BluFL1 and BluFL2 Reagents are added by highlighting these keywords in the left column and clicking Add Columns:
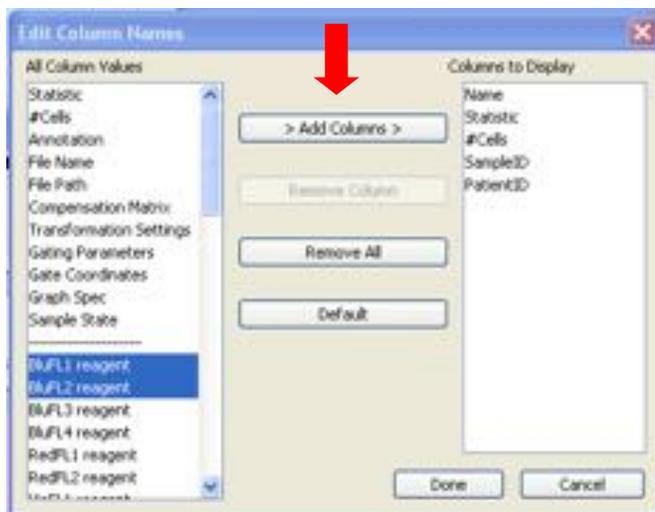
Figure 8.3.3.4

with this result:
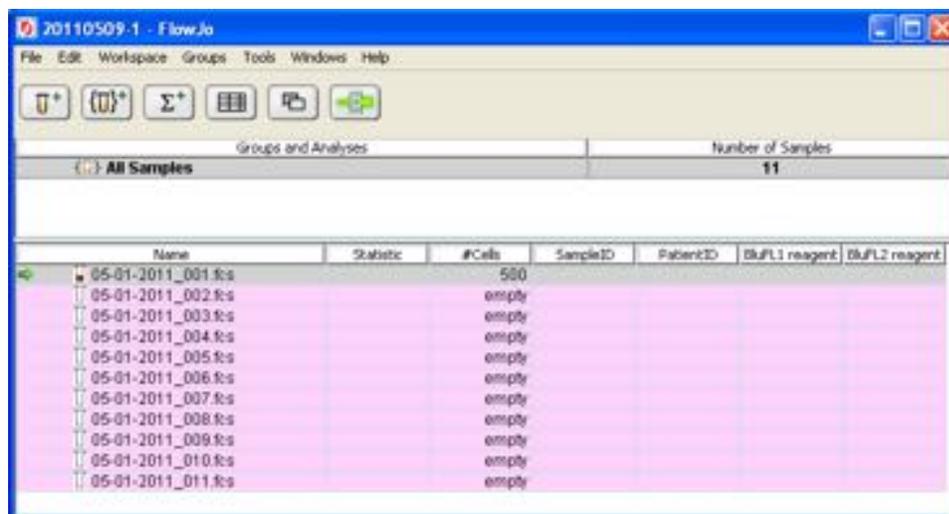
Figure 8.3.3.5

To fill in the labels for each column double-click on the first sample under BluFL1 reagent and type in the antibody/fluorochrome used, in this case, CD8 FITC. We will also label BluFL2 as CD4 PE.
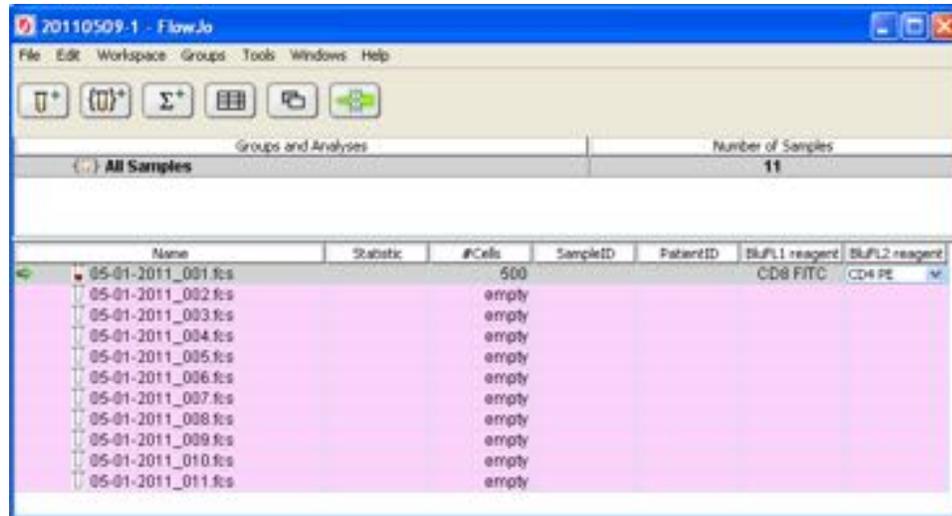
Figure 8.3.3.6

To copy the keyword to all of the EMPTY samples in the workspace, click on the keyword, then hit Control E. In the example below, CD8 FITC was selected and copied to all of the empty samples. Repeat this for each reagent label.
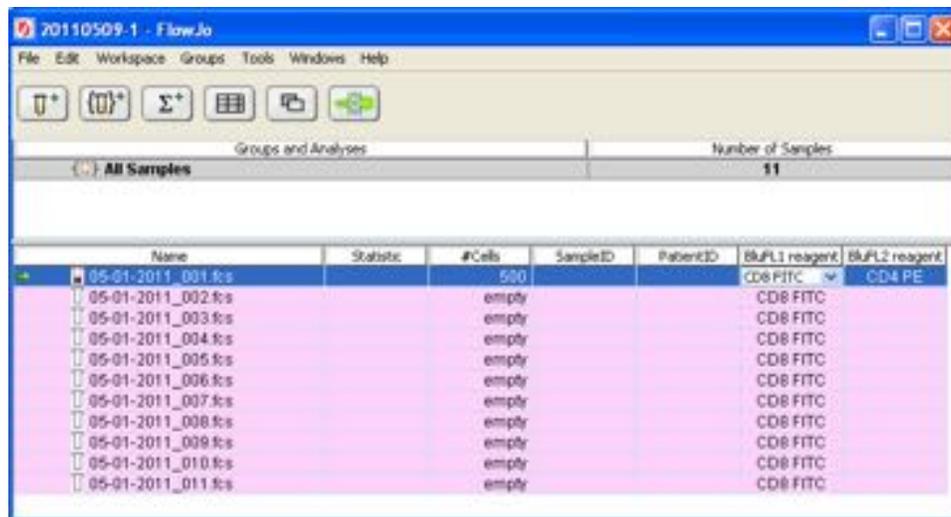
Figure 8.3.3.6

Use the "+" button in the cytometer window to add another sample to the workspace. The "+" button copies from all non-empty keyword cells from the Current Acquisition Sample (green arrow). By moving the green arrow to the last empty sample (using "up" and "down" arrows right of the wheel), a different set of keywords or reagent labels can be entered for additional samples. In the example below, 'No Drug' was added under the treatment column and using the "+" button an additional empty 'No Drug' sample was added.

Figure 8.3.3.7

To add empty samples WITHOUT column copying, use the commands in the Cytometer Menu.

SampleID field. While acquiring samples, a value may be typed in unique to each sample and it will be automatically concatenated onto the filename of the sample. For convenience, a text entry field for SampleID is located in the Collection control panel. The SampleID does not need to be unique as the system will tack on an additional digit(s) to assure filename uniqueness.

Once your workspace is filled out with enough empty samples, labeled as you would like, Save the workspace using the **File / Save…** command. Use the **Save As…** option to save the workspace with the empty samples for future use as a template.

To reuse this saved workspace to run an experiment, launch FJCE, using the file menu open the saved workspace. Close any additional workspaces and launch acquisition.

Note: If you save this workspace, and open it later, you will have the same empty sample names with the same keyword columns. The file naming model will also be saved as part of the preferences file.

8.3.4 Setup Cytometer PMT and Compensation Controls

It is beyond the scope of this user guide to discuss which experimental controls to run; therefore, it is recommended that an experienced flow cytometer operator be consulted to decide which type of control samples should be used for your experiment.

In general, an unstained population and a single-stained population for each parameter are the *minimum* number of controls required to determine fluorescence overlap into unintended channels. For demonstration purposes we are going to use an unstained along with single-stain controls on both the xP and DxP versions to demonstrate how to set compensation for fluorescent spectral overlap.

8.3.4.1 Compensation and running single stain controls for an xP upgraded cytometer

1. Set up the Workspace. The example below shows a five color experiment with single stain controls.
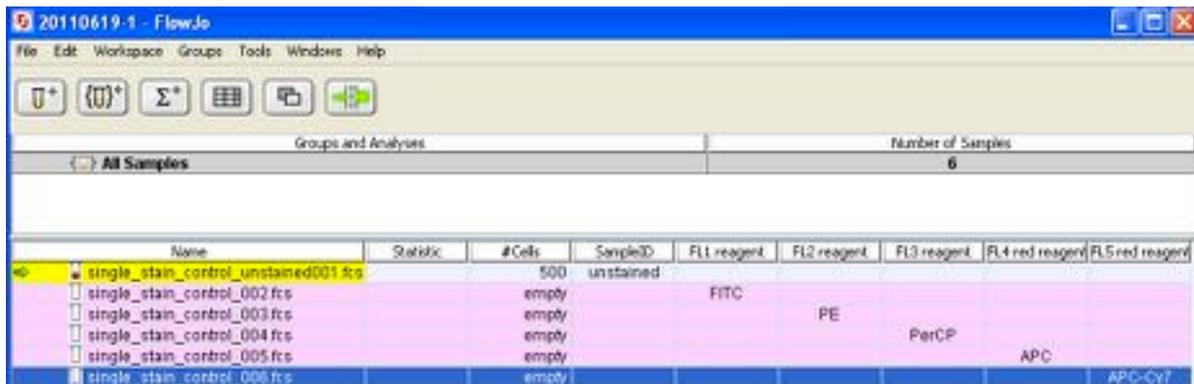
Figure 8.3.4.1

2. Load the negative (unstained) sample on the cytometer. Using the Data Scope plots and statistics panels, first adjust FSC and SSC so that the sample population(s) are visible on an FSC vs. SSC data plot.

3. Draw a gate around the population of interest: e.g., a gate on live cells that excludes debris.

4. Next, individually adjust each of the PMT voltages for the fluorescent channels so that the unstained/negative population falls within the first decade on the data plots.

5. Load the first single stained sample.

6. Using the parameter statistics panel, adjust hardware compensation on adjacent channels until Median of the positive is equal to the mean of the negative on the same axis.

7. Repeat this for each of the single stained controls.

8.3.4.2 Autocompensation on a DxP upgrade Cytometer.

Overview: The goal of running Autocompensation (AutoComp) is to acquire a set of Compensation Controls that measure the unwanted spillover of one fluorescent marker, A, into an unintended detector, B. This needs to be done for the full set of fluorescent markers used in an experiment. Once collected, an unstained (negative) population and a positive population for each fluorescent marker is dragged in the 'Compensation Window' and dropped into the appropriate box.   When all appropriate populations are entered, the compensation algorithm automatically calculates the signal 'spillover' from each channel, and a Spillover values are displayed in the matrix.

1. Set up the Workspace. The example below shows a seven color experiment with single stain controls.

Figure 8.3.4.2

2. In the cytometer window, expand the spillover compensation panel. It is important to collect the unstained and single-stained controls **without** compensation. Verify that the spillover matrix status is 'undefined' and the "Apply Compensation" checkbox is unchecked (see Figure 8.3.4.3).
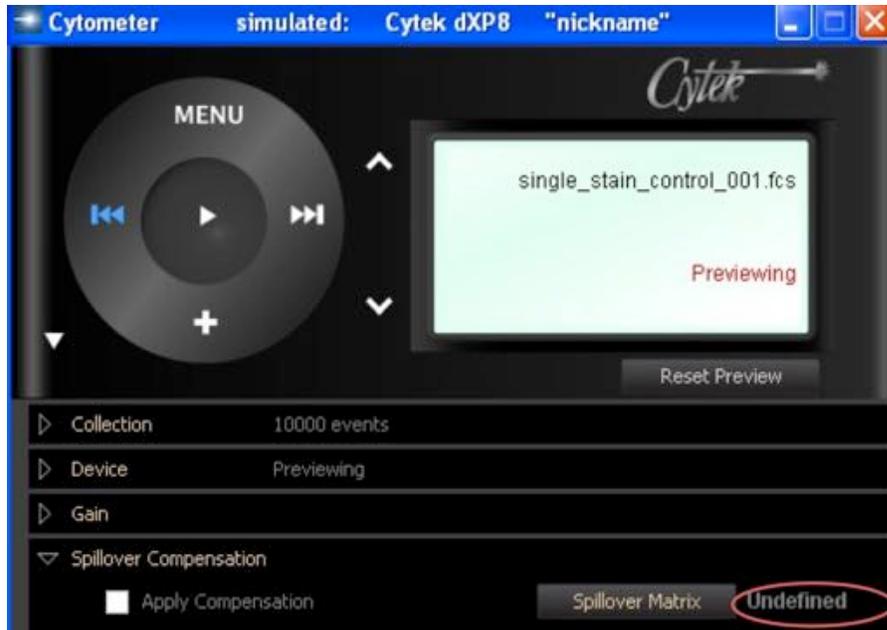
Figure 8.3.4.3

3. Adjust PMT Voltages using the negative control to set the mean of the negative population to approximately channel 30 on the plots.

4. Acquire each of the single stained control samples.

Note: These samples must be uncompensated and, therefore; in the Workspace these should not have a colored vertical bar to the left of the file name (the bar indicates a compensated sample).

5. You will now need to draw a gate around negative and positive populations for each fluorescent parameter. In the workspace double-click on the negative/unstained sample to open this file for analysis. Using the FSC vs. SSC plot draw a gate around the population of interest, e.g., to exclude debris or red blood cells, etc. Copy this gate to all samples by dragging it to the ALL Samples group in the workspace window. In this example, we will call this the 'Live' population.
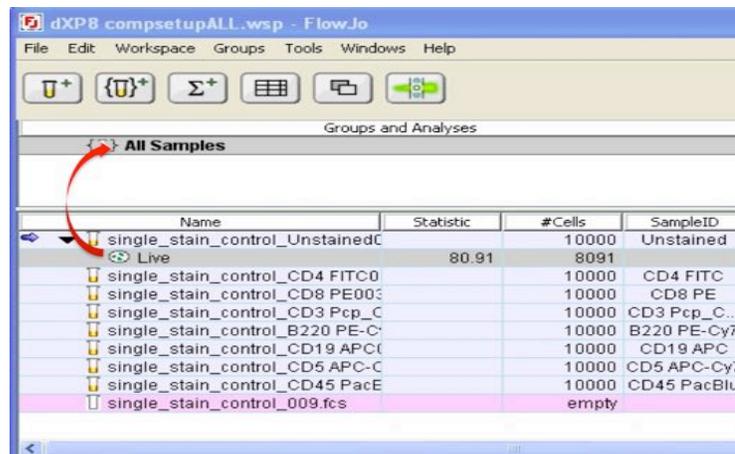
Figure 8.3.4.4

6. Now for a single-stained control, open the Live population (by double-clicking on it in the workspace) and draw a gate on the positive and negative (optional) populations.
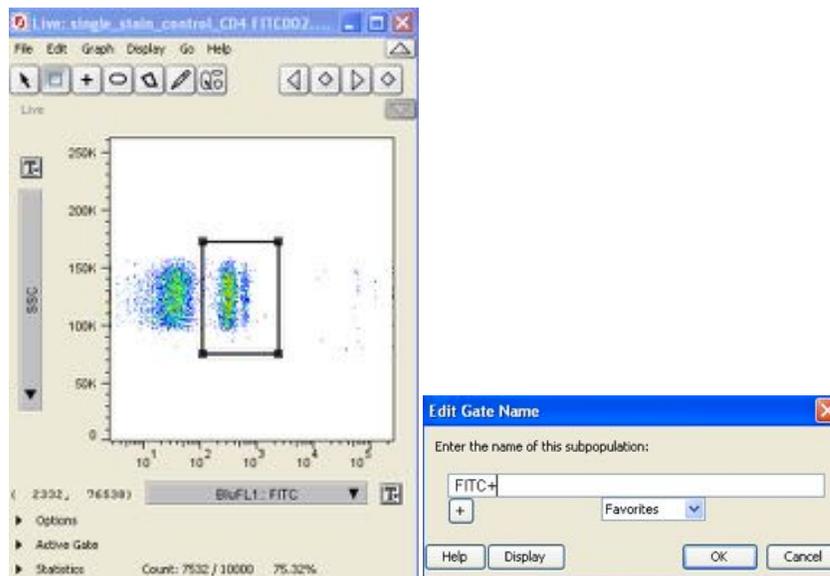
Figure 8.3.4.5

7. Repeat this for each of the single-stained populations.

8. Open the **spillover matrix** window from the Cytometer Window compensation panel.

9. Right click on the parameters not being used and select Remove parameter. Violet FL2 is not being used in the example below.
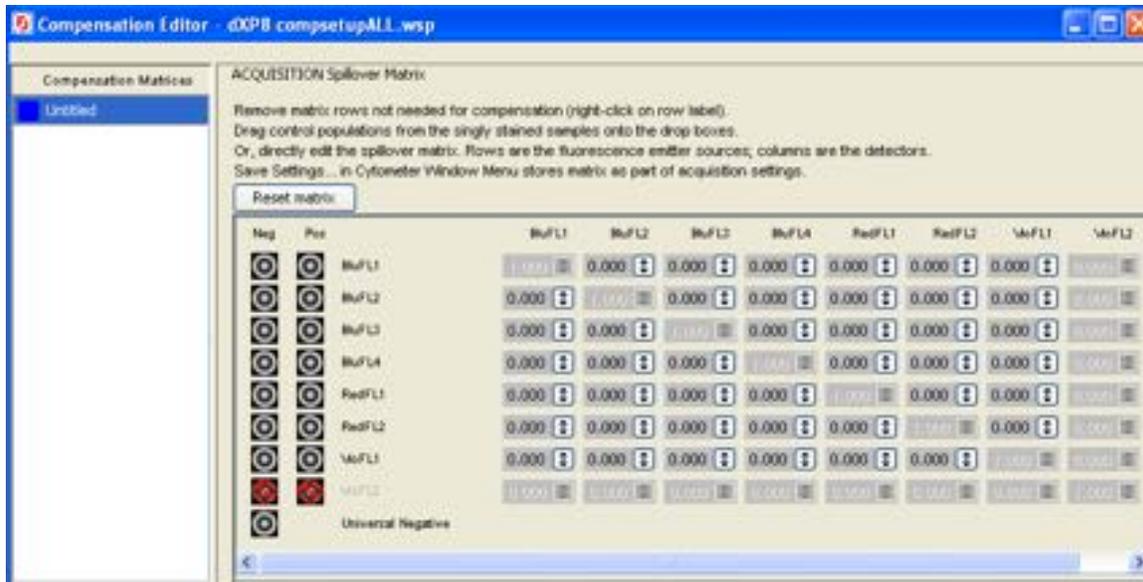
Figure 8.3.4.6

10. Add your staining control populations to the matrix. In the workspace window, select the negative (from your unstained/negative population). In our example this would be the entire Live gate from the first sample. Drag it to the universal negative target box and drop it. Green check marks should fill the Neg. column.

11. Using the same steps, drag each single stained positive sample (or population) to its respective Pos. target box, e.g., drop your FITC+ sample/population on Pos. target for BluFL1. Green checkmarks should appear for each fluorescent channel which has the Positive population applied.

12. Once you have dragged a negative and a positive population for each active channel, the spill over values will be calculated automatically and appear in the matrix to the right of the target boxes.

8.4 Record Data

8.4.1 Set the number of events to record

By now, you have setup the cytometer and the workspace with your empty samples. You will now decide how much recorded data you wish to save. You have the following options:

- # events: Will stop data collection after the instrument has processed the user-defined number of events in the given population.

- # of seconds: Will stop data recording after the user-defined number of seconds.
- Record to FCS file: If desired, you may select a subset of All Events to record to disk.
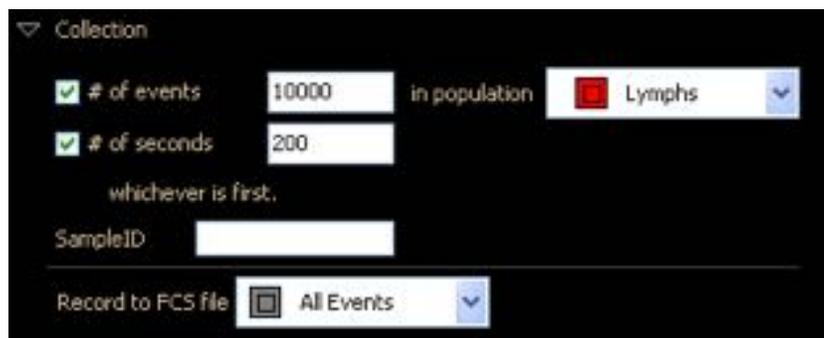
8.4.2 Acquire Data

After setting the number of events or amount of time to record, press the Acquire button in the Cytometer Window.
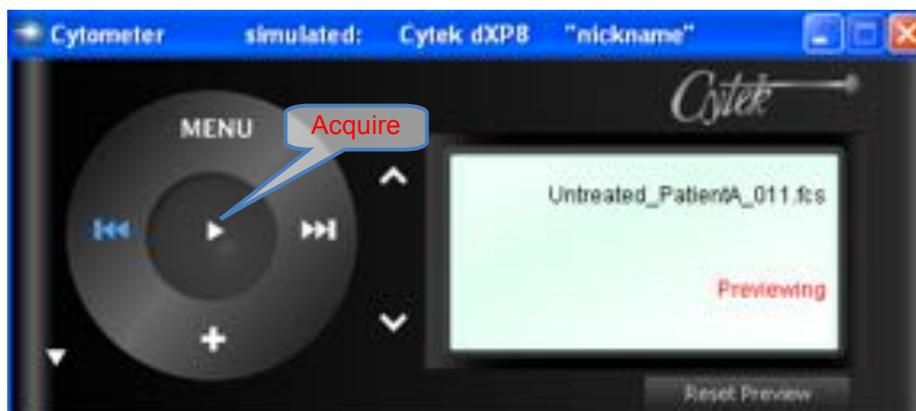
Once you have finished acquiring all of your samples you may move on to Analyzing.

8.5 Analyzing Data

It is recommended that you use FlowJo MAC or PC version for analysis. For instructions on using FlowJo for analysis please see:

FlowJo Analysis Software Manuals at http://www.flowjo.com/home/manual.html

9 Appendix A: Data Scope Gating Boundaries

9.1 Gates classifying a bounded interior

The Rectangle and Histogram ("goalpost") gates classify a bounded interior.
Where is the classification boundary?   The infinitely thin classification boundary (yellow edge below) hugs the *inside* of the drawn gate edge.

This is done so that all classified events are visible, i.e., none are covered up by the gate edge graphics (grey edge lines).
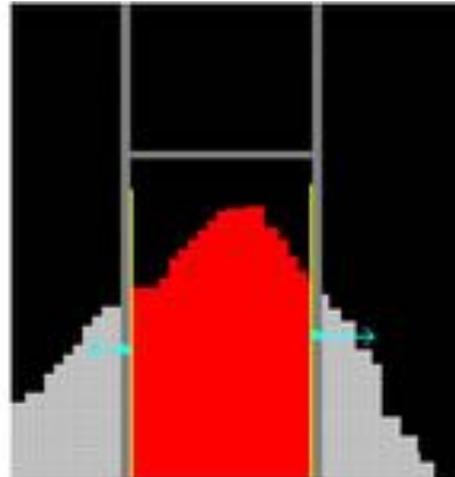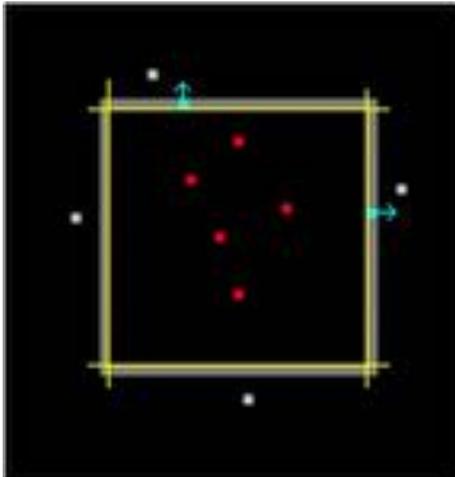
9.2 Gates that separate populations

Bimodal and quadrant gates split a parameter space into positive and negative populations

Where is the classification boundary? The splitter graphics line is drawn on the *positive population* side of the classification boundary (yellow edge below).

The "splitter" graphics line covers over *one pixel* of positive event space (with a decent void separating the (+) and (-) populations, all events will be visible)
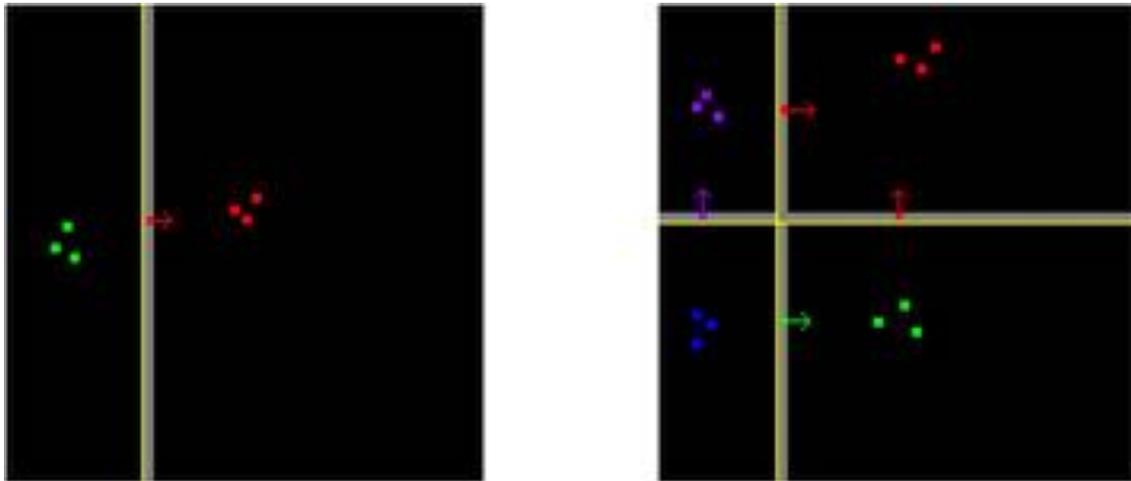
9.3 Gating negative-valued events (DxP cytometers)

A digital cytometer can produce negative values (compensation being the major source). Negative values plot to the 1st pixel row. Events >=0 begin in the 2nd pixel row.

You can set your gates to either *include* or *exclude* these events. In this example, we wish to gate events that are negative in their y-axis parameter. (row circled in yellow)

First, create a thin gate that only encloses one row of pixels. This is easier to do in the vicinity of the axis, not right on the axis.
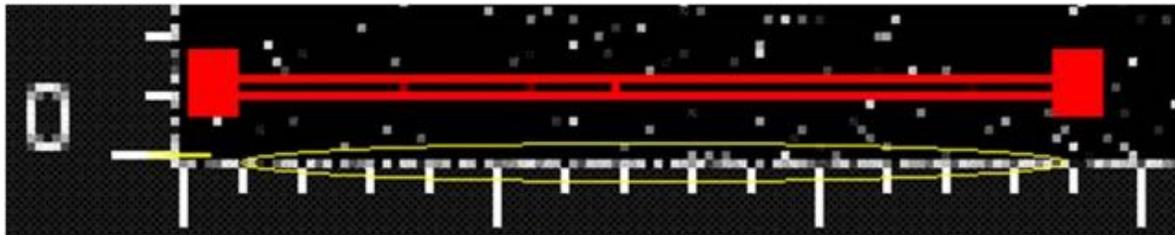
To exclude negatives:

Slowly shift the gate downward as far as it will go, then shift back up one pixel. It is easier to use the keyboard arrow keys instead of the mouse for these fine-positioning adjustments.

In this position, events plotting to the 2nd pixel row are being gated. This will include only the lowest positive-value events. Negative-value events (covered over by the lower gate boundary) are *excluded* from the gate.

To verify that low-positive events are gated, view Pop_5's parameter statistics for BluFL2. The mean and median for Pop_5 are low positive.

To *include* negative-value events:

Now, move the gate down one more pixel so that the lower gate edge is off the graph and invisible. You are now classifying events in the 1st row of pixels, which are *only* negative values. These edge events are colored as red dots (events classified into the red population).

Pop_5 now has a negative BluFL2 mean and median

9.4 Gating Boundaries SUMMARY

The dividing line between negative and positive-valued events falls between the 1st and 2nd pixel of the plot (for both Linear and Log *digital* parameters).

Position the "infinitely thin" classification boundary of the gate as desired to either include or exclude events.

Verify classification decisions by inspecting live parameter statistics for the populations

10 Appendix B: Optimizing analysis of data collected with FJCE

10.1 Setting up FlowJo 7.6 for analysis of FJCE sample data collected on analog cytometers

When analyzing data collected on an analog cytometer (models xP3, xP3+, or xP5) using FlowJo versions 7.6.2 or later, it is necessary to adjust the preference settings. This minor adjustment in the preferences settings will allow correct display and population statistics. These changes must be made BEFORE adding samples to the FlowJo workspace.

NOTE: Cytek Development has only validated population statistics using the outlined preference configuration.

10.1.1 Procedure for adjusting log display range

1. Edit the "FlowJo75.prefs" file using WordPad

   a. Launch WordPad: Start Menu > Programs > Accessories > WordPad

b. Choose File, then Open… Navigate to My Documents and open the "FlowJo75.prefs" file. Note: In the Open File Panel change the 'Files of type' option to "All Documents (*.*)" so the preference file will appear as a menu choice.
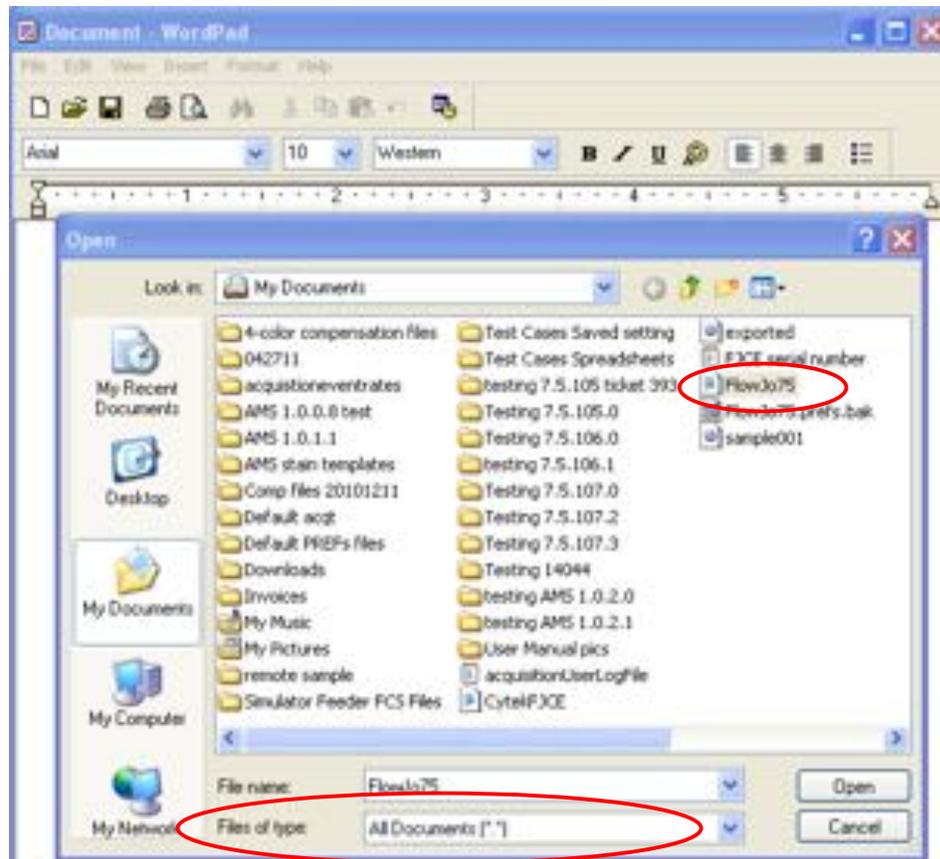
c. Once open in WordPad, use the **Find…**to search for logAllFluors

i. Hit control F (or), this opens the Find dialogue box, where you can type the search term logAllFluors
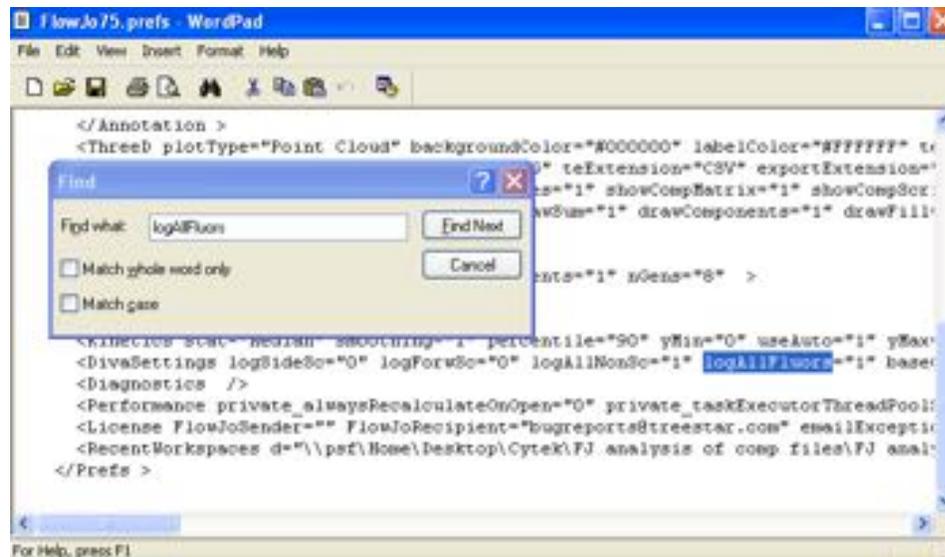
ii. Ensure that the value for logAllFluors = "1", if not, change the value to 1.

iii. Save and exit from WordPad

2. Launch FlowJo 7.6 from the application icon or program menu (i.e., NOT from a saved workspace). BEFORE opening or importing any sample files you must edit the FlowJo preferences.

i. Under the **Edit** menu, open **Preferences**, then chose the FCS3 Data Scaling button

ii. Required settings:

1. Linear channel number corresponding to left edge of log display: **1**

---

2. Decades for display for log DiVa FL params: (FL-A) **4.00** (FL-H) **4.00**

3. Then choose OK to save settings
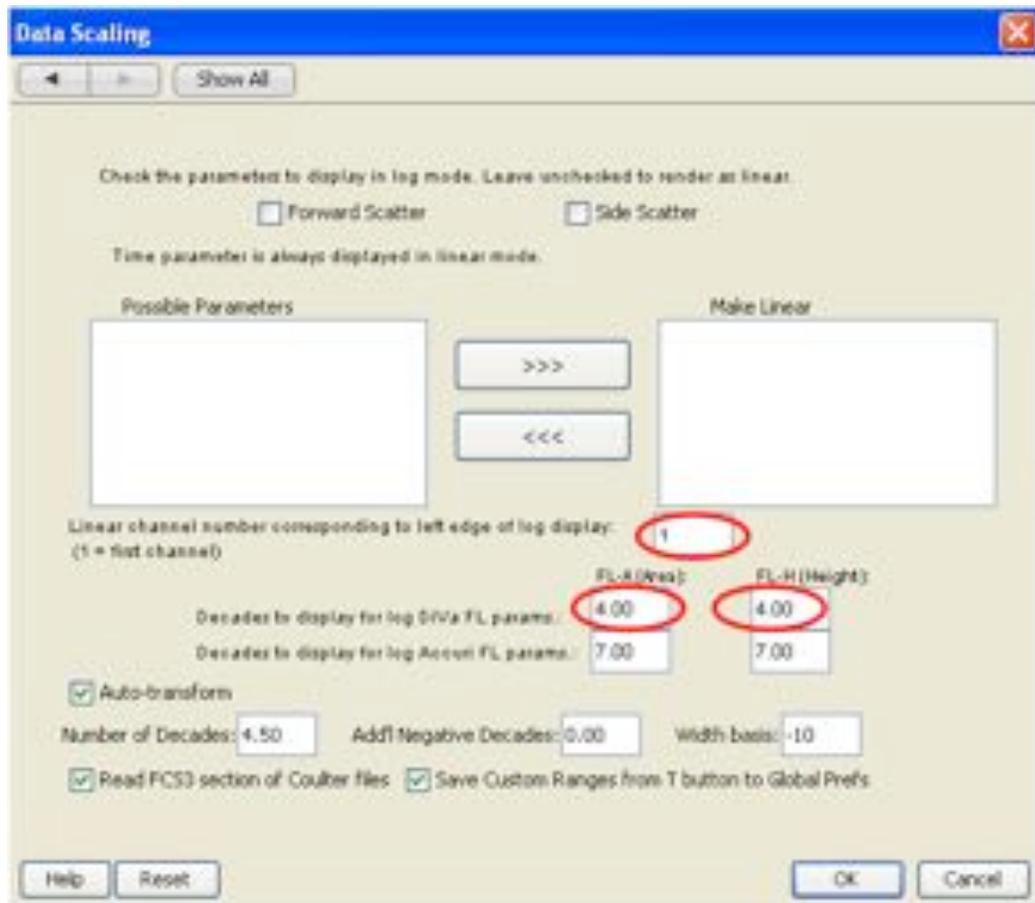
3. The preferences are now appropriate and sample data from xP cytometers can be opened properly

10.1.2 Procedure for displaying Width and Area in Linear mode

1. Width and Area parameters are always collected in linear mode. For FlowJo 7.6 analysis of xP3+ and xP5 cytometer data, you must explicitly assign a linear display to the Width and Area parameters as follows:

2. Launch FlowJo 7.6 from the application icon or program menu.

   a. Add your samples to the workspace. For this procedure, you must import at least one sample for analysis into the workspace BEFORE adjusting the preferences. This allows FlowJo to obtain a list of the parameters used.

b. Under the **Edit** menu, open **Preferences**, then chose the FCS3 Data Scaling button.

c. From the list of Possible Parameters, choose the appropriate parameters to display in linear mode and move them to the box on the right labeled "Make linear".
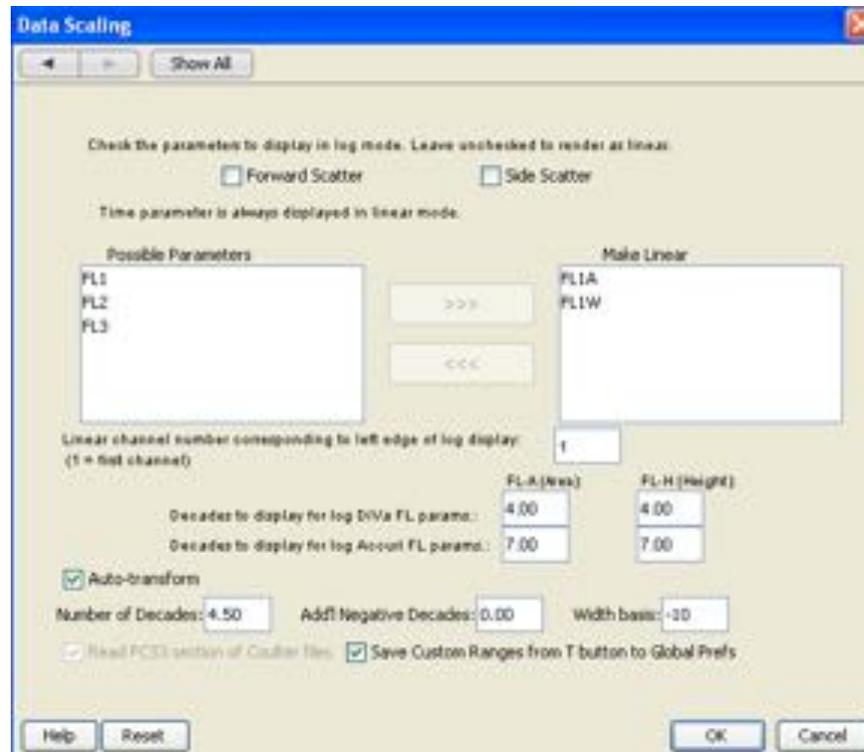
d. FlowJo will now recalculate and display these parameters appropriately as linear.

10.2 Setting FlowJo 8.8.6 and 9.2 (for Mac) preferences for appropriate display of FJCE data

10.2.1   For dXP users:

1. Launch FlowJo Mac from the application icon or menu (not from a saved document). This is needed to start up the application with default Workspace Preferences.

2. BEFORE opening or importing any data files, set the Workspace 32-bit Preferences as follows:

a. Go to Workspace > Workspace Preferences.
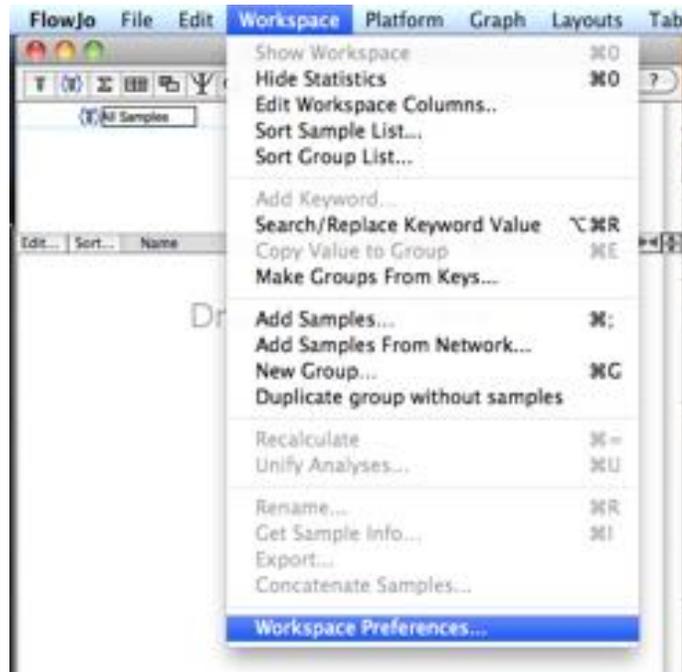
Opening preferences in this manner will only affect settings for the current workspace as noted with the following notification.

b. Under "Reading DiVa and other 32-bit Data Files" hit the "Define" button.

c. At the bottom left, turn on (check) "**Enable Transformation**" and "**Transform Height Parameters Too**"
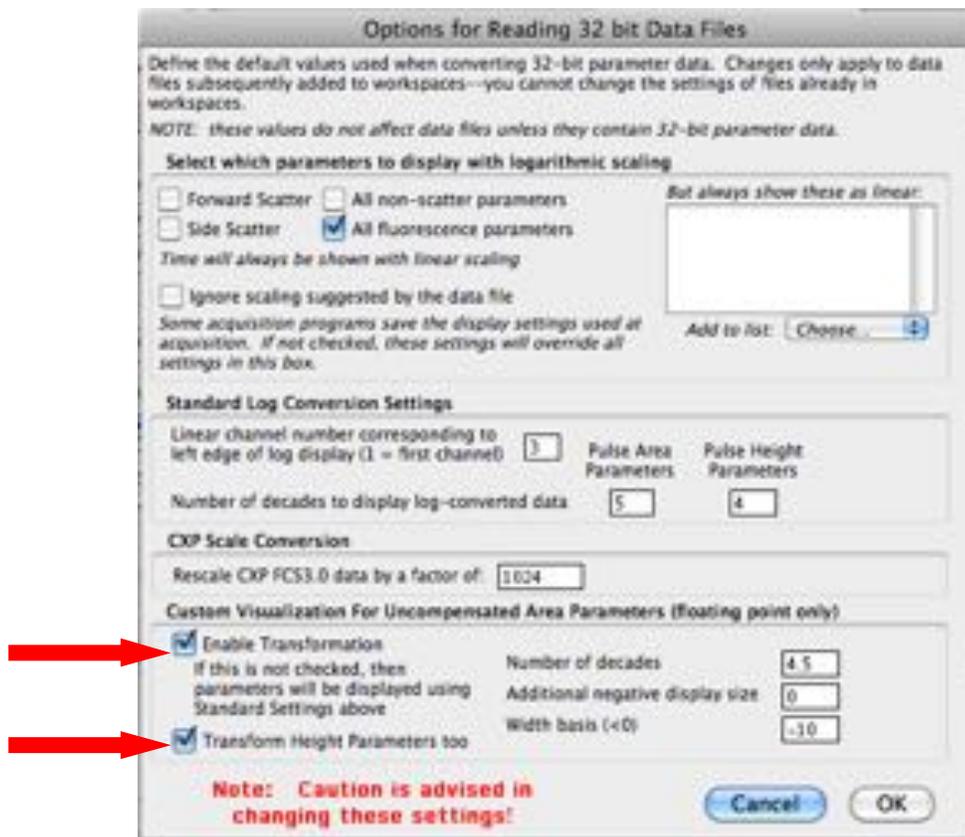
10.2.2   For xP users:

Open Workspace Preferences as described in steps 1 and 2 above. For users analyzing data from xP3, xP3+ and xP5, display settings should be as follows:

- "All fluorescence parameters" checkbox **ON**
- Under "Standard Log Conversion Settings"
  - For Lowest or left edge of log display: **1**
- Under "Then choose the number of decades to display"
  - Pulse Area Parameters: **4**
  - Pulse Height Parameters: **4**
- Enable Transformations: **OFF** (no check)
- Transform Height Parameters: **OFF**