FlowJo Collectors’ Edition
Software Users Guide
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1 About this guide

This document is divided into the following chapters:

Chapter 1, “About this Guide”.

Chapter 2, “Introduction”

Chapter 3, “Describing the System”

Chapter 4, “Installing Starting and Stopping the System”

Chapter 5, “Detailed Description of FJC Components and Their Functions”

Chapter 6, “Step by Step Instructions for Using the System”

Appendix A

Appendix B

Appendix C

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 the 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 Windows™ computer operating system. For more information, see respective Becton Dickinson flow cytometer users guide, and Windows XP user guide
(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.

- Percentage sign % represents the regular command shell prompt.

- Pipe symbol | separates mutually exclusive values for an argument.
2 Introduction

2.1 Purpose

The purpose of this users Guide is to explain the acquisition module of FlowJo 7.5 (FlowJo Collector’s edition).

- FlowJo Collector’s edition software is a collaborative effort between Treestar and Cytek to make the PC version of FlowJo acquire data on Cytek modified cytometers. Depending on the type of Cytek upgrade, xP or DxP you will add more lasers and detectors.

2.2 Scope

This manual will cover how to setup Cytek-modified cytometers using FJC. The purpose of this software training is to highlight the features of FlowJo collector’s 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 the experiments which will be run on cytometers. It does not cover biological and safety precautions associated with running flow cytometry samples.
## 2.3 System Organization

- **Abbreviation(s)**
  1. FJC or FJCE= FlowJo Collector’s Edition
  2. xP= extra parameters
  3. DxP= Digital extra parameters

- **Cytometers Supported**
  - xP3  3 Color FACScan or FACSCalibur w/o Pulse Processing
  - xP3+ 3 Color FACScan or FACSCalibur with Pulse Processing
  - xP5  5 Color FACScan or FACSCalibur (Pulse Processing Required)
  - DxP6 6 Color FACScan or FACSCalibur with Digital Electronics
  - DxP8 8 Color FACScan or FACSCalibur with Digital Electronics
  - DxP10 10 Color FACScan or FACSCalibur with Digital Electronics

- **Version number(s)**
  1. FJC B103

- **Release number(s)**
  1. 8735
3 Describing the System

FlowJo Collector’s Edition is part of a cytometer upgrade system manufactured by Cytek. This system consists of hardware and software which 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 10 colors.

3.1 Key Features

3.1.1 FlowJo Collector’s Edition can be broken down into three main components:

1. The Workspace: Used to create a sample list and to apply attributes to the samples listed
2. The Cypod: Some of Cypod uses include:
   i. Controlling the cytometer
   ii. Setting up file name and saving location
   iii. Set up acquisition preferences
   iv. Save and recall cytometer settings
3. The Datascope: Used to view live data, and setup a collection gate while previewing and recording data.

3.1.2 Inventory

FJC software is intended to be used for collecting data from a Cytek upgraded flow cytometer. 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 7.5 folder is located at C:\Program Files\FlowJo 7.5, and it is recommended this does not change.
For each Windows user there will be a separate flowjo75.pref file located in their respective My Document 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.

3.2 Environment

FJC will operate in a standard lab environment which 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 Collector’s Edition (FJC)
The latest version of FJC can be found on the Cytek development website in the knowledge base section; http://www.cytekdev.com/kb/categories.php?categoryid=3 .
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.

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: Datafiles 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 Collector’s Edition

Note: You may skip steps 1-7 in the following section if your computer was loaded with FJCE by Cytek.

INSTALLATION OF FLOWJO COLLECTOR’S EDITION FROM CYTEKDEV.COM WEB SITE

Assuming you never had FlowJo software running on you system before.

1. Launch Internet Explorer and go to http://www.cytekdev.com/kb/
2. In knowledge base, click on Browse by Category dialogue to get to FlowJo Collector’s Edition and choose FlowJo Cytek Updater v1.0.32 article
3. Follow instructions from this article to install FlowJo Updater_1.0.exe
4. To run the FlowJo Updater installer, right hand click and choose open and then run after the download. The installer will be placed in C:\Program Files folder after the installation. The Updater can be used either to install FJCE, or update existing FJCE installs.
5. To install FlowJo Collector's Edition simply navigate to C:\Program Files\ Flowjo Cytek Updater and launch FJUpdate application.

6. After the launch, a FlowJo Cytek Updater window will appear showing b102 (installer) and other patches, click on b102 (installer) and choose “Get update” to run the b102.exe installer. Following the instructions will guide you through the installation process. After having drvtxtb driver installed, [Need driver drvtxtb folder to be included in Updater]

7. launch FJCE via Flowjo shortcut on the Desktop or Flowjo application in Flowjo 7.5 folder under C:\Program Files\ directory. You should be see a dialogue asking you to enter serial number (license) if you do not have a hardware dongle attached. If you enter the serial number correctly, Flowjo workspace window will open up.

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

TO UPDATE YOUR FLOWJO WITH NEW PATCHES

Launch the FJUpdater application from C:\Program Files\FlowJo Cytek Updater to get back to FlowJo Cytek Updater window (see steps 1-4 in previous section to install Updater). DO NOT choose b102 (installer); instead, choose the appropriate program patch from the list and click “Get Update” to update the components in FlowJo b102. The patches should have the description and build number that goes with them. The most recent version will have the highest build number.
4.4 Starting up FlowJo Collector’s Edition (FJC) for acquisition

1. Turn on the cytometer, and make sure the USB cable is connected. Launch FJC by clicking on its icon in the start menu:

   ![Start Menu with FJC Icon](image1.png)

   Fig. 4.4.1

2. Or its shortcut on the desktop:

   ![Desktop Shortcut](image2.png)

3. Or by selecting it in the programs menu:

   ![Programs Menu with FJC Icon](image3.png)
4. Once you have launched FJC, a blank Workspace (WSP) will be displayed as shown in figure 4.4.2

![Fig. 4.4.2](image)

5. To connect to the cytometer and enter acquisition mode, press the acquire button. If acquire button doesn't show up in tool bar, click on Edit Pulldown menu, then Edit Buttons command. Drag the acquire button from the Edit Buttons window and drop it into the task bar.

6. You will see the Cypod data acquisition and control window open. If the Datascope window was in use when last closing the application, it will open along with the Cypod. If you don’t see the Datascope, select Cypod menu, Show Datascope.

### 4.5 Stopping and Suspending the FJC

To Quit FJC you may click on the red X in the upper left of the WSP window, press the Exit selection in the File menu or use the CTRL+Q hotkey (Fig. 4. 4.3).
FlowJo Collector’s can only be used by one user at a time for each computer. When switching users in Windows ensure you disable *Use fast user switching* as shown below. User Accounts is located in the Control Panel. This will quit programs when logging off and allow the new user logging on to be able to use FJC.
5 The Workspace

The FlowJo Workspace will serve as a sample list for listing the samples you wish to acquire along with their respective keywords.

5.1 The Workspace Window and its Icons

In figure 5.1.1 The following buttons are present by default:

- **Add Samples**: adds already acquired sample to the WSP. See FJ Analysis for detailed explanation. (Same as Add Samples in Workspace Menu)
- **New Group**: Creates a new sample group. 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. (Also appears in FJ workspace display)
Table Editor: Allows you to edit tables of data statistics. See FJ Analysis for detailed explanation. (Also appears in Windows Menu)

Layout Editor: Opens layout editor which is used for graphically representing data. See FJ Analysis for detailed explanation. (Also appears in Windows Menu)

Acquire Samples: Pressing this button will connect FJC to the cytometer, and launch the Cypod and Datascope (if datascope was viewable when last quitting FJC). If the cytometer is not connected, you will be asked to run in simulation mode.

Define or adjust compensation values: This is for setting up software compensation values in analysis mode. See FJ Analysis for detailed explanation. Please note this compensation window does not have the spillover slider controls found in the spillover matrix in the Cypod.

5.2 The Workspace Sample list Symbols and Highlight Colors

Saved data file is a data file which has already been recorded to disk using either FJC or another data acquisition software. In the workspace these files are represented by a yellow test tube, with a light blue highlight.

Previewing sample is the present sample the user is setting up to be recorded (acquired). In the workspace these files are represented by a green arrow and red test tube, with a yellow highlight.

Active Analysis sample: Arrow changes from green to purple when previewing sample is clicked to open workspace display, and apply live stats to the sample.

Recording sample (or sample being acquired): Will have a red test tube and a green highlight with an increasing #Cells column.

Paused sample: Will have a green test tube and a static #Cells column.

Empty Sample: is awaiting preview and recording and will be represented by an empty test tube, and a pink highlight.

The workspace uses various highlighting colors for rows to illustrate the state of sample and which sample has been 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

  [Add green highlight for acquiring sample, and light blue for acquired but not selected sample.]

An acquired sample which is not selected will have light blue color (figure 5.1.2 INFalpha2.fcs).
6 The Cypod

6.1 The Cypod is the area or window in FlowJo Collector’s FJC where the user can:

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 save or period of time for data to be acquired.
3. Monitor the status of the cytometer
4. Control the PMT and amplifier settings
5. Turn lasers on and off
6. Define and apply a spillover matrix (digital instruments)
7. Set up hardware compensation (analog instruments)
8. Define threshold parameter(s)
9. Setup the area and width parameter

Illustration 5.2.1 shows the Cypod in a minimized view. Each section can be expanded or minimized by selecting the disclosure triangle located to the left of each section. To expand the Cypod to see other sections select the disclosure triangle located in the lower left corner:
In the top window bar the cytometer configuration and Cytometer name are shown.

### 6.2 The Cypod Menu

In the Cypod menu (Fig 6.2.1) the user may:

1. Add one empty sample
2. Add empty samples
3. Define sample storage and naming
4. Edit Keywords
5. Define acquisition preferences
6. Open instrument settings
7. Save instrument settings
8. Display the Data Scope
9. Set the Time parameter scale
10. Show Cytometer Info.
11. Display help

12. Enter the test menu

![Cytometer simulated: Cytek dXP10 "nickname"

Fig 6.2.1

6.2.1 Add One Empty Sample

Left clicking on \**Add One Empty Sample** will add one empty sample to the WSP. This empty sample will use the file naming convention and save to folder defined in the **Sample Storage and Naming**… command area. This is the same action as selecting the ‘+’ at the bottom of the Cypod control circle.

6.2.2 Add Empty Samples…

To add multiple empty samples to the WSP, left click on the **Add Empty Samples**… command in the Cypod menu. The window in figure 6.2.2.1 will appear.
• **Number of Sample to add**: defines the number of empty samples to add to the WSP.

• **Sample Numbering**: Defines the sample number scheme for the multiple empty samples to add. This number will be added to the end of the sample name prefix.
  - **Automatic**: will start the numbering of the empty samples one digit higher than the highest number presently in the WSP
  - **Start with**: allows the user to enter the sample number they wish to start with.
  - **OK**: Adds empty samples to WSP.

• **Cancel**: Cancels add empty sample operation

• **Help**: Links to topic help section on website.

**Note**: When a “Start with” number already exists in the WSP, a “(1)” will be added to the end of the file naming prefix. As shown below

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample2(1).fcs</td>
<td>empty</td>
</tr>
<tr>
<td>sample2.fcs</td>
<td>50000</td>
</tr>
</tbody>
</table>

### 6.2.3 Sample Storage and Naming...

Left clicking on the **Sample Storage and Naming**... command will bring up the window shown in figure 6.2.3.1. In this window the operator may:
1. Define the storage folder of the data files: clicking the **browse** button opens the folder selection dialog 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.

![Select directory where acquired samples will be saved](image)

**Figure 6.2.3.2**

2. Define the File naming model: combinations of keywords (starting with $) and text may be used to define data file names. Spaces and special characters (@#$%^&*) may not be used. File naming model will be displayed on the example line as the user defines the sample name.

3. Insert keywords into the file naming model. The user may use defined FCS keywords in the dropdown menu as part of their file naming convention. Highlight the desired keyword, and select Insert Keyword. If the desired keyword isn't shown in the keyword list, select Edit Keywords to add the desired keyword.

4. The Edit Keywords window (Fig 6.2.3.3) has four sub-windows of keyword values to select which keywords will be used to define workspace columns, and which keywords appear in the Insert Keyword for the File Naming Model.

5. The first set is always added to the workspace when ‘Add as Columns in Workspace’ is selected. Selected keywords from the second set can be deleted before ‘Add as Columns to Workspace’ is selected. To delete keywords, use shift-click-click to highlight the desired range, then select ‘Remove Keywords’ in the lower right of the window. Selected keywords in the third set can be added to the keyword list in the second set by highlighting a keyword value in the third set, and selecting ‘Insert Keyword’ in the lower
right of the window. These keywords are also added to Insert Keyword list for the File Naming Model.

Custom keywords (such as SampleID) can be entered in the fourth sub-window (without a ‘$’), and added to the second window for inclusion as a column in the workspace and to the Insert Keyword list for the File Naming Model.

If a keyword value is selected for a workspace column, and used in the file naming model, then entering a keyword value in the workspace will automatically change an empty sample name from the default ‘x.fcs’, where x is a number, to ‘keyword value.fcs’. Duplicate keyword values will automatically have a number inserted to maintain unique sample names.

![Fig 6.2.3.3](image)
6.2.4 Adding Keywords to the Workspace

Keywords may be adding to the Workspace using the Add Keyword and Edit columns command located in the Workspace pulldown menu.

- The Add Keyword command allows a user defined keyword to be added to the workspace which can then be managed like other keywords.

- 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.
6.2.5 Define Acquisition Preferences

To define acquisition preferences the user left clicks on **Acquisition Preferences** command located in the Cypod menu. Figure 6.2.4.1 illustrates the Acquisition preferences window

- **Save FCS files every**: Defines how often the FCS data will be written to disk. Choices are 5, 10, 15, 30 and 60 seconds.
- **Refresh Data Scope**: Defines how often the Data Scope plots are being refreshed. Choices are 1, 2, 3, 4, and 5 times per second.
- **Refresh graphs every**: Defines the delay between updates of FlowJo WSP plots, choices are from 1 to ten seconds.
- **Limit Data Scope and preview graphs to**: Sets the maximum number of events which can be viewed in the Data Scope and FlowJo plots during preview and recording. Choices are; 50, 100, 250, 500, 1K, 2.5K, 5K, 10K and 50K events.
- **Refresh Cytometer Status**: set the frequency for updating the cytometer status in FJC. Choices are 1, 2, 3, 4, and 5 times per second.

---

Note: For detailed instructions on how to edit columns in the Workspace see section 8.3.4

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Figure 6.2.4.1
Note: Writing FCS data to disk, refreshing plots and graphs, and updating cytometer status more often will slow down FJC performance.

- **Automatically advance to next sample after auto-stop:**
  - **Box Checked:** Once the collection criteria have been met, the acquisition pointer will advance to the next available (empty) sample in the WSP. If no empty samples exist FJC will automatically create a new empty sample using the same file naming convention previously defined using the **Sample Storage and Naming...** command, and advance to that sample for previewing.
  - **Box not checked:** When the collection criteria has been met, the recording status will change to “**Recording Paused**”, FJC will wait for further commands from the user.

- **Require User Log On:** When this box is checked a log on dialog appears when FJC is started. A CSV log file with user times and dates (acquisitionUserLogFile) is kept in the My Documents folder for the user who is presently logged in.

- **Simulation Mode:** For training and demo purposes the user may run FJC in Simulation mode. When running in simulation mode FJC uses a previously saved FCS data file as the data acquisition source and “spools” the data file to simulate data coming from the cytometer.
  - **Simulation source FCS data file:** uses standard windows dialog to select a simulation data file.
  - **Simulation event rate:** sets the data event rate of the simulated data file

- **OK:** saves changes to acquisition preferences
- **Cancel:** aborts changes made to acquisition preferences and reverts to the settings prior to opening acquisition preferences window.

### 6.2.6 Open Settings....
Previously saved instrument setting files may be opened using the **Open Settings....** Command located in the Cypod menu. When left clicking on the **Open Settings...** command the dialog box in figure 6.2.5.1 will appear. Settings files will have an .acqt extension and will contain; PMT voltages, Parameter Amp modes, parameter amp values (for LIN), threshold values, area and width parameters, spill over Matrix (digital instruments), and Compensation Values (analog instruments).
6.2.7 Save Settings…
Left clicking on the Save Settings… command in the Cypod menu, will bring a Windows Dialog box similar to figure 6.2.5.1 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 present 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.

6.2.8 Show Data Scope
Left clicking on this command will display the data Scope if not presently open. When launching acquisition in FJC, the Data Scope will load in the same state (opened or closed) as it was when FJC was last quit.

6.2.9 Time Parameter Scale…

Defines the time scale for FlowJo plots and the collection time in seconds. (Figure 6.2.8.1)
6.2.10  Show Cytometer Info
Left Clicking on the Show Cytometer Info command in the Cypod Menu opens the window illustrated in figure 6.2.9.1

- **Cytek dxP10**: cytometer configuration that FJC reads during launch of acquisition. This configuration is set at 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 name you wish to give the cytometer. This name will be saved to all FCS data files acquired using it, and to the Experimenter’s Log.
6.2.11 Test Pulses

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 and parameter dump are used for diagnostic purposes.

6.3 The Cypod Action Wheel

The Record button: will transition the acquisition state from Preview to Record. By default the program is always previewing live data from the cytometer. The recording of data will begin when pressing the record button. During recording this button will turn a gray color and be unavailable to the user.

Erase and Re-Record: This function will erase and re-record a sample being recorded (saved to disk) or a sample which has already been recorded and saved to disk. If this button is pressed during the record process, the user will be presented the following warning:

Note: If you wish to erase and re-record press YES and then press the Record button again. If you do not wish to Erase and Re-record press NO and continue recording the original sample.

Next Acquirable sample: Advances the acquire pointer to the next available empty sample ready for acquisition in alpha-numeric order. Both empty and filled samples are arranged in alpha-numeric order in the WSP.
Add One Empty sample to the Workspace: This button provides a quick way to add one empty sample to the WSP. This button will use the file naming convention previously setup in the Cypod Menu area.

Workspace navigation arrows: Pressing the UP arrow will move the WSP acquisition pointer (green arrow) up one sample in the list of samples in the WSP. Pressing the DOWN arrow will move the pointer down one sample in the list of samples in the WSP.

6.4 The Acquisition Information Window

- Displays the name of the sample which the acquisition pointer is set to.
- Displays the maximum number events to be previewed
- Displays the status of acquisition; Previewing or Recording

![Acquisition Information Window](image)

Figure 6.4.1

- Reset Preview: Resets the preview counter and all DataScope plots to zero.
6.5 Defining Sample Collection:

- **# of events**: Checking this box will stop data recording on the total number of events defined in the field to the right (100000 shown in example).

- **# of Live-Gated Events**: When a gate has been defined in the datascope, # of events will change to # of live-gated events. Data recoding will now be terminated when the defined number of events is reached within the gate drawn in the Datascope.
• **# of Seconds**: Checking this box will stop data recording on the total number of seconds defined in the field to the right.

• **# of events in FlowJo Gate**: This function is being deleted please do not use.

• **Drop Gate Here**: This section is not used please disregard

• **Note**: When multiple termination criteria are defined, the first satisfied condition terminates acquisition.

### 6.6 Device: The following section shows the Cypod Device section

![Cypod Device Status](image)

**Figure 6.6.1**

- **Device Status**: Displays the status of the cytometer
  - Previewing
  - Recording data
  - Standby; STBY mode selected, or no sample pressure
- Not Ready; 5 minute warm-up, sheath empty, waste full, no 488 laser power
  - **Diff. Pressure:** Measures the differential pressure between the sheath and sample. Should be approximately 0.2 PSI for low, 1.0 PSI for high
  - **Fluid Tanks:** Monitors the level of the sheath and waste tanks.
- **Laser Excitation**: Color coded sections for each type of laser installed. If argon ion laser is installed, power and current is monitored. 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.
6.7 Gain: The following section illustrates the Gain section of the Cypod.

- **Parameter #**: The parameter number as identified in the hardware

**Note**: Figure 5.3.7 demonstrates a 4 laser 10-color system where Time=P1, FSC=P2, SSC=P3, BlueFL1=P4, BlueFL2=P5, BlueFL3=P6, BlueFL4=P7, L2-1=P8, L2-2=P9, L2-3=P10, L2-4=P11, L3-1=P12, L3-2=P13, Width=P14, Area=P15

- **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 Mode**: Select LOG or LINEAR. When log is selected scaling in the datascope will show 4 logs for analog instrument and 5 logs 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 50 volts
  - Adjusts the PMT down by 50 volts

### 6.8 Spillover Compensation (DxP digital instruments)

To view your data with compensation applied during Preview, you may apply a Spillover Matrix. This Matrix may also be saved to the recorded data file. Cypod control for this function is shown in figure 6.8.1.
When you wish to set up a compensation matrix press **Spillover Matrix.** This will open the spillover matrix window shown in figure 6.8.2. In this window you can setup, Save, Load a previously saved, and delete spillover matrices. Once you have spillover matrix values loaded into the compensation editor window, you may apply these settings to data files during acquisition by left-clicking in the check box next to "Apply Spillover Compensation" 

You may create a spillover matrix manually or automatically.

**Manual:** The sliders next to each spillover value may be used to change the spillover value

**Automatic:**

- Set PMTs to correct values
- Acquire single color controls, and negative control, and create appropriate gates if necessary
- Remove parameters in the spillover matrix window that do not have a control by right clicking on parameter
- Drag the gated negative to the Universal Negative box
- Drag the gated single color controls to the appropriate positive checkbox
- After the last positive control has been dragged, spillover values will be automatically calculated
- Click **Apply Spillover Compensation** to view live compensated data in the Datascope.
You may save your spillover matrix settings in 4 different places.

1. **FCS data file header**: When you acquire a data file with “Apply Spillover Compensation” box checked, the values listed in the compensation matrix editor will be saved to FCS data file header.

2. **FlowJo Matrix file**: A separate spillover matrix file can be saved as a .mtx file. To perform this function, go to; **file menu, Save Matrix…**, enter the desired name in the toolbar window, then choose folder to save the matrix file to.

3. **Instrument Setting file**: Saving an instrument setting file using the command located in Cypod menu section (link to menu section), This .acqt file will contain PMT, and amplifier setting as well as spillover values.

4. **The FJC Preferences File**: When exiting the program the current spillover matrix setting will be saved to the preference file located in the *My Documents* folder. It will automatically be recalled when restarting FJCE.

To apply compensation to an acquired datafile, drag it from the workspace to the Samples box in the Compensation Editor. A bar will appear next to the sample name in the workspace, and Workspace Displays will show compensated data. To delete compensation, highlight the sample in the Samples window, and select Del.
6.9 Hardware Compensation (xP Analog Instruments)

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

![Figure 6.9.1](image)

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

To adjust compensation moves the slider control shown above.

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

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

**Note:** A fundamental difference between digital and analog compensation described in the previous two sections is Hardware compensation subtracts signal prior to digitization within the cytometer, well before it becomes FCS data. Digital compensation is applied by the computer to the unaltered raw data while previewing, or after the original FCS data is saved. Digital compensation can be removed from the data file during analysis but analog hardware compensation cannot be.

### 6.10 Threshold (DxP Digital Instruments)

Setting 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. When the event exceeds the threshold amount it is then processed by the cytometer. Figure 6.10.1 illustrates the threshold controls for DxP instruments.
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 must either exceed the primary threshold value or exceed the secondary threshold parameter before the event will be detected.

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

- Adjust threshold up in increments of 50
- Adjust threshold down in increments of 50
- Adjust threshold up in increments of 10
- Adjust threshold down in increments of 10

Use the slider control to manually set threshold from 0 to 1000.

Threshold values translate to a scale from 0 to 262,000 channels. For example, a threshold value of 100 is approximately channel 26,200, 10 is approximately 2,620.

6.11 Threshold (xP Analog Instruments)

Setting the cytometer threshold sets the minimum signal level required to have the cytometer recognise an event. When an event goes through the detection area and does not meet the threshold level it is ignored. When the event exceeds the threshold amount it is then processed by the cytometer. Figure 6.11.1 illustrates the threshold controls for xP instruments.
Threshold: sets the threshold parameter. Can be set on any primary laser parameter, and is the criterion the event must meet in order to be detected.

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

- Adjust threshold up in increments of 50
- Adjust threshold down in increments of 50
- Adjust threshold up in increments of 10
- Adjust threshold down in increments of 10

Use the slider control to manually set threshold from 0 to 1000.

Note: Threshold values translate to a linear scale from 0 to 1000. For log scale, threshold values translate to a scale of channel 1-10,000. A level of 500 is approximately channel 100, and a level of 250 is approximately channel 10.
6.12 Area and Width parameters (DxP Digital Instruments)

Area and width may be selected on any available parameter on DxP upgrade cytometers, except Time. Figure 6.12.1 illustrates the area and width section of the Cypod.

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. To set this, run CEN from Biosure that have singlets, doublets, triplets, and 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. You can also use beads at a high event rate.

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.

### 6.13 Area and Width parameters (xP Analog Instruments)

When using an analog cytometer, Area and width may be selected on the primary laser’s FL1, FL2 or FL3 parameters. xP upgraded cytometers share parameters P6 and P7 with Area / FL5 (P6) and Width / FL4 (P7). Figure 6.13.1 illustrates the Area and Width section of the Cypod for xP instruments.

![Figure 6.13.1](image)

**Source Parameter:** Choose FL1, FL2, or FL3

Use the radio buttons to choose FL4 or Width and FL5 or Area.
7 The Datascope

The Datascope is the section of the acquisition module that can be used for:

1. Viewing live data
2. Setting a collection Gate

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

7.1 Datascope Event Status

Data viewing and event rate information can be viewed in the top window bar of the datascope.

![Datascope Event Status](image)

**Figure 7.1.1**

- **Datascope Last number of Events**: total number of events being displayed in each datascope plot. This number is set in the acquisition preferences section of the Cypod.
- **Events/sec**: Displays event data rate in events per second.
- **Live Gated**: displays the Datascope gated event rate in events per second.
7.2 Viewing Live Data

In the Datascope the flow cytometer user may view both Histograms and Dot Plots as shown below (figure 7.2.1)

7.2.1 To make a Histogram (figure 7.2.2):
1. Select the parameter selection bar at the bottom of the plot
2. Choose the X-Axis parameter
3. Choose Histogram

7.2.2 To Make a Dot Plot (figure 7.2.2)
1. Select the parameter selection bar at the bottom of the plot
2. Choose the X-Axis parameter
3. 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 Datascope plots
Datascope plot labels will display reagent labels when these have been added to the workspace as keyword columns as shown below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Statistic</th>
<th>#Cells</th>
<th>BluFL1 reagent</th>
<th>BluFL2 reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample2.fcs</td>
<td></td>
<td>50000</td>
<td>cd4 fit</td>
<td>CD3 PE</td>
</tr>
</tbody>
</table>
7.2.4 Gridlines
Turn the gridline option on by checking the gridline check box in the header section of the Datascope. Use the gridlines as a quick visual guide for quantifying your data’s channel location. Gridline on dot plots and histograms are shown below.

7.2.5 Plot background color selection
Toggling the Datascope background color selection switch will toggle the background color of the plots from black to white. When the plot background is white, the data will appear black (shown in figure 7.3.5). When the plot background is black, the ungated data will appear gray.
7.2.6 Working with Datascope Gates

Rectangular and Histogram gates may be drawn within the Datascope, and only one gate may be drawn within one plot. These gated events may be used for data acquisition criteria. The user has the ability to save only gated data to the FCS data file and may view only gated data in the datascope plots.

7.2.7 Drawing a live Gate

The draw live gate function allows the user to draw a histogram gate or a rectangular gate in a dot plot. To draw the gate, first select the Gate button, then click and drag around the population of interest in a plot displaying the population of interest. Examples of the histogram and dot plot gates are shown in figure 7.2.7.1.
• To resize the gates shown above, click on the squares and hold, then drag to resize.
• To delete the gate, make it active by clicking on its border then press the delete key (not backspace).

**Note:** When a gate has been drawn in a plot, the plot parameters may not be changed.

### 7.2.8 Gated events color selection

The user may use the color selector (shown in figure 7.2.8.1) to select the color of the gate.

Each gated event displayed in all data scope plots, will then be displayed in the selected color.

![Color Selector](image-url)
7.2.9 FCS File
The **FCS file** command toggles between saving all events and gated events only. When unchecked (shown in figure 7.2.6.1) all events will be saved to the FCS data file. When checked only gated events will be saved to the FCS data file. Save All events option is selected by default.

Warning: When checking Gated, only events within the gate will be saved to the data file. All events outside the gate will be discarded and not saved to disk.

7.2.10 Plot Unclassified
This command toggles between displaying all events (gated and ungated) when the box is checked (Figure 7.2.6.1) and displaying only gated events when the box is unchecked. When **plot unclassified** is unchecked, only gated events will be displayed in all datascope plots including the plot in which the gate is drawn.

Note: When un-checking the **plot unclassified** command, the user will not be able to view any events outside of the gate. To see events outside of the gate, check the plot unclassified box.
8 Step by Step Acquisition Workflow Instructions

In this chapter we will be describing how to use the FJC 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.

8.1 Start-up Cytometer

1. 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 says Ready, and the Sheath and Waste read ‘OK’ (figure 8.1.1)
2. After the cytometer has finished warming up turn on the computer and log onto windows.

8.1.1 Launch FJC and connect to the cytometer

8.1.1.1 Launch FJC in one of the following ways:
  1) through the start menu
  2) double clicking on a desktop shortcut
  3) Double clicking on the executable file in the C:\Program Files\FlowJo 7.5 folder

1. Once FJC has been launched left click on the Acquire Samples button.
   The Cypod and datascope (see note) should open.

   **Note:** Provided the datascope was open when FJC was last quit. If the datascope does not open it may be opened in the Cypod 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
   (http://www.cytekdev.com/products.php?product=Q%26b-Validation-Particles) in 1 ml of sheath fluid.
2. Load test tube onto cytometer and set flow rate to low.
3. Set the scatter parameters to LIN 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.

Detailed instructions on how to run the Q&b procedure can be found on Cytek’s website (https://www.cytekdev.com/pages.php?pageid=20&from=qb.php%3Faction%3DStart#NewSetup)

8.3 Set-Up Experiment

8.3.1 Name your sample and select a folder where the data file will be saved to.

1. In the Cypod Menu select Sample Storage and Naming… The following window appears;

![Acquisition File Naming Model](image)

2. Left Click on the **Browse** button to select or setup a folder in which to save FCS data files to.
3. Type the file name which you wish to use. Spaces and special characters are not allowed. If you wish to use keywords as part of the file name, select the desired keyword from the pull-down menu, then left click on insert keyword.
4. Click OK to save Acquisition file naming model. Future empty samples added to the WSP will have the file naming model previously setup.

### 8.3.2 Add Empty Samples to the Workspace (WSP)

1. Add an empty sample by selecting **Add One Empty Sample** in the Cypod **Menu** or by left clicking the **+** on the Cypod wheel.
2. You may add multiple samples by selecting **Add Empty Samples…** in the Cypod **Menu**.

![Add Empty Samples To Workspace](image)

In the example figure above, 10 empty samples will be added to the WSP. The numbering at the end of the file name prefix will automatically start with one digit higher than the highest numbered sample in the WSP. If you wish to start the number at a number other than the next highest, choose **Start with** instead of **Automatic**, then enter the number you wish to start with.

The figure below shows the WSP with the 10 empty samples added to the WSP when the OK button is left clicked.
8.3.3 Setting up a Workspace of Empty Data files

The following steps will allow setting up the WSP with data file names which may be changed after the empty files have been added to the WSP and before data has been recorded to them.
1. Set up a column in the workspace using the $smno keyword.
   a. Go to Menu in the Cytopod, Edit Keywords,
   b. Delete all the default keywords in the middle window using shift-click (hold the shift key down, click on the first and last keywords, then select Remove Keywords).
c. Select $SMNO in the bottom window, then select **Add Keyword**. You will now see one keyword, $SMNO, in the middle window.

```
<table>
<thead>
<tr>
<th>Start</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SMNO</td>
<td></td>
</tr>
</tbody>
</table>
```

```
<table>
<thead>
<tr>
<th>CELLS</th>
<th>Sample (tube or well) label.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$COM</td>
<td></td>
</tr>
<tr>
<td>$CTEN</td>
<td></td>
</tr>
<tr>
<td>$DSP</td>
<td></td>
</tr>
<tr>
<td>$INST</td>
<td></td>
</tr>
<tr>
<td>$OP</td>
<td></td>
</tr>
<tr>
<td>$PROJ</td>
<td></td>
</tr>
<tr>
<td>$SMNO</td>
<td></td>
</tr>
<tr>
<td>$SRC</td>
<td></td>
</tr>
<tr>
<td>$SYS</td>
<td></td>
</tr>
</tbody>
</table>
```

Note: Select **Add as Columns**. This will add all the $PnS keywords, as well as $SMNO to the workspace.

```
<table>
<thead>
<tr>
<th>Name</th>
<th>Statistic</th>
<th>#Cells</th>
<th>$SMNO</th>
</tr>
</thead>
<tbody>
<tr>
<td>single_saint.fcs</td>
<td></td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>
```

---

**Note:** If you don’t want all the $PnS keyword columns in the workspace, go to the Workspace menu, select Edit Columns, select all the $PnS you want to delete using shift-click, then select Remove Column. You should now just have a single keyword, $smno in the workspace.
2. Go to the Cypod Menu, select Sample Storage and Naming...
3. Click the expansion arrow to see available keywords for sample file naming, select $SMNO,
4. Select Insert Keyword so it appears in the File Naming Model, then click OK.
5. In the Cypod Menu select Add Empty Samples,
6. Select the number of empty samples to add, click OK.

**Note:** If you save this workspace, and open it later, you will have the same empty sample names with the same single keyword column. Also, the file naming model will be saved as part of preferences.
8.3.3.1 Naming Samples Using Keyword Values

After setting up the WSP with empty data files as described in 8.3.3, sample file naming may now be done by entering keyword values.

Type in a keyword value in the keyword column, the value will be automatically transferred to the sample name, without moving the position of the sample in the workspace.

*The example below shows “lymph1, lymph2…lymph10” used as the $SMNO keyword, and how the WSP will look when this keyword is used as part of the filename.*

If you use the drop down menu to enter a keyword value, then the sample position in the workspace will automatically move to its alphabetized position.

*For example, if you have 8 $smno empty samples, and you are on the first of the 8, and you use the dropdown menu to enter ‘aaax’, then the sample will move down after the last $smno, since ‘$’ comes before ‘a’ in the ASCII code. If you type in ‘aaax’ for a keyword value, the sample will remain in the same place, before the rest of the $smno samples.*

---

**Warning:** When entering values for a keyword value, it is important to press Enter, because no other operations are possible until Enter is pressed.

**Note:** Be sure to use ‘Save As’ when saving this workspace after acquisition is complete to preserve the empty sample workspace, so it can be used again.
8.3.4 Add Keyword Column to the WSP

Adding keyword information to your empty samples can help identify details of each sample during analysis. To add keyword columns to the WSP select Edit Columns... located in Workspace pull-down menu as shown below.

After selecting the Edit Columns... command the following dialog box appears.
The keywords available for display as columns in the WSP are listed in the left column. Keyword columns which will be displayed in the WSP 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](http://www.isac-net.org/index.php?option=com_content&task=view&id=101&Itemid=46)

An example to add all reagent label keywords for an 8 color experiment plus area and width parameters follows:
Step 1: Shift-Left click to select multiple reagent labels in the left column

Step 2: Left click **>Add Columns** to move the selected keywords to the right **Columns to Display**

Step 3: Left Click the **Done** button, and then verify the reagent labels in the right column are now being displayed in the WSP.

**8.3.4.1 Type Keywords value for the columns added to the Workspace (WSP)**

Type in the Values you wish for each column or select from the list of previous values added. See below
8.3.5 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. For demonstration purposes we are going to use an unstained along with single-stain controls on both the xP and DxP versions.

8.3.5.1 Running single stain controls for an xP upgraded cytometer

1. Set up the Workspace (WSP) in section 6.1.4., The example below shows a five color experiment with single stain controls.
   Show WSP with color single stain controls

2. Adjust PMT Voltages to set the negatives in the first decade.

3. Adjust hardware compensation on adjacent channels until Median of the positive is equal to the mean of the negative on the same axis.

8.3.5.2 Running single stain controls on a DxP upgrade Cytometer.

1. Set up the Workspace (WSP) in section 6.1.4., The example below shows a seven color experiment with single stain controls.

2. Adjust PMT Voltages to set the mean of the negative population around channel 30.

Note: Alternatively you may use Cytek 6-peak QC beads for this and set the blank bead to channel 30.
3. Acquire each of the single stained control samples.
4. Draw a gate around the negative population
5. Draw gates around each of the single stained populations
6. Open the spillover matrix window in the Cypod
7. Right click on the parameters not being used. Violet FL2 is not being used in the example below

8. Drag the negative population to the universal negative target. Green check marks should fill the Neg. Column.
9. Drag each single stained positive sample to its respective Pos. Column target box. Green checkmarks should appear for each fluorescent channel which has the Positive population applied.
10. Once you have dragged a universal negative and a positive population for each active channel, the spill over values should compensate automatically.

8.4 Record Data

8.4.1 Set the number of events to record
Once 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:
• **Total events**: Will save the user-defined number of total events. In the “Total Events” graphic, data recording will terminate after 100,000 total events or 200 seconds which ever occurs first.

• **Gated events**: Will save the user defined number of events with the datascope gate. In the “Gated Events” graphic, data recording will terminate after 100,000 datascope gated events or 200 seconds which ever occurs first.

• **Timed acquisition**: Will stop data recording after the user defined number of second.

<table>
<thead>
<tr>
<th>Collection</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td># of events</td>
<td>100000</td>
<td></td>
</tr>
<tr>
<td># of seconds</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Collection</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td># of Live-gated events</td>
<td>100000</td>
<td></td>
</tr>
<tr>
<td># of seconds</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

### 8.4.2 Acquire Data

After setting the number of events or amount of time to record, press the Acquire button in the Cypod.

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 a detailed description of how to you FlowJo for analysis please see:
• For the MAC http://offsite.treestar.com/downloads/flowjo_v8_reference.pdf
• For the PC http://offsite.treestar.com/downloads/flowjo_v7_reference.pdf