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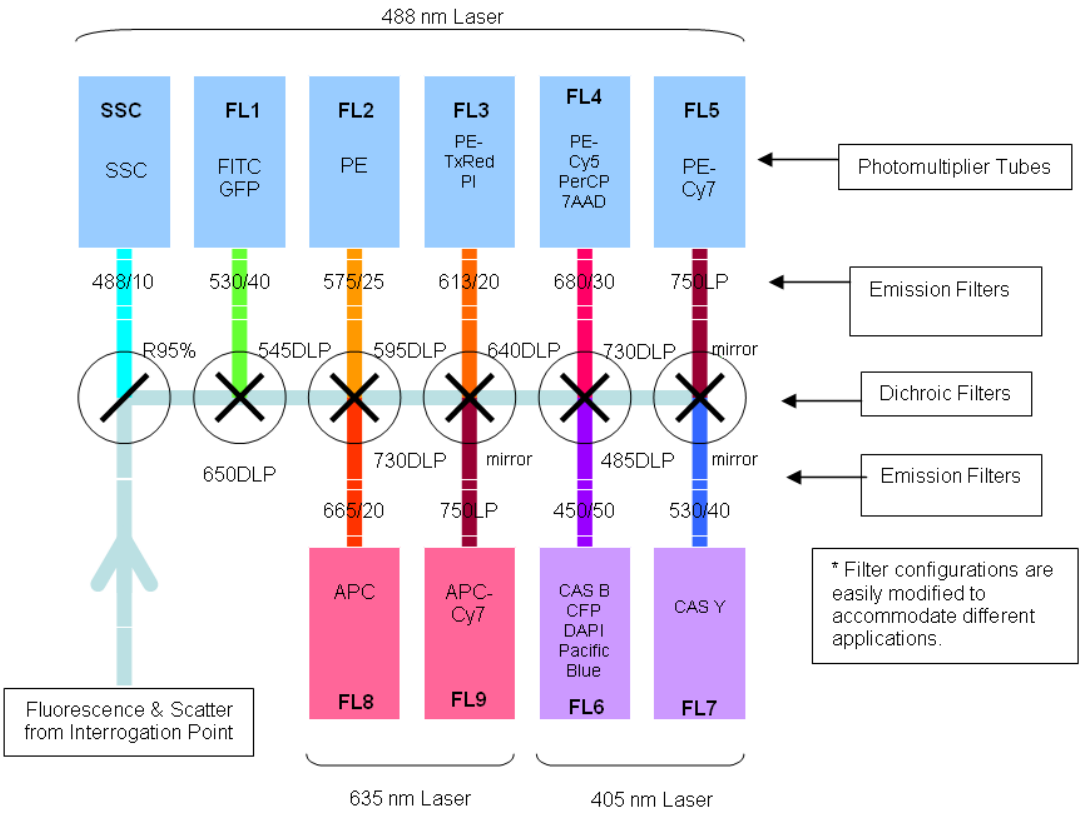
# 1. Instrument Layout




**Optical layout of a CyAn.** Each group of PMT detectors is indicated by color. Blue detectors measure light from the blue laser (488 nm), Red detectors measure light from the red laser (635 nm) and Violet detectors measure light from the violet laser (405 nm).

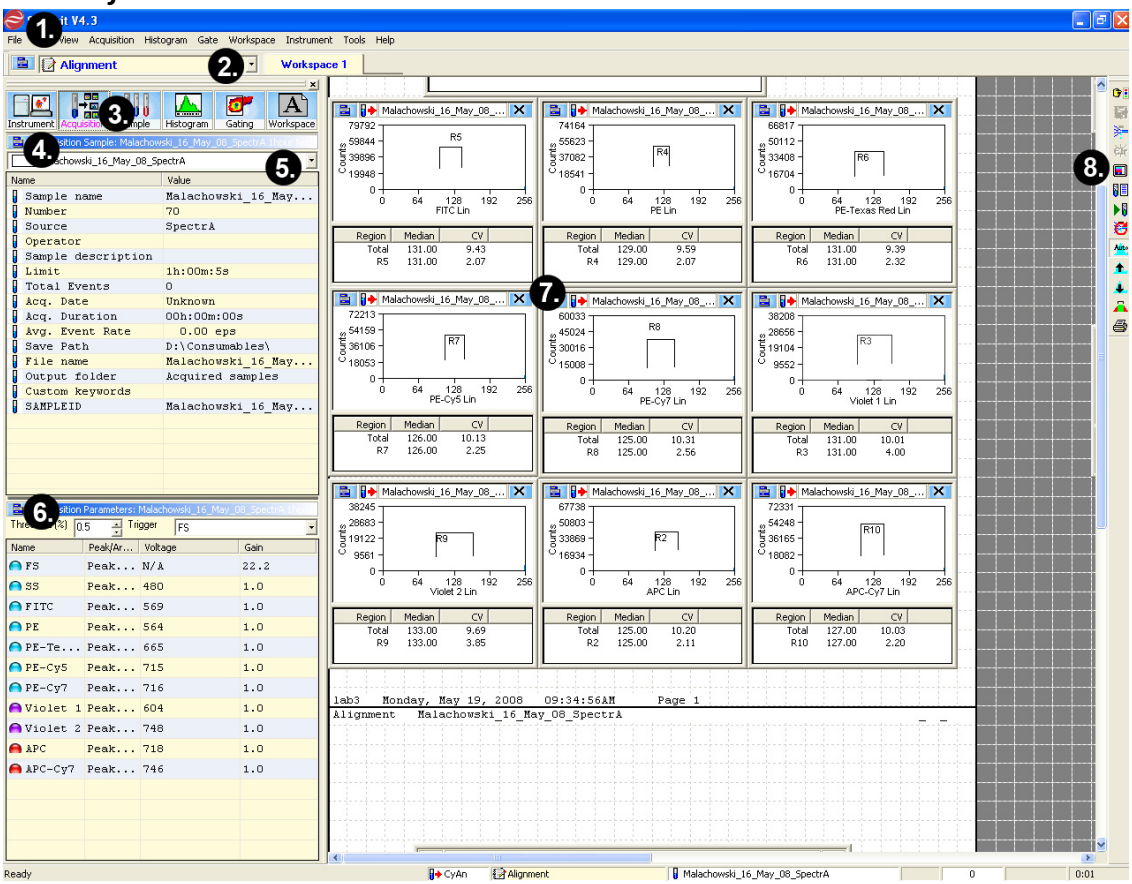
### *Detector names and commonly used fluorochromes and dyes*

| | |
|---|---|
| **FITC (FL-1)** | FITC, Alexa 488, GFP, CFSE, YFP, DyLight 488 |
| **PE (FL-2)** | PE, dsRED |
| **PE-TxRed (FL-3)** | PI, PE-Alexa 610, PE-TxRed |
| **PE-Cy5 (FL-4)** | PE-Cy5, PE-Cy5.5, PerCP, 7AAD |
| **PE-Cy7 (FL-5)** | PE-Cy7 |
| **Violet 1 (FL-6)** | Pacific Blue, V450, DAPI, Live/Dead Violet |
| **Violet 2 (FL-7)** | Pacific Orange, Live/Dead Aqua |
| **APC (FL-8)** | APC, Alexa 647 |
| **APC-Cy7 (FL-9)** | APC-Cy7, APC-Alexa 750 |

## 2. SUMMIT OVERVIEW

Summit software is a Windows ® based application that has a series of menus, hot keys and buttons, which allow you to acquire data in FCS format. With Summit software you can monitor and control the instrument, define protocols, configure compensation settings and workspaces.

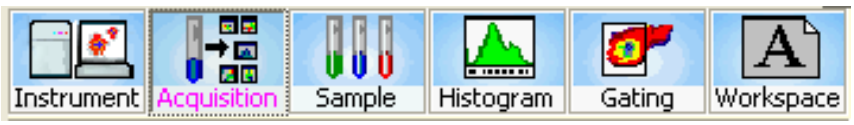
### *Summit Software Screen Overview*




1. Summit software Main Menu
2. Protocol List
3. Summit Control Panel (to gain access to additional screens)
4. Additional Menu
5. Sample List
6. Additional Menu
7. Workspace (with blank histograms)
8. Toolbar Icons

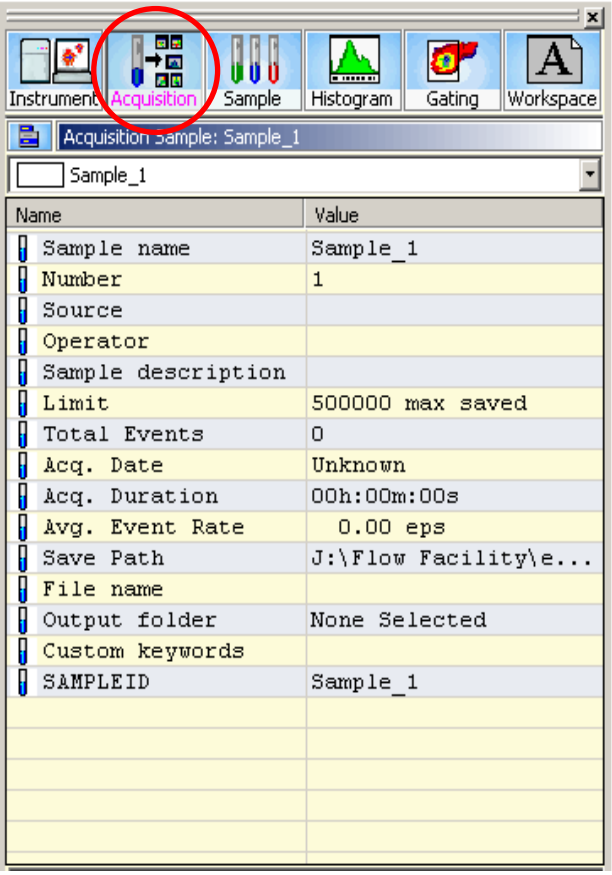
### *Summit Software Control Panel*

Most of the operations in Summit software can be accessed through the Summit Software Control Panel. The panel is located on the left side of the screen and has a series of buttons across the top. You can select each of these buttons to get information related to a particular topic. Each tab contains submenus that have options specific to that menu.



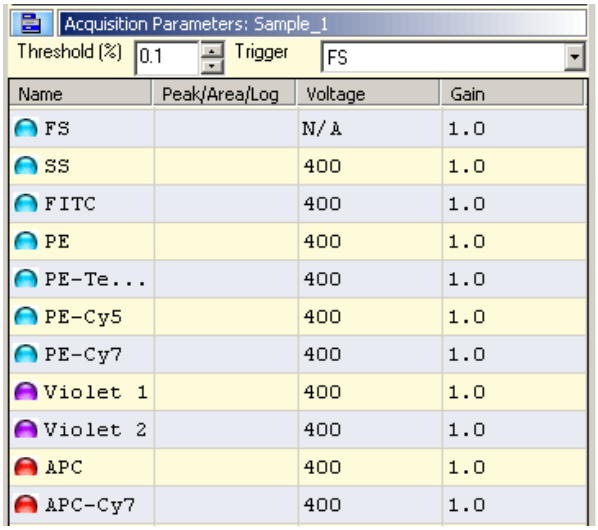
### *Acquisition Tab*

The **Acquisition tab** allows you to set the threshold at which an event will be detected by the instrument, set the event triggering parameter, designate parameter names, specify the data types that will be collected and set the voltage and gain to be applied to each parameter. From Acquisition tab you can also set up specific sample run information and view sample run statistics.




**Acquisition Tab *(Upper Panel)*:**

File save path, gate limits, and output folders and file name headers are selected here.

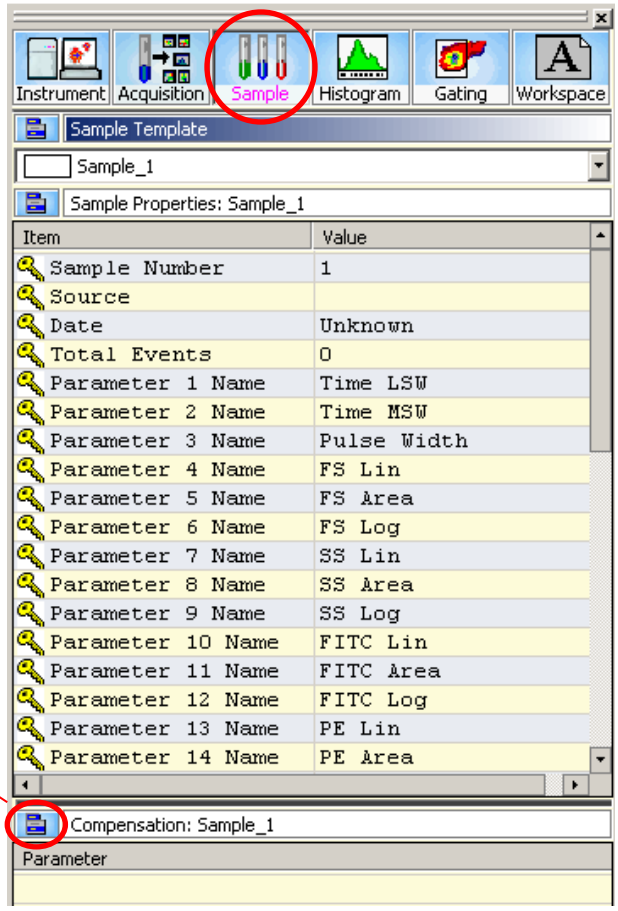



**Acquisition Tab *(Lower Panel)*:**

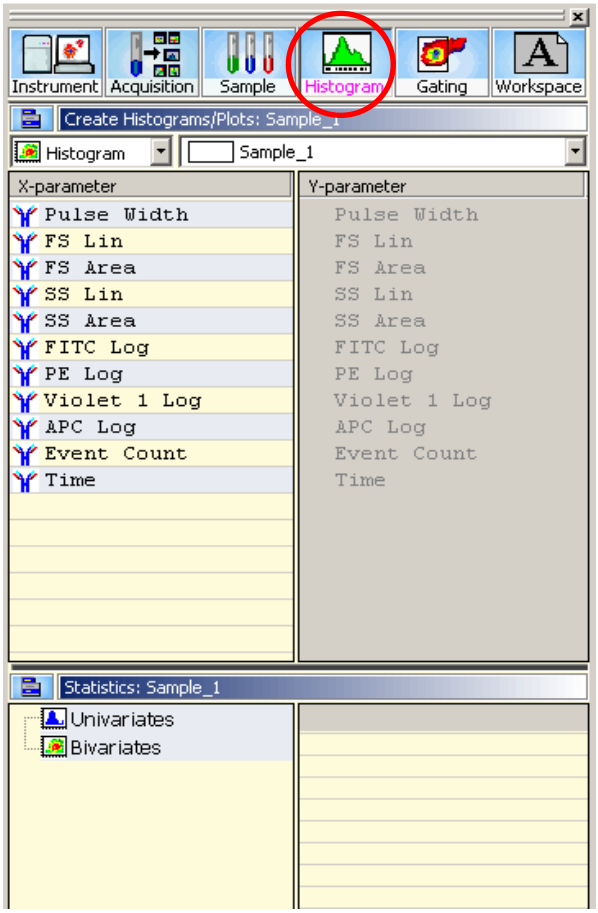
Use this panel for selection of parameters to measure, parameter aspects (peak/area/log), voltages, and gain for the FSC, threshold amount and trigger.

### *Sample Tab*

The **Sample tab** (right) displays the parameters of the selected sample, and allows you to change the list of parameters visible on screen. Select this tab if you want to perform compensation. (*For a detailed description on how to do auto-compensation see chapter 9).*




Select here for **Compensation**.




### *Histogram Tab*

Histograms and dot plots are created in the **Histogram tab** (left). The **Create Histograms/Plots** panel displays all of the parameters that are enabled in the **Acquisition tab**.

You must create histograms and dot plots in order to display the data you acquire. Prior to creating dot plots and histograms you must enable the parameters you would like to collect.

*(See chapter 7 on how to create histograms)*.

***Toolbar Icons***

**User Toolbar Settings**
**Save Acquisition *(F3)***
**Acquisition On/Off *(F2)***
**Refresh Data (*Ctrl+Z)***
**CyAn Controls**
**Show Sample List *(F8)***
**Replay Sample**
**Toggle Cycle Mode**
**Auto Scale Data**
**Scale Data Down**
**Scale Data Up**
**Toggle Color Gating**
**Print Workspace Windows**

## 3. DATA STORAGE AND DATA FILES

***Personal Folders***

All saved FCS3.0 data files acquired on the CyAn must be saved to your personal data folder located on the **J Drive.** The **J Drive** is named **Polkton Microbiology Groups (J:)** and your personal data folder will have the same name as your Onyen. Your data folder can be found in the **J Drive > Flow Facility > yourOnyen.** *Note: J Drive storage is for temporary use only. Files will be deleted after 3 months. It is user responsibility to download and backup their data files from the J Drive.*

***Database***

A database (*.sum file*) is essential to run Summit. It contains the **protocols** and **links** to all the files that are saved in that particular database. Databases can be re-used and it is recommended to save your database locally, such as in a folder on the D Drive. Alternatively, a new database can be made each time you begin an experiment (this minimizes the problems with software bugs). *Note: You delete old databases; you can acquire samples just fine with only one active database at a time, it is the* protocol *that will contain your instrument settings.*

***Protocol***

A protocol (*.plo file*) is the collection of parameters, voltages, gates and histograms used to run your acquisition inside the database. Save your protocols either locally on the desktop or in your J Drive folder. Saving a protocol is the easiest way to access setting and gates during subsequent acquisitions. Saved protocols can only be re-used for experiments measuring exactly the same parameters – do not add or subtract parameters from the protocol after it has been saved. Feel free to have different protocols for various assays, panels or cell types.

***Data Files***

FCS 3.0 files saved by Summit contain all the measured signals that you saved for any given sample. They can be re-opened in Summit or opened in FlowJo for data analysis.

## 4. SAVING DATA

Set up Summit to save your data, make the necessary adjustments in the **Acquisition tab**.

***Save Path for data files***

Save your .fcs data files into your data folder on the J Drive. Use **Save Path** in the top panel of the **Acquisition tab** to create a new folder inside of your data folder.

***Size of sample file***

Select the Limit row to adjust the maximum file size. 500,000 events is default. *Note: It is recommended to select a Gate Limit based on your gated singlets. Usable data will be contained in your singlets and having a Gate Limit on singlets will control for variable amounts of debris and doublets between samples. The size of your data file will be related to the abundance of cells in your sample that you are interested in.*

***Auto-save command***

To ensure you remember to save your files, it is **highly** recommended to turn on **Auto-Save**. Go to the drop down menu **Acquisition** and select **Auto-Save**. This makes the software request a save each time you stop acquisition. *Note: You can also command Summit to save by pressing F3.*

***Files to save***

As well as saving your experimental samples, save your unlabeled samples and any other controls (e.g. compensation, Fluorescence Minus One(s), biological controls) as these will be vital in analyzing your data after acquisition.

## 5. START UP

If the green lights on the fluidics cart are on, then the CyAn and the lasers are already on. If the green lights on the fluidics cart are off you will need to turn the CyAn on.

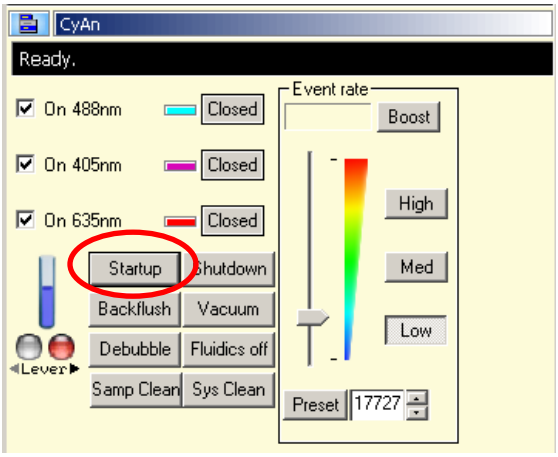
1. Check **Sheath and Waste** containers if needed. Swap Waste container with an empty one if needed *(add bleach to the bottom seam, ~10% final concentration)*. Keep the lid loose to prevent back pressure. Fill the Sheath container with Sheath Solution *(Sheath Solution is 1X PBS at MEJB, dH20 at LCCC and Thurston*). Do not let the Sheath container run dry.

2. Login to the computer with your Onyen, open Summit.

3. Make a new database (or open a previously used one).

4. Select the **Instrument tab** and click **Startup.**

***Note:*** *Lasers are configured to turn on when Summit is opened and* ***Startup*** *is selected. If ever needed, lasers can be turned on manually by selecting the check boxes.*




5. After Debubble you will be prompted to remove the tube and close the lever to initiate a backflush procedure. *Note: Allow the instrument 30 minutes to warm up if you are the first user of the day. You may login, startup and then logoff 30 minutes prior to your scheduled acquisition.*

6. (Optional) Perform a pre-acquisition cleaning procedure to ensure the CyAn is clean before your acquisition and to assess that the backflush and sample lines are unclogged. *Note: Cleaning after your acquisition is mandatory. See Chapter 10 for the cleaning procedure.*

***Note: All samples are required to be filtered just before acquisition on the CyAn.***

## 6. CHOOSING AND NAMING PARAMETERS

Data parameters must be selected before data can be acquired. Parameters are chosen and named in the **Acquisition tab**.

***Parameter activation***

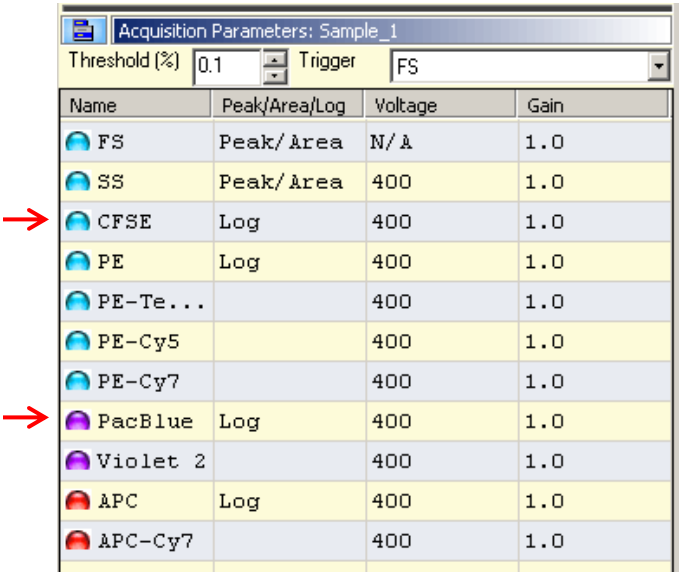
To activate a parameter, double click in the **Peak/Area/Log** field. Select the check box that pertains to the data type that you want to acquire.

| | | |
|---|---|---|
| Peak/Area | = FS & SS parameters | Linear Scale |
| Log | = Fluorescent markers | Logarithmic Scale |
| Area | = Doublet discrimination | |
| Pulse Width | = Doublet discrimination | |

Note: For cell cycle analysis record the fluorescent parameter being used (e.g. PI or 7-AAD) in Linear mode (either "Peak" or "Area". *Note: "Area" gives a brighter signal than "Peak").*

***Parameter Name***

To change the name of a parameter, double-click the name of the parameter in the Name column, type a new name and then press ENTER. *(This way the data plots will match your experimental staining).*



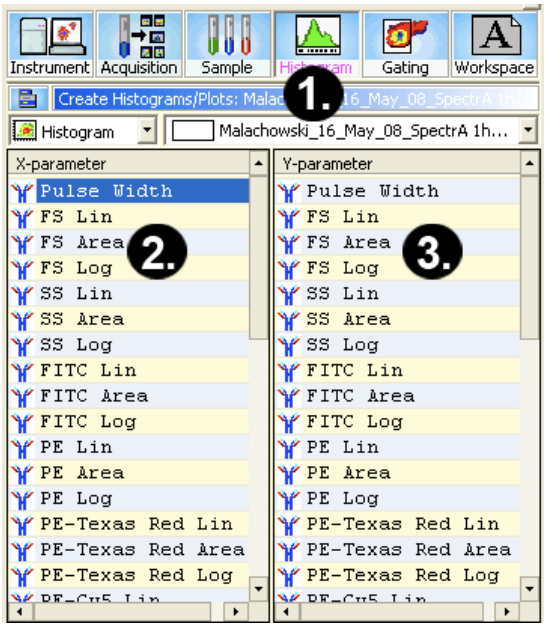

Parameters can be renamed if desired.

## 7. CREATING HISTOGRAMS

*The list of parameters available for making histograms is populated by the parameters that were selected under the Acquisition tab.*

To create a **single parameter histogram**, double-click on the X-axis parameter for the histogram you would like to create. The frame for the histogram will appear in the Workspace on the right of the screen.

To create a **double parameter histogram**, click once on the X-axis parameter and twice on the Y-axis parameter. The newly created frame for the dot plot will appear in the workspace.




1. Histogram Tab
2. X-axis Parameters
3. Y-axis Parameters

*Note: During initial setup of a new protocol it is suggested to create a single parameter histogram for each measured parameter in order to optimize voltage settings. See next section.*

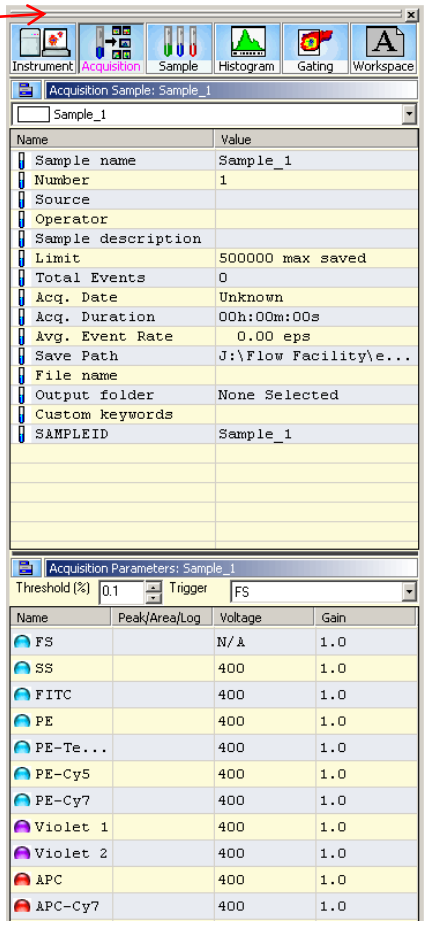
## 8. EXAMPLE OF PROTOCOL SETUP

Instrument settings need to be optimized for each experiment. An unlabeled sample is required to set the correct sensitivity for each parameter used in the experiment. It is recommended to always record and save the unlabeled negative control for each experiment.

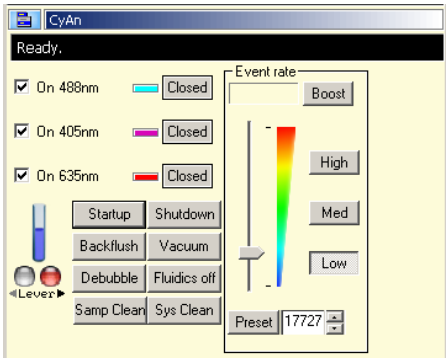
1. Open Summit, creating a new database or opening a previously used database.
2. Move **Control Panels** and **Samples Viewer** to the right computer monitor. *(This will give more space on the left monitor for viewing data and histograms).*

**Control Panel.** Select ***View > Control Panel*** to show. To move the Control Panel click and drag the two grey lines at the top of the panel.

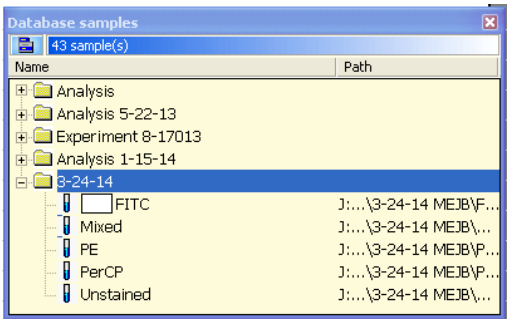
Click here to move.




**CyAn Controls.** Select Cyan controls in the **User Toolbar** or go ***Instrument > CyAn > Control Panel.***

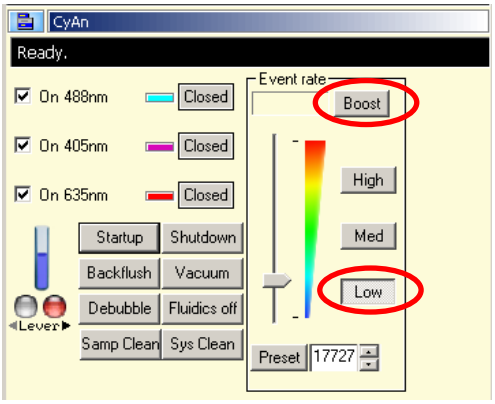



**Samples View.** Press F8 or select ***View > Samples*** to pull up this view.

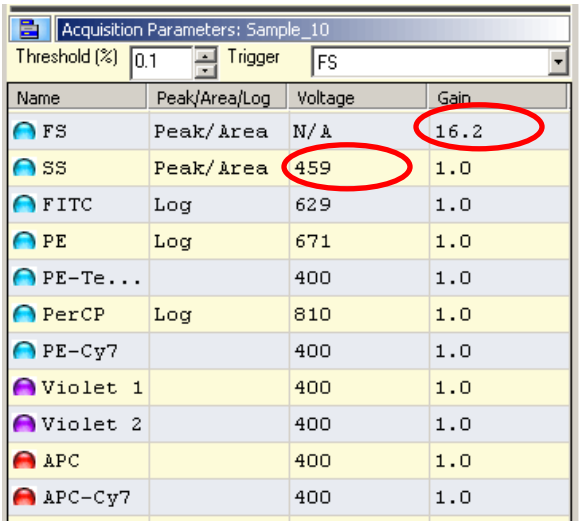



3. Under the **Acquisition tab** select the **Save Path** field and designate a new folder within your data folder on the **J drive** to save acquisitions.
4. In the **Acquisition tab** select the **Output folder** field, activate the drop-down menu and create a new output folder in the **View Samples** window to monitor the samples being acquired during your session. *Note: Do this if you are re-using a database. New databases will already generate a single new output folder.*

5. (Optional) Perform a pre-acquisition cleaning procedure to ensure the CyAn is clean before your acquisition and to assess that the backflush and sample lines are unclogged. *Note: Cleaning after your acquisition is mandatory. See Chapter 10 for the cleaning procedure.*
6. **Activate parameters** to be analyzed. *See Chapter 6; Choosing and Naming Parameters.*
7. Create a **FSC-A x SSC-A** histogram. (A **FSC-Lin x SSC-Lin** is acceptable, too). This is your initial scatter plot. *See Chapter 7; Creating Histograms*.
8. Create a **FSC-Lin x FSC-A** histogram. This is your doublet discrimination plot.
9. Create additional histograms as needed to view the data. *Note: During initial setup of a new protocol it is suggested to create a single parameter histogram for each measured parameter in order to optimize voltage settings.*
10. In the **CyAn Controls** set the **Event Rate to Low**.
11. **Place an unlabeled sample** on the CyAn. Select FS as the trigger.
12. Optional; **Boost the sample** for ~ 1 sec. *Note: default instrument settings are for a 0.7 sec boost of the sample which will deliver sample quickly to the flow cell. Always press Ctrl+Z to clear the data after a boost.*

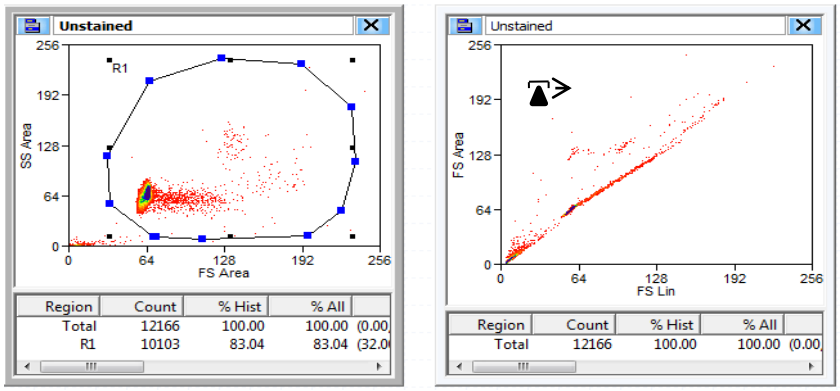



13. Activate **Cycle Mode** in the Toolbar Menu. *(This will refresh the data every 200-300 events and is very useful when adjusting detector settings).*
14. Select the **Acquisition tab**, double-click the Gain field and **adjust the FSC Gain** up or down.
15. Double-click the SSC Voltage Field and adjust **SSC voltage up or down**.
16. Aim to place the main population on-scale in the FSC x SSC plot and separated from debris. *Note: Scatter settings can vary significantly between different cell types!*
17. **Optimize the voltages of the fluorescent parameters**. Adjust fluorescent parameter voltages so that the unlabeled control is in the 'first log decade'.

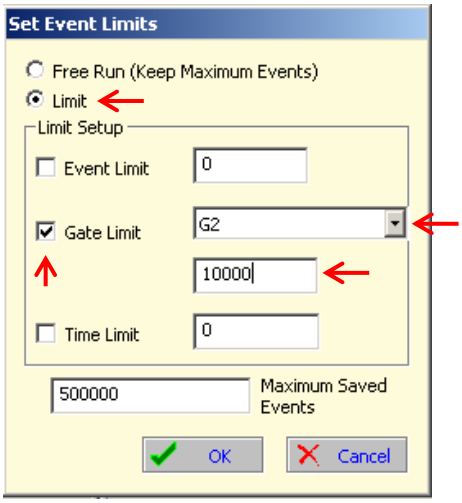



18. Once scatter and fluorescent settings are adjusted, **turn off Cycle Mode** and let some events populate the graphs.
19. Press F2 to pause acquisition.

20. **Create a gate** by right-clicking on the **FCS x SSC plot** and choosing the gating tool. *(This first gate will contain cells of interest and ideally exclude debris).*
21. **Apply this gate to the FSC-Lin x FSC-A plot**. To do this, right click the gate itself and choose '**Set Gate'** (the cursor will change to a histogram peak symbol) and double-click inside the **FSC-Lin x FSC-A plot**. *(This will become a doublet discrimination plot).*



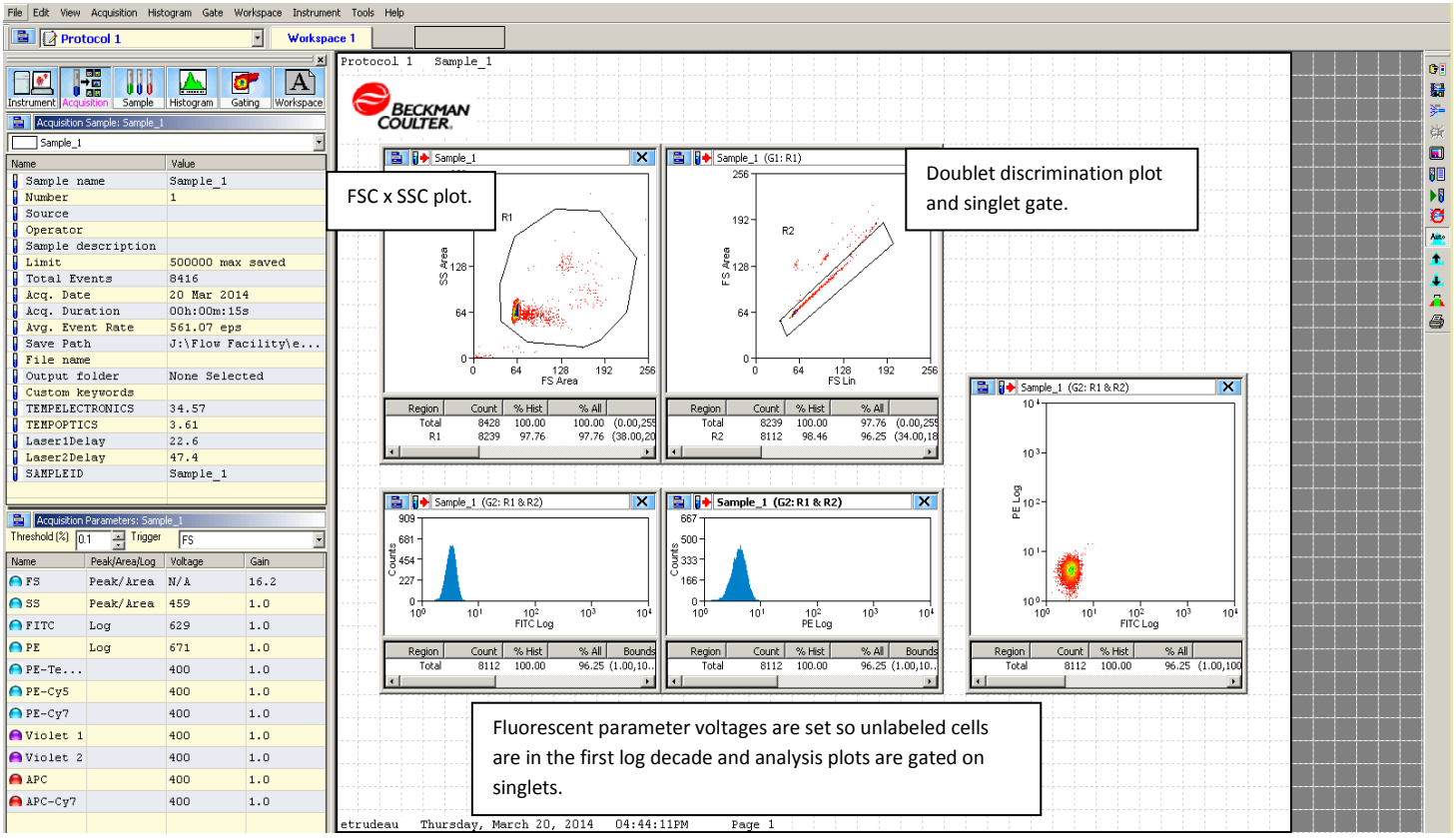

22. **Right click inside the FSC-Lin x FSC-A doublet discrimination plot**, choose the gating tool and **draw a gate on the singlets** (generally at a 45$^{o}$ angle on this plot) and avoid clumps and doublets. See example p14.
23. **Apply the newly created Singlet Gate to all other plots**. To do this, right click inside the Singlet Gate, choose 'Set Gate' (select 'OK' when asked about combining regions). Click once in each analysis plot and then click twice in the final plot. The Singlet Gate will be applied to all 'clicked' plots.
24. **Set a Gate Limit.** After having identified the singlet gate go to **Acquisition tab** and double-click the **Limit field.** In the 'Set Event Limits' dialog box, select 'Limit', check 'Gate Limit', identify the gate that is your singlet gate and type in the number of singlets you would like to record for each sample. Hit 'OK'. *(Note: Data of interest will be contained in the identified singlets. Recording with a gate limit based on singlets is a way to standardize data files and control for variable amounts of debris and doublets between samples. The number of events to record will depend on your sample and prevalence of the population of interest).*



25. ***Recommended;*** *Before recording data* ***examine each single color control*** *or* ***fully stained panel*** *to ensure that the brightest signal for all fluorochromes are on-scale. Adjust fluorescent voltages if needed.* ***Once voltages are set do not change them during the acquisition.***
26. **Ensure that Auto-Save is enabled**. Go to the drop-down menu Acquisition and **select Auto-Save**.
27. Flow rate is adjusted via the slider bar on the instrument control panel. For most applications flow rates of 1,000-20,000 are standard. (*Stricter criteria apply when acquiring cell cycle data).*
28. **Save the protocol** (for future use) and proceed with sample acquisitions. To prevent carryover be sure to backflush and gently rinse the outside of the probe with $dH_20$ between samples.

***Note: All samples are required to be filtered just before acquisition on the CyAn.***

***Below: An example of scatter gating, singlet gating and PMT settings based on unlabeled control.***



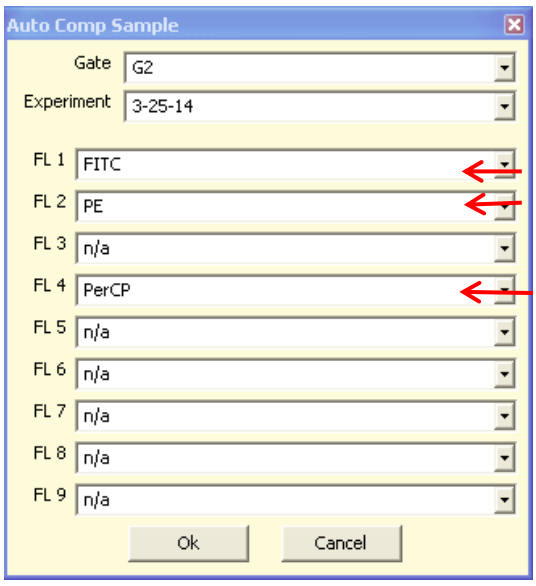
## 9. COMPENSATION

In multiparameter flow cytometry experiments it is common for the spectal emission of one fluorochrome to overlap into a PMT channel dedicated for measuring the emission of another fluorochrome. Compensation is the mathematical process of measuring each fluorochrome's emission and spillover as a single-color control and adjusting for the amount of spectral spillover. Compensation matrices are derived after measuring each single-color control and calculating the amount of spectral overlap that is present. The resulting compensation is applied so that fluorescence detected in a particular detector derives solely from the fluorochrome that is being measured. Compensation may be calculated based on your single color controls before acquisition. Alternatively, samples can be acquired uncompensated but it is essential that your single-color controls are recorded and saved so that compensation may be applied in the subsequent analysis.

***Auto-compensation***

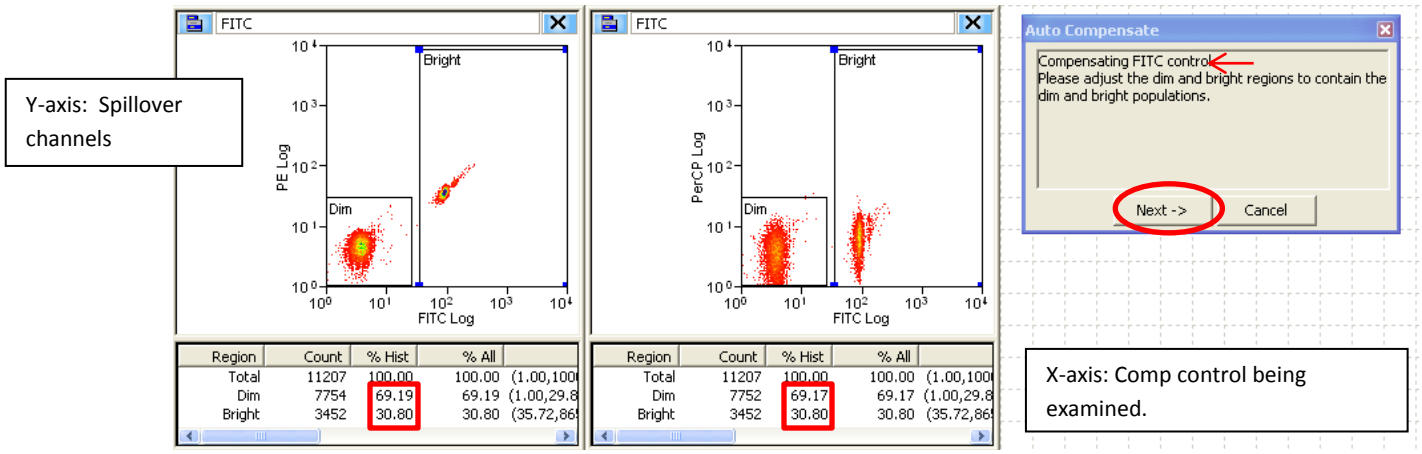
Before performing auto-compensation be sure to have optimized the voltages for each detector and have saved a copy of each single-color control. Once optimized do not change the detector voltages during that day's acquisition as this will directly impact the spillover values. *(Note: Summit requires the presence of a well-defined positive and negative population in each compensation control).*

1. Click the **Sample tab**.
2. In the **Sample Compensation** panel (the bottom half of the Sample tab), click the small, blue icon in the upper-left corner and select **Auto Compensate** from the list.
3. The **Auto Comp Sample** dialog box appears.
4. Select a gate from the **Gate** field, if applicable.
5. In the **Experiment** field, select the folder that contains your single-color controls.
6. **Allocate** each FCS file for **each single color control** to its **own FL detector** *(see Ch 1, Instrument Layout).* Click **OK**.




**Above:** For compensation assign the .fsc file for each single-color control to the appropriate FL detector.

7. A new Workspace labeled **AutoComp** is created and a set of dot plots is made. Also, the **Auto Compensate** wizard appears. *(In the compensation plots, the X-axis represents the single-color control being examined and the Y-axis represents the spillover channels in the experiment).*



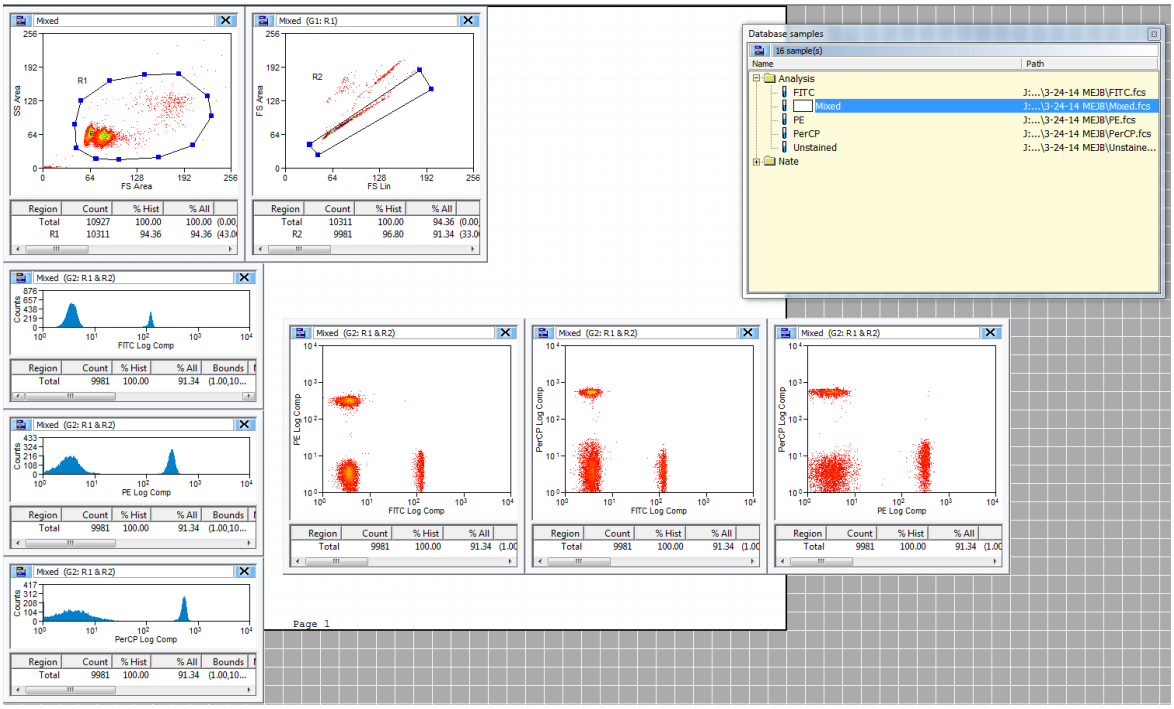

8. If needed, **adjust the Dim and Bright regions** to contain the **positive and negative populations** for the single-color control being examined. *(Note: Examine the % Hist statistic for each histogram adjusting so that >5% of the data appears in each region).*

9. **Click 'Next'** to proceed to the next single-color control in the experiment.

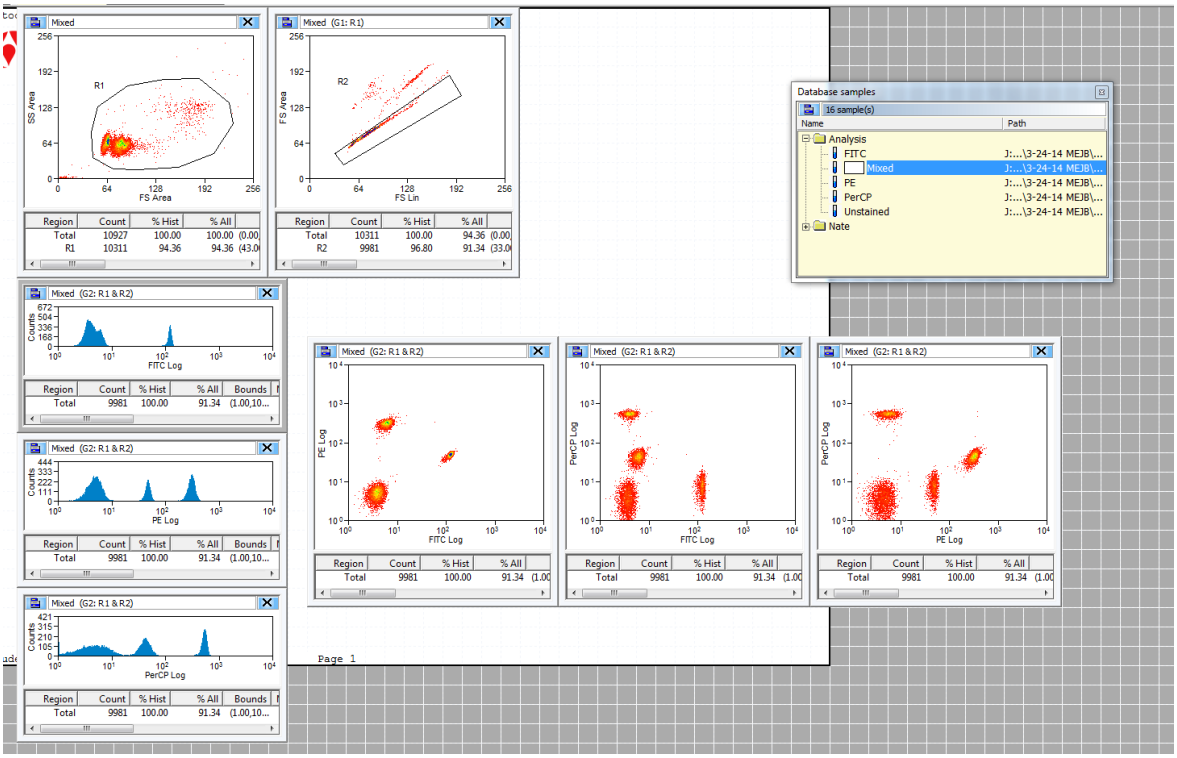
10. **Repeat steps 8 and 9** until all single-color controls have been compensated. When auto compensation is complete, the compensation matrix will contain the appropriate values (in the bottom panel of the **Sample tab**).

***Note:*** *Do not analyze uncompensated data. Accurately compensated data will be displayed properly while uncompensated data can be erroneous.*

**Below: Properly compensated dataset (3 color experiment; FITC, PE and PerCP acquired on a CyAn and analyzed in Summit 4.3).**




**Below: The same dataset as above but uncompensated**.



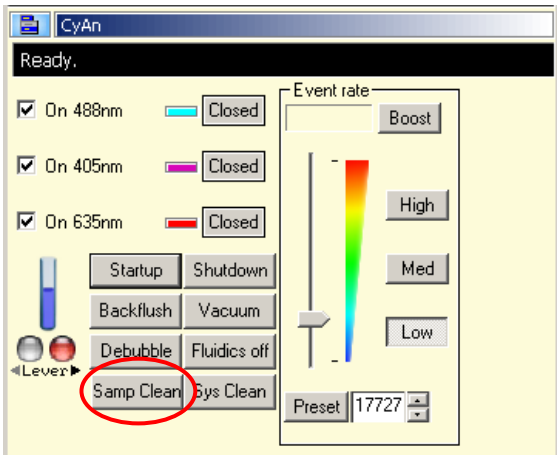
## 10. CLEANING PROCEDURE AND SHUTDOWN

Instrument cleaning is mandatory after acquistion and takes 5 minutes. Cleaning involves **three backflushes** (using 1% Contrad-70, FACSClean and $dH_20$) followed by **three 'Sample Clean'** functions (using 1% Contrad-70, FACSClean and $dH_20$).

***Cleaning procedure when there is another user after you:***

The **1% Contrad-70** is color-coded **Yellow**; the **FACSClean** is color-coded **Red** and the **$dH_20$** is color-coded **Blue**.

1. Fill **the short, yellow-labeled tube** with **1% Contrad-70** and place on the probe. Close the lever to initiate the backflush. *(Note: the short tube does not engage the sensor at the top of the probe. As a result no sample tube is detected when the lever is closed and a backflush is performed).*
2. **Repeat Step 1** using **the short, red-labeled tube** with **FACSClean**.
3. **Repeat Step 1** using **the short, blue-labeled** tube filled with **$dH_20$**.
4. Click the **'Samp Clean'** button on the **CyAn Control** panel.

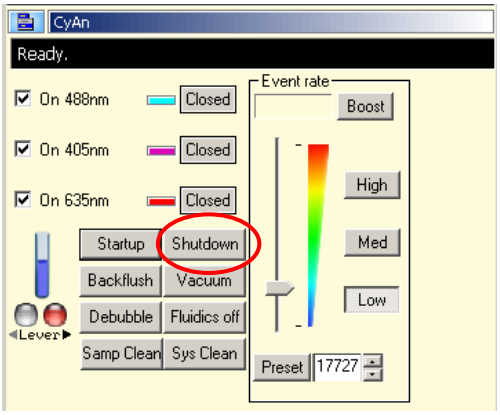



5. Fill **the tall, 5 ml polypropylene, yellow-labeled tube** with **1% Contrad-70** **¾ full** and place on the probe. Close the lever to initiate a **Sample Clean function**. *(This will run cleaning solution at maximum speed for 1 minute).*
6. Remove the cleaning solution and close the lever to backflush.
7. **Repeat Steps 4 – 6** using **the tall, 5 ml polypropylene, red-labeled tube** with **FACSClean** **¾ full**.
8. **Repeat Steps 4 – 6** using **the tall, 5 ml polypropylene, blue-labeled tube** with **$dH_20$** **¾ full**.
9. Leave the tube of **$dH_20$** on the probe (about ½ full, you may need to add water) with the lever open.

10. Close Summit and logout.

***Cleaning procedure when you are shutting the instrument down:***

1. **Follow steps 1 – 8 as above**.
2. Click the **'Shutdown'** button on the **CyAn control panel**.
3. Summit will prompt you for a tube of cleaning solution. Place **the tall, 5 ml polypropylene, red-labeled tube** with **FACSClean ¾ full** on the machine and close the lever.
4. Summit will now prompt you for a tube of water. Place **the tall, 5 ml polypropylene, blue-labeled tube** with **$dH_20$ ¾ full** onto the machine and close the lever.




5. Leave the tube of **$dH_20$** on the probe (about ½ full, you may need to add water) with the lever open.
6. Close Summit and logout.

## 11. TROUBLESHOOTING

- Tube (or microtiter tube within a tube) is loaded but no events are detected
  - ✓ Check if tube is detected (seals well) and **Auto-Sample** is checked
  - ✓ Increase the pressure differential (event rate) with the slider bar in the Instrument tab
  - ✓ Check for a clog – Click **Samp Clean** and install a tube with 3mL (below the tape line) of **$diH_2O$**. If the volume is not dropping, contact the Flow Core
  - ✓ Check for a software error – restart Summit, create a new database, or a new protocol

- Pressure or Vacuum error
  In the Instrument tab click the **Startup** button and follow the software prompts

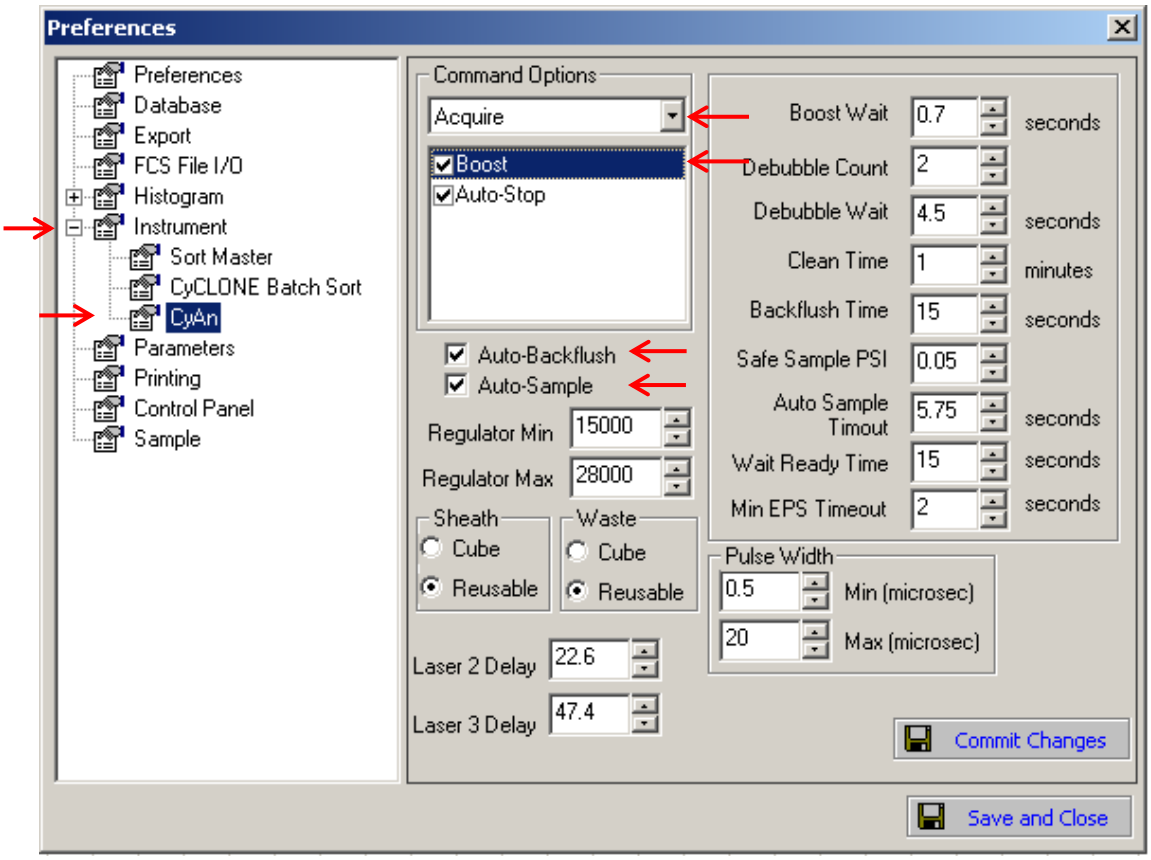
- DCM error message
  Restart the computer

- FSC/SSC profile and/or fluorescent parameters look abnormal
  In the Instrument tab click the **Debubble** button or check for a clog (see aforementioned issue). Check/Adjust the **Threshold** setting

- Sample is running but no events are detected

Ensure the pressure slider bar is up high enough. Also, pause acquisition and try triggering on SSC instead of FSC.

- **Auto-Backflush/Auto-Sample**: Edit menu > Preferences > Instrument > CyAn > Check appropriate option




**Full restart**: Shutdown Cytometer, SMS, and PC. Restart in same order.

If the above troubleshooting does not resolve an issue then please notify the Flow Cytometry Core Facility staff immediately at 919-966-1530 (or email staff members) to describe the issue with the CyAn.