

# **BD FACSDIVA TUTORIAL**

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### Computer and Software Login

Login on windows: Username: facsuser

Password: BDIS

- Start the software: double-click the shortcut icon on the desktop.
- Login on DIVA using your own login name and password.
- Wait for the cytometer connection and click "Use CST Settings".

Status	Laser Com	pensation Parameters	Rati
Parameter	Vol	tage	
FSC	654		
SSC	349		
FITC	535		
PE	554		
PerCP-Cy5-5	802		

**NOTICE:** To verify the workstation has successfully connected to the cytometer, check that the Cytometer window displays the message "Cytometer Connected" or "The system is ready" at the bottom of the window. If the message reads "Cytometer Disconnected," switch the cytometer power off, wait 10 seconds, and then switch the power on. Restart the computer.

### Setting Up Your Experiment

Before you start, make sure that you have all the necessary windows open. Go to the "View" menu and make sure the following boxes are checked:

- Browser
- Cytometer
- Inspector
- Worksheet
- Acquisition Dashboard

### Creating a New Experiment

- Click on the "New Experiment" icon in the browser toolbar.
- To create an experiment based on a saved template, choose Experiment >New Experiment. The Experiment Templates dialog appears where you can select your unit and the template.
- Click the "New Specimen" button to add a specimen and tube to the experiment; click once on the plus sign (+) next to "Specimen\_001" to expand it.
- In the browser, click the icon to the far left of the tube named "Tube\_001". The pointer changes to green.

Note that experiment names cannot contain commas or periods. The experiment modification date is the date of the last data collected.

	BD FA	CSDiv	a Software -	Administr	ator (D
File	Edit	View	Experiment	Populations	Worksh
		🗸 Too	olbar		
		🗸 Sta	tus Bar		
		🗸 Bro	wser	Ctrl+Sł	nift+B
		Pla	te	Ctrl+Sł	nift+Z
		🖌 Cyl	ometer	Ctrl+Sł	nift+N
		🗸 Ins	pector	Ctrl+Sł	nift+P
		🗸 Wo	rksheet	Ctrl+Sł	hift+₩
		🗸 Acc	quisition Dashbo	ard Ctrl+Sh	nift+C
		Bie	xponential Edito	or Ctrl+Sh	nift+E
		Re:	et Positions		

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📴 Browser - 6-color gating

B Experiment 001

Cytometer Settings

Specimen\_001



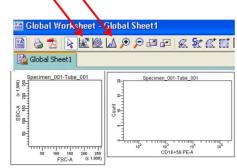
#### Selecting Parameters

To delete parameters you do not need, click the selection button next to each unneeded parameter. Hold down the Ctrl key to select more than one. When you are finished selecting click Delete. Select H and W for the FSC parameter.

Parameter	Voltage	Log	A	н	W	1
• FSC	250		×			^
• SSC	300		4			
Alexa Fluor 488	500					
DsRed	500	Image: A start of the start				
PE-Cy5	500		~			
PE-Cy7	500	Image: A start and a start				
<ul> <li>Indo-1 (Violet)</li> </ul>	500					
• DAPI	500	<b>V</b>	~			~
Add			0	Delete	>	-

#### Creating a Dot Plot and Histogram

Create a FSC-A vs. SSC-A plot on the global worksheet. Select the "Dot Plot" icon on the Worksheet toolbar and click once in the upper left corner of the worksheet field. A default size plot is drawn. Create a histogram for each fluorescent parameter, selecting "Histogram" icon on the Worksheet toolbar and click on the worksheet field.



#### **Creating a Gate**

Create a gate by selecting the "Polygonal Gate" button and drawing it in the dot plot. Be sure you finish your gate at the same point you started drawing it. This gate (P1) will let you gate on a single cell population and exclude aggregates, dead cells and debris.

#### Selecting Parameters for Dot Plot Axes

Change the parameters on your dot plot by left clicking on them and selecting the one you need in the pop-up menu.

To gate it on the P1 gate, right click inside the FSC-A vs. SSC-A dot plot and in the popup menu select "Show Populations", then "P1".

Show Population Hierarchy	Ctrl+G	
Create Statistics View	Ctrl+R	
Show Populations	Þ	All Events
Scale to Population	•	P1
Show Gate	<b>.</b>	
Bring to Front	•	
Send to Back	•	
Order Populations by Count		
Duplicate	Ctrl+D	
Paste	Ctrl+V	
😹 Cut	Ctrl+X	
Сору	Ctrl+C	
Delete	Delete	

#### Setting Up Voltages Based on an Unstained/Negative Control

Your first tube should be an unstained control. Make sure that the pointer on the far left from the tube name is activated.



Place the tube on the Sample Injection Port (SIP). Press the "Low" and the "Run" buttons on the instrument. Click the "Acquire Data" button on the "Acquisition Dashboard".





Adjust the voltages so you can see the population of interest in the center of the FSC-A vs. SSC-A dot plot. Adjust the voltages so you are able to see both the ascending and descending slopes of the peak for each fluorescent parameter.

#### Adjusting Area Scaling for FSC

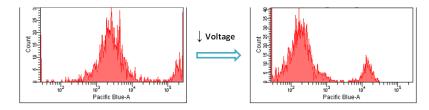
If your cells are bigger than lymphocytes you must adjust the area scaling factor for FSC. Area scaling adjusts area measurements to be the same magnitude as height measurements. Create a histogram for each FSC-A, FSC-W, FSC-H parameter. Check area scaling for forward scatter: go to the "Laser" tab in the "Cytometer" window and

Status Parameters Threshold ease	Compensation Ratio	
Name	Delay	Area Scaling
Blue	0.0	0.9
355 UV	43.1	5 0.7
Red	64.5	5 0.8
FSC Area Scaling: 0.72 🕃 🚹 🕊		

adjust the "FSC Area Scaling" value until the population of interest (P1) on the FSC-A, FSC-H and FSC-W Histograms is aligned.

#### **Checking the Single Color Tubes**

Create a histogram for each single color. Check each one to insure all the data that you are going to collect will fall within the scale. If the positive population appears out of scale you must decresase the parameter voltage. Make sure that the single-stained color tube that you are acquiring is not brighter for other parameter than the one that is on the tube.



### **Recording your data**

When your settings are done you should be ready to start acquiring your samples. First you need to define the number of events you want to record – you can do so in **"Events To Record"** by setting the number you wish. In **"Stopping Gate"** you have the option to choose one gate, and the acquisition will

vents Elapsed Time	Stopping Gate Events	Threshold Rate	Current Activity Active Tube/Well
00:00:00	0 evt	0 evt/s	Tube_001
	-		Basic Controls
Restart			
Restart	Record Data	Acquire Data	♦ Next Tube
	$\smile$		Acquisition Setup
Stopping Time (sec): 0	ord: 30000 evt 🕽 🗸 Stopping	Il Events ) V Events To Re	Stopping Gate: 🚺 🗖 A
Stopping Time (sec):		Events V Events To Re Events V Events To Dis	
			Storage Gate:
<b>v</b>			
			Storage Gate:

only stop when the number of events is reached in that specific gate. The "**Storage Gate**" should always be set to 'All Events'. Press "**Record Data**" when you want to start to record your acquisition.



If you wish, in the "Inspector" window you can check the "Tube" tab to name the tube and to view

certain keywords and settings saved with recorded data. Use the "Labels" tab to enter parameter labels for each fluorochrome. Labels are displayed on plot axes and in statistics views.

Inspector - 3/16+56/45/4/19/8				
Tube Labels ,	Acq. Cytometer Settings Keywords			
FITC	CD3			
PE	CD16+CD56			
PerCP-Cy5-5	CD45			
PE-Cy7	CD4			
APC	CD19			
APC-Cy7	CD8			

### Acquiring Compensation Controls and Calculating Compensation

FACSDiva software has a feature that allows you to calculate compensations automatically. To use this feature all your single color tubes must have a good positive peak (as previous histogram shows). If you don't have nice positive peaks you should calculate the compensation manually.

#### **Calculating Compensation Automatically**

The Compensation Setup feature in BD FACSDiva software is designed to automatically calculate spectral overlap values for an experiment. Select "**Compensation Setup**" from the "**Experiment**" menu; then select "**Create Compensation Controls**".

BD FAC	SDiva Software - Administrator	(Diva 6.0	0 4 Blue, 2 355UV, 2 Red 1)		Create Compensation Controls		
File Edit	View Experiment Populations Wor	ksheet Cy	tometer HTS Help				
	💽 📰 New Folder	Ctrl+N			<ul> <li>Tubes</li> </ul>	⊖ Plate	
	New Experiment	Ctrl+E					
	餐 New Specimen	Ctrl+M			Include separate unstained control tube/w	ell	
	🗗 New Tube	Ctrl+T		Г	Fluorophore	Label	
	😂 New Cytometer Settings				FITC	Generic	^
	Import Cytometer Settings				PE	Generic	
	New Global Worksheet				PerCP-Cy5-5	Generic	-
	New Plate	Ctrl+Y			PE-Cy7	Generic	- 3
	Open Experiment	Ctrl+O			APC	Generic	-
	Close Experiment	Ctrl+W				Generic	
	Experiment Layout			L	APC-Cy7	Generic	~
	Compensation Setup	•	Create Compensation Controls				
			Modify Compensation Controls		Add Delete Labels	OK Cance	el
			Calculate Compensation				

**Notice:** In the Create Compensation Controls dialog, you can add only parameters that are listed in the Parameters tab. To change to another fluorochrome for any parameter, edit cytometer settings in the Cytometer window or Inspector before you create compensation controls.

Current Activity ctive Tube/Well	Threshold Rate	Stopping Gate E		
Tube_001	0 evt/s	0 evt	00:00:	00
lasic Controls		•		
♦jj Next Tube	Acquire Data	Record Data	Restart	
equisition Setup				
	Events 👻 Events To Reco	ord: 30000 evt	Stopping Time (sec):	0 01
topping Gate: 📕 All	Events v Events To Reco		<ul> <li>Stopping Time (sec):</li> </ul>	0 \$
topping Gate: 📕 All				0 (¢]
topping Gate: All torage Gate: All				

Click once on the plus sign (+) next to the **Compensation Controls Specimen** to expand it. Select "**Unstained Control**" by clicking on the icon to the left of the tube name. Place the tube with the unstained sample on the Sample Injection Port (SIP). Click the "**Acquire Data**" button, wait a few seconds until the threshold rate is stable, and then click

the "**Record Data**" button on the Acquisition Dashboard. After you are done recording, adjust the P1 gate o include your population of interest. Then you may right click on the P1 gate and select "Apply to All Compensation Controls".



Record data for all tubes in the Compensation Control Specimen. Adjust P1 and P2 (positive events) gates as needed.

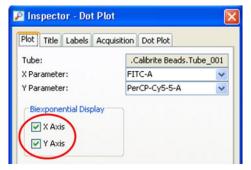
Navigate to "File" > "Experiment", then "Compensation Setup", and then select "Calculate Compensation". The computer will calculate compensation for all parameters. In the window that appears select "Link & Save. Switch back to Global Worksheet by clicking on the top left icon. Now you are ready to collect data for your samples.



Notice that the pointer on the far left of the tube for which you are about to record the data should be selected.

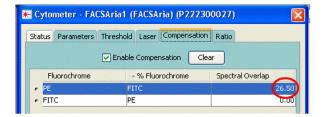
#### Calculating Compensation Manually

- To determine settings, the means for each fluorescence positive population are compared to the means for its negative population.
- This section describes how to determine spillover coefficients for the FITC fluorochrome. The same procedure is used for the remaining fluorochromes in the experiment.
- Create a dot plot and change the plot axes to display the appropriate fluorochromes (e.g. FITC vs PE). Use biexponential display to view populations with negative

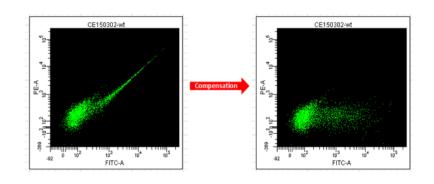


events: click on the Plot Inspector, select the Biexponential Display checkboxes for the X and Y axes.

On the **Cytometer** window select the **Compensation** tab. Increase the spectral overlap value until you see the positive population correctly compensated on your FITC vs PE dot plot.





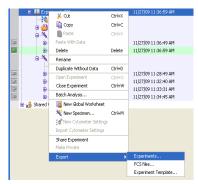


 After you finish compensating you need to apply these compensation values to the settings of your experiment. Right click one of your compensated tubes and select Copy Spectral Overlap. Then, right click Cytometer Settings (under your experiment title) and click Paste Spectral Overlap with Zeros.

Experiment 1	5ettings	3/5/15 11:54:13 AM	Experiment 1	3/5/15	11:54:13 AM
Global Work	sheets		Cytometer Settings	X Cut	Ctrl+X
🗉 🐚 Tube 1	🖌 Cut	3/4/15 3-12-53 PM Ctrl+X	🖃 🌂 Specimen 1	Copy	Ctrl+C
🕀 📊 Tube 2 🕀 🙀 Tube 3	Copy	Ctrl+C	🕀 🕞 Tube 1	Paste	Ctrl+V
🕀 📊 Tube 4	Paste	Ctrl+V	🕀 🕞 Tube 2	Delete	Delete
🕀 🥵 Shared View	Delete	Delete	🕀 — 🚺 Tube 3	Copy Spectral Overlap	
	Apply Analysis Tem	plate	🕀 🖓 Tube 4	Paste Spectral Overlag	0
	Copy Spectral Over	<b>7</b>	Shared View	Paste Spectral Overlag	o with Zeros
	Paste Spectral Ove			Print	
	Paste Spectral Ove	rlap with Zeros			
	Rename			Export	

### Exporting your data

After you finish recording your last sample, you can now export your data. Right click on the name of the experiment you wish to export and select "**Export**". There are three options: export experiment; export FCS files; or export experiment template.



#### **Experiment**

When you use the "Export experiment" option FACSDiva will create a folder named after the title of

your experiment, and place the .fcs files in there along with the .xml file that contains the map of the experiment. The whole experiment including worksheets, gates, samples and specimens is saved.

Select "**Export**" > "**Experiment**" and in the window that appears click the "**Browse**" button. Select your destination folder, press OK and then click "**Export**" to save your files.

Export Experiments	
Delete experiments	after export
<ul> <li>Directory Export</li> </ul>	) Zip File Export
Directory: :\Documents and Setti	ings\LSRII\Desktop Browse
Experiment	Date
Experiment_001	11/27/09 11:36:5 🔼
	✓
	OK Cancel



#### FCS files

Use this option if you plan to analyze your acquired data later using analyzing other software (such as FlowJo). By default, FACSDiva exports the .fcs files into a folder named after the title of your experiment. The compensation, if applied in FACSDiva, is stored in the .fcs files and can be edited or stripped during further data analysis. No information on gating is recorded. When ready to analyze the data, add the files to the FlowJo workspace or create a New Experiment in FACSDiva and use the "import FCS files" option.

Select "Export" > "FCS files". In the window that appears make sure you select the "FCS 3.0" option and press OK. You do not need to change any of the other parameters. In the

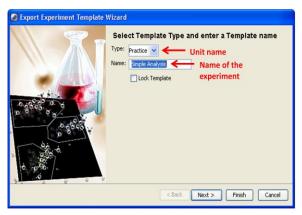
File Version 🖌	
FCS2.0 💿 FCS3.0	
Parameter	Parameter Type
FSC-A	● Linear ○ Log ○ None
FSC-H	● Linear ○ Log ○ None
55C-A	● Linear ○ Log ○ None
FITC-A	🔵 Linear 💿 Log 🔵 None
FITC-H	🔿 Linear 💿 Log 🔿 None
PE-A	🔿 Linear 💿 Log 🔿 None
е-н	🔿 Linear 💿 Log 🔿 None
APC-A	🔿 Linear 💿 Log 🔿 None
APC-H	🔿 Linear 💿 Log 🔿 None
lime	⊙ Linear O Log O None
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next window click "Browse", select your destination folder and press "Save".

#### **Experiment template**

Use this option if you have acquired some data and need to repeat a similar experiment in the future. Experiment templates include specimens, tubes, cytometer settings, labels and worksheets, but do not include the recorded data. Any experiment can be saved as a template.

Select "Export" > "Experiment Template". The Export Experiment Template Wizard appears.



For the template type select your Unit. Click "Finish". Your Experiment Template is now saved.

Templates are stored outside the Browser to simplify the Browser display. To open an experiment template you need to create a new experiment using the menu command: click "File" > "New **Experiment**". The Experiment Template dialog appears. Choose the tab corresponding to your unit and select the desired template. Click OK.