1. Log into PPMS (gray screen) with Emory credentials



2. Launch the Diva software from the icon in the Windows taskbar, select your lab from dropdown menu and hit **OK** to log in (no password)



3. In the Diva Browser window, click the file icon to create a new Experiment.



BD FACSDiva Software - Administrator (ORAC Emory Univ 6B 3R 5YG 8V 6UV)

- Rename the experiment by right-clicking on the experiment name in the Browser or edit the name in the Inspector window.
 Note: Include the date in the experiment name for data retrieval purposes
- 5. The open book icon to the left of the experiment name indicates the experiment is open. Note: to edit or acquire in any experiment in Diva, you must have the open book icon (double-click to open).

Click **Cytometer Settings** beneath the experiment name to display the settings in the Inspector window.



6. Select Parameters

- a. Go to the **Parameters** tab in the Inspector window and select appropriate detectors using check boxes:
 - i. FSC and SSC: typically visualized looking at the Area (A) of the signal

Cytometer - LSRFortessa (FACSymphony A5) (1)									
I,	Sta	tus Parameters Threshold L	aser Compensation	Ratio				_	
		Parameter	Voltage	Log	A	н	w		
		FSC	478					^	
		SSC	201			\checkmark	\checkmark		

- ii. Use Log (L) for FSC and SSC if acquiring bacteria or small particles
- iii. Collect Height (H) and/or Width (W) for scatter parameters in addition to Area (A) to enable doublet discrimination

*	* Cytometer - LSRFortessa (FACSymphony A5) (1)									
Status Parameters Threshold Laser Compensation Ratio										
		Parameter	Voltage	Log	А	н	W			
	e	FSC	478		\checkmark	\checkmark		^		
	e	SSC	201		\checkmark	\checkmark	\checkmark			

- b. Select Fluorescent Parameters (same window as step 4)
 - i. It is best practice to delete all fluorescent parameters first and then add back only the necessary parameters:
 - 1. Highlight all fluorescent detectors: click on dot to the left of the first fluorescent detector (the first detector listed underneath SSC), hold down the shift key, scroll down and click on the dot to the left of the last detector
 - 2. Select delete (bottom right-hand corner of browser window)

Parameter Voltage FSC 512 SSC 208 B 515/20 448 B 610/20 459 B 670/30 423 B 710/50 465 B 780/60 445 R 670/30 373 R 710/50 383 R 710/50 335 V 431/28 326	Log	A		
FSC 512 SSC 208 8 515/20 443 8 610/20 459 8 670/30 423 8 710/50 465 8 780/60 464 R 670/30 373 R 770/30 383 R 710/50 383 R 780/60 335 V 431/28 320				
 SSC 208 208				
B 515/20 448 B 610/20 459 B 670/30 423 B 710/50 465 B 750/30 445 R 70/30 733 R 710/50 833 R 710/50 335 V 431/28 320				
B 610/20 459 B 670/30 423 B 710/50 465 B 750/30 445 B 780/60 464 R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382				
B 670/30 423 B 710/50 465 B 750/30 445 B 780/60 464 R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382				
B 710/50 465 B 750/30 445 B 750/60 464 R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382				
B 750/30 445 B 780/60 464 R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382				
B 780/60 464 R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382				
R 670/30 373 R 710/50 383 R 780/60 335 V 431/28 382 V 431/25 362		\leq		
R 710/50 383 R 780/60 335 V 431/28 382 V 471/5 260				
K 780/60 S35 V 431/28 S82 V 431/15 S60				
• V 431/28 382				
V 596/15 - 424				
V 500/15 +54				
V 670/30 466				
V 710/50 510				
V 740/35 494				
V 780/60 433				
UV 379/28 354				
UV 515/30 403			H	
UV 586/15 505				
UV 670/30 543				
UV 740/35 515				
UV 820/60 545				
• YG 586/15 368				
YG 610/20 393				
YG 670/30 391				
YG 710/50 323				

- ii. Add back detectors for fluorochromes being used
 - 1. Click the Add button (bottom left of window in above step)
 - Select the bandpass filter from the drop-down menu to add a fluorochrome to the list of parameters being collected. Consult the Instrument filter set attached to the SOP for commonly used fluorochromes and corresponding bandpass filters <u>Important</u>: The letter before the bandpass filter designates the laser excitation

ytometer Settings							
Parameters Threshold Ratio	Compens	sation					
Parameter		Voltage	Log	Α	н	w	
 FSC 		512		\sim			^
• SSC		208		\sim			1
 B 515/20 		448					1
• B 610/20	~	459					
UV 740/35 UV 820/60	^						
YG 586/15							
YG 610/20 YG 670/30							
YG 710/50							
YG 780/60	_						
B 610/20	×						

- The number of fluorochromes in your experiment should match the number of fluorescent detectors in the Parameters tab of the Browser <u>Exception</u>: when using open detectors for autofluorescence detection or when acquiring data for spectral analysis
- 4. For most applications, fluorescent detectors should be set to a Log (L) scale (default). For DNA content/Cell Cycle analysis, a linear scale should be used.

nsp /tor	nector - Cytometer Settings meter Settings							
Par	ameters Threshold Ratio Co	mper	nsation					
	Parameter		Voltage	Log	Α	н	w	
•	FSC		512		\sim			^
•	SSC		208		\sim			1
•	B 515/20		448					1
•	B 610/20	~	459					
	UV 740/35	^						
	UV 820/60 VG 586/15							
	YG 610/20							
	YG 670/30							
	YG 710/50							
	YG 780/60							
	B 610/20	~						

- 7. Optional set fluorescent voltages based on bead target values if values have been previously established (see separate SOP: Standardization with bead target values)
- 8. Set up Compensation (experiments with > 1 color). For one color experiments, skip to step 10.
 - a. Go to Experiment -> Compensation -> Create Compensation Controls

BD FACSDiva Software - Administrator (ORAC Emory Univ 6B 3R 5YG 8V 6UV)											
File	Edit	View	Expe	riment Populations Work	sheet Cyto	met	er HTS Help				
-	1		ei i	New Folder	Ctrl+N						
9	Brows	rowser - Ex		New Experiment	Ctrl+E		23		ρ	Inspector -	Cytor
2	Image: Second		×	New Specimen	Ctrl+M					C. da	-112
-			1	New Tube	Ctrl+T				ľ	Lytometer 5	ettings
L			;¢°,	New Cytometer Settings						Parameters	Thre
-				Import Cytometer Settings	;		Date			Daram	atar
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				Close Experiment	Ctrl+W	20 1	1:31:13 AM				
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		•					0.1.0				1
		•		Compensation Setup	>	,	Create Compensa	tioi	n C	ontrols.	
	(• 📃	Apper	nd Testing	3/16/:		Modify Compens	atic	n	Controls	
	(3-00	Exper	iment_17March2021	3/17/:		Calculate Compe	nsa	tio	n	
		(in the second s	9 C	obal Worksbeets							
	÷.,	🔒 Shar	red Vie	2W							

b. Select checkbox for Unstained control to use a universal negative

Create Compensation Controls						
Tubes	○ Plate					
	3					
Include separate unstained control tube/wel						
Fluorophore	Label					
	Generic					
• B 610/20	Generic					
	·					
L						
Add Delete Labels	OK Cancel					

- c. Confirm parameters in list match single stain controls to be recorded and click **OK**
- 9. Run Compensation Controls

a. Expand the Compensation Controls Specimen and select the Unstained tube by clicking on the arrow to the left of the tube name (arrow should turn green). A Normal worksheet corresponding to the unstained control tube will appear in the worksheet window.



- b. Set the instrument to **RUN** and flow rate to **LO** or **MED** (panel on front left of instrument)
- c. Remove DI water tube on SIP by moving the support arm to either side and pulling the tube down
- d. Install the unstained compensation control tube and move support arm under the tube
- e. Click Acquire on the Acquisition Dashboard to preview the data without recording a file

🔢 Acquisition Dashboard			×
Current Activity			
Active Tube/Well	Threshold Rate	Stopping Gate Events	Elapsed Time
Unstained Control	0 evt/s	0 evt	00:00:00
Basic Controls			
øij Next Tube	🛛 Acquire Data 🛛 📕 Re	cord Data	rt
Acquisition Setup			
Stopping Gate:	vents 🧹 Events To Record:	5000 evt 🗸 Stop Tim	e (sec): 0 🚔 🕇
Storage Gate:	vents $\ arsigma$ Events To Display:	1000 evt 🗸 🗸	
Acquisition Status			
Processed Events:		Electronic Abort Rate:	
Threshold Count:		Electronic Abort Count:	

f. Adjust the FSC and SSC voltages to get cells or beads on scale in the Cytometer window -> Parameters tab

*	Cytometer - LSRFortessa (FACSymphony	A5) (1)	-1000				×
	Status Parameters Threshold Laser Con	mpensation R	-800				_
	Parameter	Voltage	-600	A	н	w	
	FSC FSC	512 🚔 🕇					^
	• SSC	208	-400	\checkmark			
	• B 515/20	448					
	• B 610/20	459	-200				
			-0				

- g. Stop acquisition and remove unstained tube from SIP.
- h. <u>While remaining on the unstained control</u> tube, preview each single color control and check that 1) a positive population exists and 2) all positive populations are not so bright that they appear off-scale (events will be visible to farthest point to the right of the histogram if off-scale).
- i. Lower the appropriate fluorescent voltage in the **Cytometer** window -> **Parameters** tab while acquiring if population is too bright.



FACSymphony Setup Version: 1.01



j. Once all compensation controls have been previewed and voltage adjustments made, acquire and record all compensation control tubes. There should be file icon beside each tube indicating it contains data.

	Experiment_17March2021 Cytometer Settings Global Worksheets Compensation Controls Cytometer Settings	3/17/21 11:00:54 AM
	🍺 🚡 Unstained Control	3/17/21 11:00:54 AM
	🖶 🐻 B 515/20 Stained Control	3/17/21 10:53:37 AM
	🗄 - 🐻 B 610/20 Stained Control	3/17/21 11:00:40 AM
G	🖫 🍰 Shared View	

- i. For each tube, ensure the following:
- ii. The P1 gate contains the beads or cells of interest while excluding debris, and
- iii. The P2 (?) gate snaps into the correct place over the positive population after the file has been recorded. The P2 gate may be adjusted by manually dragging over the positive population if necessary.



- k. Calculate compensation
 - i. Go to Experiment -> Compensation Setup -> Calculate Compensation

BD FACSDiva Software - Administrator (ORAC Emory Univ 6B 3R 5YG 8V 6UV)

File	Edit View	Expe	riment	Populations Works	heet Cyto	meter HTS Help	
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9	Browser - Ex	F	New E	xperiment	Ctrl+E	23	🖉 Inspector - Un
60	ang 🐼 🖬	1	New S	pecimen	Ctrl+M		
-		12	New T	ube	Ctrl+T		Tube Labels /
		: 4	New C				Experiment 17
	1	14	New C	ytometer settings		Date	ExponsionC_171
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	🕀 🔚	6	New P	late	Ctrl+K	20 11:01:48 AM	CST BEADS LO
	🕀 - 📃					20 11:19:49 AM	CYTOMETER C
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		ഷ്ട്രഹ	(tometer	Settings	Ľ	1	prix

ii. Choose "Apply Only" option

10. Create Experimental Specimens and Tubes

a. A specimen must first be added to an experiment before tubes can be added

<u>Note</u>: The filename will be a combination of the specimen and tube name, with the specimen name appearing first in the file name, followed by an underscore and the tube name

i. Highlight the experiment name and select the syringe icon in the Browser window to add a specimen to an experiment



- ii. Expand the specimen. By default, a single tube is automatically added to a specimen when created
- iii. To add more tubes to a specimen, highlight the specimen and click the tube icon in the upper left corner of the Browser window

📴 Browser - Experiment_17March2021								
🛎 🖀 🌾 💕 🗳 📓 🖂 🗸 -								
Name Name	Date							
🗐 🦂 Administrator								
🕀 📔 Analysis Simon 14th August 2020	8/14/20 2:11:40 PM							
🖶 🔚 Experiment_004	11/16/20 11:01:48 AM							
🖶 🔚 Experiment_005	11/16/20 11:19:49 AM							
🖶 🔚 Experiment_006	11/16/20 11:22:10 AM							
🖶 🔚 Experiment_007	11/16/20 11:31:13 AM							
NANT2017_SEATL22_Day29_6Jan2021	1/8/21 10:37:31 AM							
🖶 🔚 ANBL 19P 1_89979 1_Pre-T_5 Jan 2021	1/8/21 11:28:16 AM							
🖶 📔 Exp_R66096400085_template_test	2/24/21 11:16:41 AM							
Experiment_008	3/16/21 8:52:52 AM							
🕀 🔚 Append Testing	3/16/21 11:48:52 AM							
🖨 🛄 Experiment_17March2021	3/17/21 11:00:54 AM							
Cytometer Settings								
🕀 🛗 Global Worksheets								
🖶 🖄 Compensation Controls								
🛱 🌂 Specimen_001								
🕞 👘 🗍 Tube_001								
🗄 📲 🥵 Shared View								

iv. Rename specimen and tubes if desired either in the Browser window or Inspector window

Emory Pediatrics/Winship Flow Cytometry Core Updated: 18 Mar 2021

📓 BD FACSDiva Software - Adı	minist	trator (ORAC En	nory Univ 6B 3R 5YG	8V 6UV)				
File Edit View Experiment Po	pulat	ions Workshee	t Cytometer HTS	Help				
- 10 - 10 - 10 - 10	-				_			_
Browser - Experiment_17Ma	rch20	21		×		lnspector - Tube_0	001	23
🖆 🗑 餐 ᢪ 💓 📓 🔤 📐	/ -				I	Tube Labels Acq.	Keywords	
					Ш	Name:	Tube 001	
Name Name	V		Date		P	Global Worksheet:		
	th Aug 1_Pre 85_te arch20	just 2020 -T_5Jan2021 mplate_test 21	8/14/20 2:11:40 PM 1/8/21 11:28:16 AM 3/16/21 11:48:52 AM 2/24/21 11:16:41 AM 11/16/20 11:01:48 AM 11/16/20 11:10:49 AM 11/16/20 11:22:10 AM 11/16/20 11:32:13 AM 3/16/21 8:52:52 AM 3/17/21 11:35:51 AM	н м м м		Total # of Events: Record Date: Record Start: Record End: Record Liser: Institution: Cytometer Name: Cytometer Serial #; Laser Delay: Area Scaling:	Image: Contract of the second secon	
Gobal Worksr Gobal Worksr Compensation Specimen_00 Tube_001 Def Cleaning	Cont 1	Cut		Ctrl+X		FSC Area Scaling: Window Extension:		
B Shared View	lù G	Copy Paste Delete		Ctrl+C Ctrl+V Delete				
		Apply Analysis Copy Spectral Paste Spectral Paste Spectral	Template Overlap Overlap Overlap with Zeros					
		Rename						
	: 4 °	Duplicate With Batch Analysis New Cytomete Import Cytome	er Settings eter Settings	Ctrl+D				

- b. Add as many specimens and tubes as necessary for experiment
- c. Add cleaning tubes
 - i. Go to Experiment -> New Specimen

BD FACSDiva Software - Administrator (ORAC Emory Univ 6B 3R 5YG 8V 6UV)

The Edit view	expe	iment Populations works	neer Cyru	песег пто пер
🚽 – 🛐 🧰 💌	Ċ	New Folder	Ctrl+N	
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File Edit View Experiment Populations Worksheet Cytometer HTS Help

ii. Select **Cleaning** from the Specimen template menu

Panel Templates		
General		5
Name	Date	Name: Cleaning
Blank Panel		
Cleaning	11/30/20 11:01 AM	
	_	
Name: Cleaning		Copies: 1
		OK Cancel

- iii. A Cleaning specimen with 3 tubes is automatically added to the experiment:
 - 1. Bleach
 - 2. Clenz
 - 3. Water

Browser - Experiment_17March2021										
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Name	Date									
📮 🦂 Administrator										
🖶 🔚 Analysis Simon 14th August 2020	8/14/20 2:11:40 PM									
🖶 🔚 Experiment_004	11/16/20 11:01:48 AM									
🖶 🔚 Experiment_005	11/16/20 11:19:49 AM									
🖶 🔚 Experiment_006	11/16/20 11:22:10 AM									
🖶 🔚 Experiment_007	11/16/20 11:31:13 AM									
Image:	1/8/21 10:37:31 AM									
🖶 🔚 ANBL 19P 1_89979 1_Pre-T_5Jan 2021	1/8/21 11:28:16 AM									
🖶 🔚 Exp_R66096400085_template_test	2/24/21 11:16:41 AM									
🖶 🔚 Experiment_008	3/16/21 8:52:52 AM									
🕀 🔚 Append Testing	3/16/21 11:48:52 AM									
🖨 🛄 Experiment_17March2021	3/17/21 11:00:54 AM									
Cytometer Settings										
🕀 🔁 Global Worksheets										
🕀 📉 Compensation Controls										
🖨 📉 Specimen_001										
🕞 👘 🗍 Tube_001										
🕞 🛛 🗍 Tube_002										
🕑 🛛 🗍 Tube_003										
🕞 👘 🗍 Tube_004										
🔲 🗎 📉 Cleaning										
BLEACH										
CLENZ										
💌 🥡 WATER										
🕀 😼 Shared View										

- 11. Edit the Experiment Layout (optional)
 - a. Go to **Experiment** -> **Experiment Layout**

BD FACSDiva Software - Administrator (ORAC Emory Univ 6B 3R 5YG 8V 6UV)

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b. Create labels for parameters

Creating labels adds additional information to the FCS file regarding the markers and fluorochromes in each panel and makes downstream analysis easier, for example "CD4 PE YG 586/15" is added to the detector label in the FCS file instead of just "YG 586/15"

- i. Go to **Labels** tab
- ii. Click column header to add labels for all tubes at one time or alternatively click individual cells to rename labels for individual tubes
- iii. Type the name of the desired label in **Label** box (top left-hand corner of **Experimental Layout** window while the cells are highlighted, e.g. CD4 PE, and hit enter

🛛 Experi	iment Layout									
Labels	Keywords Acquisition Click to	header to sel	ect all tubes							
Ouick Entry										
Lab	el CD4 FITC V									
	Name	Label	Label							
•	Experiment_17March2021									
	🔓 Unstained Control	B 515/20	B 610/20							
•	🖷 🖟 B 515/20 Stained Control	B 515/20	B 610/20							
	🔓 🔓 B 610/20 Stained Control	B 515/20	B 610/20							
•	Specimen_001									
•	🧻 Tube_001	B 515/20 CD4 FITC	B 610/20							
	🥛 Tube_002	B 515/20 CD4 FITC	B 610/20							
	🥛 Tube_003	B 515/20 CD4 FITC	B 610/20							
	↓ Tube_004	B 515/20 CD4 FITC	B 610/20							
	🔨 Cleaning									
•	J BLEACH	B 515/20 CD4 FITC	B 610/20							
•	···· J CLENZ	B 515/20 CD4 FITC	B 610/20							
•	WATER	B 515/20 CD4 FITC	B 610/20							

- c. Set acquisition criteria
 - i. Go to the Acquisition tab in the Experiment Layout window
 - ii. Set events to record for each tube by clicking on column header to select all tubes at a time, clicking on individual cells, or holding down **Ctrl** on the keyboard and selecting multiple cells at a time
 - iii. With cells selected, type desired number of total events to record in the **Events to Record** box at the upper left-hand corner of the experimental layout under the words "Quick Entry"



(💽 E	Call Experiment Layout											
La	Labels Keywords Acquisition											
	Quick Entry Events to Record 100,000 Global Worksheet Storage Gate											
		Name	Events to Rec	Global Worksh	Stopping Gate	Storage Gate	Stopping Time					
	•											
	•	X Compensation Controls										
	•	🎧 Unstained Control	5,000		All Events	All Events	0					
	•	🥻 B 515/20 Stained Control	5,000		All Events	All Events	0					
	•	🔚 🐻 610/20 Stained Control	5,000		All Events	All Events	0					
	•											
	•	🗍 Tube_001	100,000		All Events	All Events	0					
	•	🗍 Tube_002	100,000		All Events	All Events	0					
	•	🗍 Tube_003	100,000		All Events	All Events	0					
	•	🔤 🗍 Tube_004	100,000		All Events	All Events	0					
	•	🦂 Cleaning										
	•	🗍 BLEACH	1,000,000		All Events	All Events	180					
	•	🗓 CLENZ	1,000,000		All Events	All Events	180					
	•	WATER	1,000,000		All Events	All Events	180					

- d. Select **OK** (bottom right-hand corner of window) to exit the Experiment Layout
- 12. Set up the Global Worksheet to visualize data while acquiring
 - a. Switch from the normal worksheet to the global worksheet by clicking the Worksheets view button in the Worksheet toolbar (top left-most button)

<u>Note</u>: Plots in the *global worksheet* show data for the tube that is selected with the current tube pointer. Plots in the *normal worksheet* are tube-specific (only data recorded in the selected tube can be displayed.



b. Create plots or histograms by selecting an option in the Worksheet toolbar and then clicking in the worksheet. Change parameters displayed on axes by right-clicking on each parameter name.



c. Create regions in plots and histograms using the tools in the Worksheet toolbar



d. Gate histograms on populations of interest by right-clicking inside the white space on the desired plot to be gated and selecting the wanted region from **Show Populations**



e. Create a population hierarchy by right-clicking on any plot and choosing Population Hierarchy





- 13. Acquire samples
 - Set acquisition limits by either going to Experiment -> Experiment Layout -> Acquisition tab to set limits for all tubes at once or set the limits manually, before recording each tube, by editing values in the Acquisition Dashboard

🔢 Acquisition Dashboard			×
Current Activity			
Active Tube/Well	Threshold Rate	Stopping Gate Events	Elapsed Time
Tube_001	0 evt/s	0 evt	00:00:00
Basic Controls			
ø i Next Tube	📕 Acquire Data 📕 Re	cord Data	art
Acquisition Setup			
Stopping Gate: All E	Events 🗸 Events To Record:	: 100000 evt \sim Stop Tim	ne (sec): 0 📮 🕇
Storage Gate:	vents Events To Display:	: 1000 evt 🗸	
Acquisition Status	P2		
Processed Events:		Electronic Abort Rate:	
Threshold Count:		Electronic Abort Count:	

- b. To overwrite a previously recorded file/tube, start acquiring with the desired tube to be overwritten selected, select **Record** in the Acquisition Dashboard, and select **Overwrite** when prompted
- c. To append (add) to a previously recorded file/tube, start acquiring with the desired tube to be appended to selected, select **Record** in the Acquisition Dashboard, and select **Append** when prompted



- 14. Clean Instrument (transfer data while cleaning see step 15)
 - a. Use the cleaning specimen created in Step 10c and following the cleaning section in the SOP
 - b. Put the instrument in **Standby** with tube of DI water on the SIP (arm under tube)
 - c. Check the instrument schedule online and if you are the last user of the day, shut down the instrument by hitting the green power button on right-hand side of instrument.

15. Transfer data

- a. Go to File Explorer in the Windows toolbar and navigate to **Desktop** -> **FCSfiles shortcut** -> folder with today's date
- b. Open Chrome and log into OneDrive (bookmarked). Drag your experiment folder to OneDrive and ensure all files have transferred before logging out of OneDrive and closing the browser.

16. Log out of Diva



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17. Log out of PPMS

