

STANDARD OPERATING PROCEDURE

Title: BD FACSCelesta™ Flow Cytometer Operation and Data Analysis

SOP#: M-146

Version #: 1

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1. PURPOSE

This procedure will describe how to stain cells that will be analyzed on the BD FACSCelesta™ flow cytometer, how to operate the BD FACSCelesta flow cytometer™, and how to analyze the flow cytometry data using FACSDiva.

2. SCOPE

This procedure will describe sample preparation and operation of the BD FACSCelesta™ flow cytometer and data analysis using FACSDiva.

3. RESPONSIBILITIES

It is the responsibility of the person(s) preparing these reagents to be familiar with lab safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure steps as written and to document any deviations, problems, and observations during an experiment in their notebook.

3.1 Please refer to the following resources located in the Shared Drive, crtp-acl (\\at-s-is2.ncifcrf.gov), for guidance on this procedure:

3.1.1 BD-FACSCelesta™-Users-Guide

4. EQUIPMENT

4.1. BD FACSCelesta™, BD Biosciences

4.2. Vortex Genie 2 (Fisher, Cat. #12-812)

4.3. Rainin Pipet plus LTS Pipettor, adjustable 100-1000 µL

4.4. Rainin Pipet plus LTS Pipettor, adjustable 20-200 µL

4.5. Rainin Pipet plus LTS Pipettor, adjustable 2-20 µL

4.6. Rainin Pipet plus LTS Pipettor, adjustable 0.1-2 µL

4.7. Sorval® Legend RT Bench Top Centrifuge and buckets

4.8. Mini-Centrifuge, VWR

5. **MATERIALS**

5.1. Polypropylene 15 ml Conical Tube (Falcon BLUE MAX Jr. Cat. #352099)

5.2. Polypropylene 50 ml Conical Tube (Falcon BLUE MAX Jr. Cat. #352070)

5.3. Rainin Space Saver LTS Reloads Pipette Tips. 1000 µL (Rainin, Cat. #GPS-L10)

5.4. Rainin Space Saver LTS Reloads Pipette Tips. 250µL (Rainin, Cat. #GPS-L250)

5.5. Rainin Space Saver LTS Reloads Pipette Tips. 20 µL (Rainin, Cat. #GPSL-1000)

5.6. Eppendorf Centrifuge Tubes, 1.5 ml (VWR, Cat. # 20901-551)

5.7. Polystyrene 10 mL pipettes, (FALCON, Cat. #356551)

5.8. Polystyrene 25 mL pipettes (Diagger, Cat. number #1BSPS25)

5.9. BD FACSDiva™ CS&T Research Beads (BD Biosciences, Cat. #655050)

5.10. Brilliant Violet 421™ Goat anti-mouse IgG (minimal x-reactivity) Antibody (BioLegend, Cat. #405317)

5.11. BV421 Goat anti-rabbit IgG Polyclonal Antibody (BD Biosciences, Cat. #565014)

5.12. Falcon Round Bottom 12 x 75 mm, 5 mL polystyrene FACS tubes, (Corning, Cat. #352058)

5.13. Rabbit (DA1E) mAb IgG XP® Isotype Control #3900 (Cell Signaling, Cat. #3900S)

5.14. Mouse (E5Y6Q) mAB IgG2a Isotype Control (Cell Signaling, Cat. #61656)

5.15. Mouse (E7Q5L) mAB IgG2b Isotype Control (Cell Signaling, Cat. #53484)

5.16. Mouse (G3A1) mAB IgG1 Isotype Control (Cell Signaling, Cat. #5415)

5.17. Mouse (E1D5H) mAB IgG3 Isotype Control (Cell Signaling, Cat. #37988)

5.18. Deionized H₂O

5.19. Cyto-Fast™ Fix/Perm Buffer Set, 10X (Biolegend, Cat. # 426803)

- 5.20. True-Nuclear™ Transcription Factor Buffer Set (BioLegend, Cat. #424401)
 - 5.21. Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™), (BD Biosciences, Cat. #553142)
 - 5.22. Human BD Fc Block™, (BD Biosciences, Cat. #564219)
 - 5.23. 10X PBS without calcium/magnesium (Invitrogen, Cat. #10010023)
 - 5.24. Bovine Serum Albumin, heat shock fraction, pH 7, > 98%, (Sigma, Cat. #A7030-100G)
 - 5.25. EDTA, 0.5 M, pH 8.0, RNase-Free, (Invitrogen, Cat. #AM9260G)
 - 5.26. BD FACSTFlow Sheath Fluid (BD Biosciences, Cat. #342003)
 - 5.27. Fixation Buffer, BioLegend (Cat. #420801)
 - 5.28. 16% Methanol-free paraformaldehyde, Thermo Fisher Scientific, (Cat. #28908)
 - 5.29. Contrad 70 Liquid Detergent, Decon Labs (Cat. #89233-152)
 - 5.30. Concentrated NaOH
 - 5.31. Concentrated HCl
 - 5.32. Appropriate cell line(s) that include as a minimum:
 - 5.32.1. A positive control that is known to express the target
 - 5.32.2. A negative control that is known to not express the target.
- Note: Be sure to confirm the presence or absence of engineered fluorophores in the target cell lines used to evaluate antibody binding (i.e. GFP)**

6. TEST SAMPLES

- 6.1. Monoclonal antibody or antibodies (mouse or rabbit).

7. PROCEDURE

7.1. Preparation of Cell Samples for External Cell Surface Target Staining (For Preparation of Cell Samples for Internal Cytosolic or Nuclear Target Staining go to Section 7.2)

- 7.1.1. Prepare FACS buffer as follows:
 - 7.1.1.1. Add 100 mL of 10X PBS to a 1L glass bottle.
 - 7.1.1.2. Add 30 g of BSA.
 - 7.1.1.3. Add 2.0 mL of EDTA (Stock 0.5M)
 - 7.1.1.4. Bring to 1L with DI H₂O

- 7.1.1.5. Mix until dissolved.
- 7.1.2. Prepare the appropriate blocking buffer based upon the cell type as follows:
- 7.1.2.1. Dilute Human Fc Block™ or Mouse BD Fc Block™ 1:50 in FACS buffer to a final concentration of 10 µg/mL solution.
- 7.1.3. Prepare 4% Paraformaldehyde **Stock Fixation Buffer**
- Note: Paraformaldehyde is toxic and is a suspected carcinogen. Use caution when handling.**
- 7.1.3.1. In the chemical hood, pre-heat 150 ml 10% FBS/1X PBS in a 250 ml beaker to 60°C on a hot plate with magnetic stirrer.
- 7.1.3.2. Add 50 ml 16% paraformaldehyde to the warm PBS and leave stirring for 20 min at 60°C.
- 7.1.3.3. Add concentrated NaOH until the solution reaches pH ~11.
- 7.1.3.4. Allow the solution to cool down to room temperature.
- 7.1.3.5. Adjust pH to 7.2 – 7.3 using first concentrated HCl, and then diluted HCl for fine adjustment.
- 7.1.3.6. Prepare aliquots and store at -20°C. Thaw at room temperature right before use.
- 7.1.4. Prepare 1% Paraformaldehyde **Working Fixation Buffer**
- 7.1.4.1. In the chemical hood, dilute **Stock Fixation Buffer** (4% Paraformaldehyde) 1:4 in 1X PBS.
- 7.1.4.2. Mix well.
- 7.1.5. Transfer 1.0 – 2.0 x 10⁶ total cells to a FACS tube for target protein staining. Transfer 1.0 – 2.0 x 10⁶ cells to FACS tubes for control staining according to Table 1 below.

Sample #	Cell Line Name	Primary Antibody	Secondary Antibody
1	Test	CPTC Test IgG	BV421 (Goat anti-IgG)
Control #	Cell Line Name	Primary Antibody	Secondary Antibody
1	Test	Buffer	Buffer
2	Test	Isotype Control	BV421 (Goat anti-IgG)
3	Test	Buffer	BV421 (Goat anti-IgG)

Table 1

- 7.1.6. Centrifuge cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.1.7. Add 1.0 mL of FACS buffer to wash cells.
- 7.1.8. Centrifuge cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.1.9. Repeat steps 7.1.6. and 7.1.7. to wash cells.
- 7.1.10. Add 100 μ L of 1X Fc blocking buffer. Gently shake the tubes to mix.
- 7.1.11. Incubate samples on ice in the dark for 20 minutes.
- 7.1.12. Add 1.0 mL of FACS buffer to each tube.
- 7.1.13. Centrifuge at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.1.14. Dilute the test primary antibody (CPTC tag) in 1X Fc blocking buffer to the appropriate concentration. Typical concentrations range from 0.1 μ g/mL – 10 μ g/mL.
- 7.1.15. Dilute the isotype control antibody in 1X Fc blocking buffer at a matched concentration as the test primary antibody.
- 7.1.16. Add 100 μ L of respective primary antibodies to the appropriate samples and controls as shown in Table 1.
- 7.1.17. Incubate samples on ice for 0.5 hr – 1.0 hr.
- 7.1.18. Add 1.0 mL of FACS buffer to wash cells to remove the excess primary antibodies.
- 7.1.19. Centrifuge cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.1.20. Repeat steps 7.1.17. and 7.1.18. 2X to remove the excess primary antibodies.
- 7.1.21. Dilute the appropriate BV421 labeled secondary antibody (anti-mouse or anti-rabbit IgG) in Fc blocking buffer to the appropriate concentration. Typical concentrations range from 0.1 μ g/mL – 10 μ g/mL.
- 7.1.22. Add 100 μ L of secondary antibody to the appropriate samples and controls as shown in Table 1.
- 7.1.23. Incubate the samples for 30 minutes at room temperature.
- 7.1.24. Add 1.0 mL of FACS buffer to wash cells to remove the secondary antibody.
- 7.1.25. Add 100 μ L of **Working Fixation Buffer** to each sample and gently mix to fix cells.

- 7.1.26. Fix cells at room temperature for 30 minutes.
- 7.1.27. Centrifuge cells at 1500 rpm for five minutes. Discard supernatant in the appropriate waste container in the chemical hood.
- 7.1.28. Add 1.0 mL of FACS buffer to each sample and centrifuge cells at 1500 rpm for five minutes. Discard supernatant in the appropriate waste container in the chemical hood.
- 7.1.29. Wash cells with 1.0 mL of FACS buffer. Aspirate.
- 7.1.30. Add 200 uL of FACS buffer and store at 4°C until flow cytometry analysis.

7.2. Preparation of Cell Samples for Internal Cytosolic or Nuclear Target Staining

- 7.2.1. Transfer 1 – 2.0 x 10⁶ total cells to a FACS tube for target protein staining. Transfer 1 – 2.0 x 10⁶ cells to FACS tubes for control staining according to Table 1 below.

Sample #	Cell Line Name	Primary Antibody	Secondary Antibody
1	Test	CPTC Test IgG	BV421 (Goat anti-IgG)
Control #	Cell Line Name	Primary Antibody	Secondary Antibody
1	Test	Buffer	Buffer
2	Test	Isotype Control	BV421 (Goat anti-IgG)
3	Test	Buffer	BV421 (Goat anti-IgG)

Table 2

Note: If the target is cytosolic use the Cyto-Fast™ Fix/Perm Wash Buffer Set. If the target is nuclear, use the True-Nuclear™ Transcription Factor Buffer Set.

- 7.2.2. Dilute Cyto-Fast™ Perm Wash solution (10X) to 1X in diH₂O. Add 10 mL of Cyto-Fast™ Perm Wash solution (10X) to 90.0 mL of diH₂O. **Or**, dilute the True-Nuclear™ Perm buffer (10X) to 1X in diH₂O. Add 10 mL of FOXP3 Perm buffer (10X) to 90 mL of diH₂O. Place on ice.
- 7.2.3. Wash the cells 1 mL of ice cold 1X PBS buffer.
- 7.2.4. Centrifuge the cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.2.5. Repeat steps 7.23 and 7.24 (total 2 washes)

- 7.2.6. If the target protein is cytosolic, use the Cyto-Fast™ Fix/Perm Buffer (1X). In the chemical hood, add 150 uL of Cyto-Fast™ Fix/Perm Buffer to each FACS tube. Mix well and incubate for 20 minutes.
- 7.2.7. If the target protein is nuclear, use the True-Nuclear™ Fix Concentrate (4X). In the chemical hood, first dilute the True-Nuclear™ Fix Concentrate (4X) to 1X in True-Nuclear™ Fix Diluent. Carefully add 1.5 mL of True-Nuclear™ Fix Concentrate (4X) in 4.5 mL of True-Nuclear™ Fix Diluent. Mix well. Add 1 mL of diluted True-Nuclear™ Fix to each FACS tube.
- Note: Both fixing solutions contain paraformaldehyde. Use caution when handling.**
- 7.2.8. Centrifuge the cells at 1500 rpm for 5 minutes.
- 7.2.9. Decant or pipette fixing solution into the appropriate waste container in the chemical hood.
- 7.2.10. Wash the cells with 1 mL 1X CytoFast™ Perm/Wash or True-Nuclear™ Perm buffer.
- 7.2.11. Centrifuge the cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.2.12. Add 8.0 mL of 1X CytoFast™ Perm/Wash or True-Nuclear™ Perm buffer.
- 7.2.13. Mix well and dispense 2.0 mL of cell suspension into four FACS tubes according to Table 2 above.
- 7.2.14. Centrifuge the cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.2.15. Dilute the test primary antibody (CPTC tag) in 1X Cyto-Fast™ Perm/Wash buffer or True-Nuclear™ Perm buffer. Typical concentrations range from 0.1 ug/mL – 10 ug/mL.
- 7.2.16. Dilute the isotype control antibody in 1X Cyto-Fast™ Perm/Wash buffer or True-Nuclear™ Perm buffer at a matched concentration to the test primary antibody. Add 100 uL to the appropriate cells.
- 7.2.17. Incubate 0.5 hr – 1.0 hr on ice in the dark.
- 7.2.18. Add 1 mL of 1X Cyto-Fast™ Perm/Wash buffer or True-Nuclear™ Perm buffer to the cells. Mix well.
- 7.2.19. Centrifuge the cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.2.20. Repeat steps 7.2.18. and 7.2.19.

- 7.2.21. Dilute the appropriate secondary antibody (BV421) in Cyto-Fast™ Perm/Wash buffer or True-Nuclear™ Perm buffer to the appropriate concentration. Typical concentrations range from 0.1 ug/mL – 10 ug/mL.
- 7.2.22. Add 100 uL of secondary antibody to each sample. Incubate the cells for 30 minutes at RT in the dark.
- 7.2.23. Add 1.0 mL of 1X PBS to remove the secondary antibody.
- 7.2.24. Centrifuge the cells at 1500 rpm for 5 minutes. Aspirate supernatant.
- 7.2.25. Repeat steps 7.2.23 and 7.2.24 2X.
- 7.2.26. Resuspend cells in 200 uL of 1X PBS.
- 7.2.27. Cover samples with aluminum foil and store at 4°C for one week until flow cytometer data acquisition.

7.3. Preparation of BD FACSCelesta™ Instrument

- 7.3.1. Turn on the computer and log into the operating system.
 - HP Computer Login: **BDAdmin**
 - Password: **BDIS#2\$\$**
- 7.3.2. Turn on the cytometer main power.
- 7.3.3. Start BD FACSDiva Software.
- 7.3.4. Enter the log-in information and then click OK.
 - Diva Software Login: **Administrator**
 - Password: **Password#2**
- 7.3.5. Ensure that the software is connected to the cytometer by checking the bottom of the Cytometer window. If necessary, select Cytometer -> Connect. The software can be slow to connect sometimes. This is normal.
- 7.3.6. Verify that the system status on the control panel is set to standard fluidics mode.
- 7.3.7. Check the System Status LED button status.
 - 7.3.7.1. Green – No action
 - 7.3.7.2. Yellow – Sheath and or waste tanks need attention.
 - 7.3.7.3. Red – Refill the sheath fluid tank and empty the waste tank.

- 7.3.7.4. If necessary, fill the sheath tank with sheath fluid to the fill line marked on the inside of the sheath tank.
- 7.3.7.5. If necessary, empty waste container by carefully pouring waste liquid into the red Hazardous Chemical Waste container in Room C1003.
- 7.3.8. Check the sheath filter located on the sheath tank for air bubbles.
 - 7.3.8.1. Place a plastic beaker on the floor next to the sheath tank.
 - 7.3.8.2. Position the filter in a vertical position with the vent line pointing up above the beaker.
 - 7.3.8.3. Gently tap the filter body to dislodge the bubbles and force them to the top.
 - 7.3.8.4. Press the small button at the end of the vent fitting line until a steady stream of fluid empties from the filter into the beaker.
- 7.3.9. Check the sheath line at the back of the instrument for air bubbles.
 - 7.3.9.1. Place a plastic beaker on the bench at the back of the instrument.
 - 7.3.9.2. Open the roller clamp to bleed off any air in the line into the beaker.
 - 7.3.9.3. Close the roller clamp.
- 7.3.10. Press RUN and HI on the cytometer fluids control panel.
- 7.3.11. Install a FACS tube containing 3 mL of DI water on the Sample Injection Port (SIP).
- 7.3.12. Allow the solution to run on HI for 15 minutes.
- 7.3.13. Press the STANDBY button on the fluids control panel.
- 7.3.14. Place a FACS tube containing no more than 1 mL of DI water on the SIP.
- 7.3.15. Allow lasers to warm for at least 30 minutes.

7.4. Perform Cytometer Set-Up and Tracking (CS&T) performance test

- 7.4.1. In a FACS tube, add 1 drop of CS&T beads to 350 uL of filtered sheath fluid.
- 7.4.2. Vortex.

- 7.4.3. In the FACSDiva software, select Cytometer -> CST
- 7.4.4. Select the correct lot ID from the list that corresponds to the lot ID for the CS&T beads that you are using.
- 7.4.5. Set the flow rate to LO on the cytometer fluidics control panel.
- 7.4.6. Verify that the sample fine adjust is set to 250.
- 7.4.7. Press RUN.
- 7.4.8. Remove the diH₂O FACS tube from the SIP and install the Cytometer Set-up and Tracking (CS&T) bead tube on the cytometer.
- 7.4.9. Verify that Check Performance is selected from the Characterize list and click Run in the CS&T workspace.
 - 7.4.9.1. Click OK to confirm that the CS&T tube has been installed.

Note: Plots are displayed in the CS&T workspace and the performance check is run. The performance check will take ~ 15 minutes.

- 7.4.10. Install a FACS tube with 1 mL of DI water onto the cytometer and press STANDBY.
- 7.4.11. Review the CS&T results. If the performance check passes proceed with step 7.5. If the performance check passes with warnings or fails, troubleshoot by repeating steps 7.1.9. to 7.1.17 to remove air from the system. It may be necessary to repeat these steps multiple times to completely remove any trapped air from the system.
 - 7.4.11.1. Repeat CS&T.
 - 7.4.11.2. If CS&T does not pass, place a service call.
- 7.4.12. Select File -> Exit
- 7.4.13. Click Use CS&T Settings.
- 7.4.14. Instrument is now ready for cytometer acquisition.

7.5. Data Acquisition and Experiment Set-Up

- 7.5.1. Click the **New Folder** button on the Browser toolbar and rename it with the project name.
- 7.5.2. Select the folder and click the **New Experiment** button and rename it with the experiment name.

- 7.5.3. Click the **New Specimen** button to add a new specimen and rename it with the name of the cell line that will be processed.
- 7.5.4. Click the **New Tube** button to create the following tubes:
 - A. Unstained
 - B. Isotype Control
 - C. No Primary/Secondary Ab Only
 - D. Test Primary Antibody (CPTC Tag)
- 7.5.5. Double click on the Experiment Name to open the experiment. An open book icon will appear. Only one experiment can be open at one time in the browser.
- 7.5.6. Select the **Cytometer Settings** icon.
- 7.5.7. Click the Parameters tab to view the current parameters.
- 7.5.8. Delete all the parameters **except** for FSC, SSC, and BV421.
- 7.5.9. For both the FSC and SSC parameters, select H and W.
- 7.5.10. For the BV421 parameter, select **Log**.
- 7.5.11. Click the **Dot Plot** button on the Worksheet toolbar.
- 7.5.12. Click inside the global worksheet work area to create the plot FSC-A (x-axis) and SSC-A (y-axis).
- 7.5.13. Click on the Global Worksheets icon in the browser.
- 7.5.14. In the Inspector, change the size of the worksheet by increasing the Number of Pages from 1 to 2 for both the Vertical and Horizontal dimensions.
- 7.5.15. Click the Histogram Plot button on the Worksheet toolbar.
- 7.5.16. Click inside the global worksheet work area to create the histogram plot. Change the x-axis to BV421 parameter.
- 7.5.17. Set the current tube pointer to the unstained control tube.
- 7.5.18. Set the flow rate to **LOW and RUN**.
- 7.5.19. Gently vortex the unstained cells tube and install it on the cytometer SIP.
- 7.5.20. In the dashboard, set the Events to Display to **500**.
- 7.5.21. Start acquiring data.

- 7.5.22. In the global worksheet area, view the FSC-A (x-axis) and SSC-A (y-axis) plot. Verify that the cell populations are completely on-scale by creating a rectangular P1 gate.
- 7.5.23. Create a population hierarchy by right-clicking on any dot plot and selecting "Show Population Hierarchy." View the percentage of cells that are on-scale. If needed, use the Cytometer window to adjust the FSC and SSC PMT voltages to place the cells on-scale. In general, voltages for FSC should be ~ 300-350 V and 250-300 V for SSC. View the unstained cells in the histogram plot so that the cells are $< 10^3$.
- 7.5.24. Use the polygon gating tool to create a gate to remove the cell debris. Select the gate and then right click to select "Drill Down" to create another dot plot.
- 7.5.25. On the second plot, select FSC-A on the x-axis and select FSC-H on the y-axis. Use the polygon gating tool to create a gate to select the cell singlets. Select the gate and then right click to select "Drill Down" to create another dot plot.
- 7.5.26. Stop acquiring data after appropriate voltage adjustments and plots are made.
- 7.5.27. Remove the unstained cell tube from the SIP.
- 7.5.28. Install the CPTC tag test antibody sample (Sample #1 from Table 2.) tube on the cytometer SIP. This should be the sample that contains cells stained with the brightest fluorescence.
- 7.5.29. Start acquiring data.
- 7.5.30. View the BV421 histogram plot and check that the positive populations are onscale ($< 10^5$).
- 7.5.31. Stop acquiring data when the appropriate voltage adjustments have been made.
- 7.5.32. Remove the test primary antibody BV421 sample FACS tube from the SIP.
- 7.5.33. Place a FACS tube of diH₂O on the SIP and place the instrument in standby.
- 7.5.34. On the acquisition dashboard, change the events to record to 10,000 events.

7.6. Data Recording

- 7.6.1. Install the Unstained Cells tube on the SIP and set the current tube pointer to the unstained control tube.

- 7.6.2. Click **acquire data** and wait for events to be displayed. Verify that the events appear as they did during data acquisition.
- 7.6.3. Click **Record Data**.
- 7.6.4. After data has been recorded for the first tube, click **Next Tube** on the dashboard.
It is important that the current tube pointer (green) is selected on the correct sample during data recording.
- 7.6.5. Repeat steps 7.6.1 – 7.6.4 for the remaining sample tubes. Verify that a data file with the date and time has been recorded for each tube. This file should appear next to each tube name.
- 7.6.6. Remove the final tube from the SIP.
- 7.6.7. Place a FACS tube of diH₂O on the SIP and place the instrument in standby.
- 7.6.8. Select File -> Export -> Experiments to export the .fcs files to the appropriate folder on the Shared Drive.
- 7.6.9. Perform daily cleaning as described in section 7.8.1 after data for all samples has been recorded.
- 7.6.10. Place a FACS tube of ~ 1 mL of 20% Contrad 70 on the SIP.
- 7.6.11. Close the FACSDiva software.
- 7.6.12. Turn off the flow cytometer.

7.7. Data Analysis

- 7.7.1. If needed, open the experiment with recorded data in FACSDiva.
- 7.7.2. Change the global worksheet to a normal worksheet by clicking on the worksheet button in the toolbar.
- 7.7.3. Set the current tube pointer to the Unstained Cells tube.
- 7.7.4. Create a histogram plot in the normal worksheet.
- 7.7.5. Drag the file inside the histogram plot in the normal worksheet.
- 7.7.6. Drag the files for each of the remaining tubes to create an overlay plot in the normal worksheet.
- 7.7.7. Right click the dot plot and select **Show Population Hierarchy**.
- 7.7.8. Format the population hierarchy to show only the population names and colors.
- 7.7.9. Move the population hierarchy to a clear area in the worksheet.
- 7.7.10. Right click any plot and select **Create Statistics View**.

- 7.7.11. Verify the gates are set properly and the population hierarchy shows the appropriate gating structure for each sample.
- 7.7.12. Compare the statistics for each sample in the experiment to analyze the data.

7.8. Maintenance

7.8.1. Maintenance during use

7.8.1.1. Cleaning the fluidics

- 7.8.1.1.1. Prepare a 10% bleach solution by adding 50 mL of concentrated bleach to 450 mL of diH₂O. Mix well. Prepare fresh every two weeks.
- 7.8.1.1.2. Prepare a 20% Contrad 70 solution by adding 100 mL of Contrad 70 to 400 mL of diH₂O. Mix well. Prepare fresh every two weeks.
- 7.8.1.1.3. Press **RUN and HIGH** on the cytometer control panel.
- 7.8.1.1.4. Install a FACS tube containing 3 mL of a 10% bleach solution on the SIP with the support arm to the side (vacuum on) and let it run for one minute.
- 7.8.1.1.5. Move the FACS tube support arm under the FACS tube (vacuum off) and allow the cleaning solution to run for five minutes with the sample flow rate set to **HIGH**.
- 7.8.1.1.6. Repeat steps 7.8.1.1.4. – 7.8.1.1.5. using diH₂O and 20% Contrad 70 solution.
- 7.8.1.1.7. Press standby on the fluidics control panel.
- 7.8.1.1.8. Place a FACS tube containing ~ 1.0 mL of 20% Contrad 70 solution on the SIP.

7.8.1.2. Shutting Down the Cytometer

- 7.8.1.2.1. Place a FACS tube of 1 mL of diH₂O on the SIP.
- 7.8.1.2.2. Turn off the flow cytometer.
- 7.8.1.2.3. Close the FACSDiva software.
- 7.8.1.3. Always check that the SIP tube contains ~ 1 mL of 20% Contrad 70. This will prevent the introduction of air into the fluidics system.

7.8.2. Bi-yearly maintenance

- 7.8.2.1. Remove the sheath filter
- 7.8.2.2. Press the **quick-disconnects** on both sides of the filter assembly.
- 7.8.2.3. Remove the filter assembly.
- 7.8.2.4. Connect the two fluid lines.

Caution: Do not run detergent, bleach, or ethanol through the sheath filter. They can break down the filter paper within the filter body, causing particles to escape into the sheath fluid, possibly clogging the flow cell.

- 7.8.2.5. Empty the sheath container and rinse it with DI H₂O.
- 7.8.2.6. Fill the sheath container with at least 1 Liter of BD FACSClean solution.
- 7.8.2.7. Empty the waste container, if needed.
- 7.8.2.8. Open the roller clamp by the fluidics interconnect and drain the fluid into a beaker for five seconds.
- 7.8.2.9. Remove the DI H₂O tube from the SIP.
- 7.8.2.10. Prime the instrument twice:
 - 7.8.2.10.1. Press the **PRIME** button on the fluidics control panel.
 - 7.8.2.10.2. When the STANDBY button light is amber, press the PRIME button again.
- 7.8.2.11. Install a FACS tube with 3 mL of BD FACSClean solution on the SIP and put the tube support arm underneath the tube.
- 7.8.2.12. Press **RUN and HIGH** on the cytometer fluid control panel. Ensure that the sample fine adjust is set to 250. Run for 30 minutes.
- 7.8.2.13. Press the **STANDBY** fluid control button and depressurize the sheath container by lifting the vent valve.
- 7.8.2.14. Empty the waste tank to avoid the mixing of Bleach and BD Detergent Solution.
- 7.8.2.15. Repeat steps 7.8.2.5 to 7.8.2.14 with 1.5% BD Detergent Solution Concentrate.
- 7.8.2.16. Repeat steps 7.8.2.5 to 7.8.2.14 with DI water.

7.8.2.17. Replace the sheath filter and refill the sheath tank with sheath fluid to the maximum volume indicated by the fill line seen inside the sheath tank.

7.8.2.18. Install a FACS tube with ~ 1 mL of 20% Contrad 70 onto the SIP.

8. REFERENCE DOCUMENTS

8.1. BD FACSDiva™ Software Reference Manual

8.2. Operator Course Workbook for BD FACSCelesta™