Flow Cytometry Analysis Using the Becton Dickinson FACS Calibur

The availability of user-friendly analytical instruments such as the Becton Dickinson (BD) FACS Calibur allows investigators direct access to flow cytometers. Because these instruments are investigator-operated, scheduling is simplified and is not limited to normal working hours, making larger experiments, such as screening of new monoclonal antibodies, more practical. This unit details the operation of a FACS Calibur flow cytometer for cell analysis. The operation of the BD FACS Calibur and accompanying BD CELLQuest software version 3.0 is described, but the unit is general enough to be helpful for users of all flow cytometers. The FACS Calibur replaces both the FACSCan and FACSort. It has the same basic instrument design and continues to utilize the CELLQuest software. Thus, the information presented here is also applicable to older BD instruments.

In this unit, particular emphasis is placed on data acquisition rather than data analysis. Discussions of flow cytometry terminology and analysis of data can be found in *UNITS 5.1 & 5.2*, and other uses of flow cytometry can be found in *UNITS 5.5-5.8*. FACS Calibur and CELLQuest operation protocols are described in increasing order of complexity. Following a brief description of the FACS Calibur/CELLQuest system, single-color analysis using fluorescein isothiocyanate (FITC)-conjugated antibodies is described (see Basic Protocol). Support Protocol 1 then describes how to check instrument performance and sensitivity. Single-color (FITC) analysis with simultaneous live/dead discrimination using propidium iodide (PI) is also described (see Support Protocol 2). Alternate Protocol 1 describes simultaneous two-color analysis using FITC- and phycoerythrin (PE)-conjugated antibodies; this is followed by Support Protocol 3, which outlines two-color FITC/PE analysis with simultaneous live/dead discrimination using PI. Alternate Protocol 3 covers simultaneous three-color immunofluorescence with FITC, PE, and the red fluorescent dyes. Finally, Alternate Protocol 4 covers simultaneous four-color immunofluorescence with FITC, PE, red fluorescence dyes, and APC.

DESCRIPTION OF THE FACS Calibur/CELLQuest SYSTEM

The BD FACS Calibur/CELLQuest system is a dual-component flow cytometer comprised of (1) a wet unit that includes laser excitation source, fluidics, and emission detectors, and (2) a computer with CELLQuest software for data acquisition and analysis. For each cell analyzed, the system allows correlation of any single-parameter measurement (i.e., light scatter or fluorescence intensity) with up to four other parameters, thus permitting the analysis of all cells or only cells with defined characteristics.

The BD FACS Calibur together with the CELLQuest software comprise a system that permits rapid, highly sensitive, multiparameter flow cytometric analysis. As many as five separate characteristics (“parameters”) can be measured simultaneously on each cell that passes through the laser beam (which has a 488-nm excitation wavelength and is generated by a single air-cooled, argon-ion laser). These five parameters are FSC, SSC, FL1, FL2, and FL3. With the optional second laser, the FACS Calibur is capable of measuring an additional fluorescence parameter, FL4. At the intersection of the beam with a stream of cells, laser light is scattered by the cell in all directions, but is measured by detectors in the forward (FSC) and orthogonal, or 90° side scatter (SSC) orientations. Although influenced by other factors, light that is scattered forward correlates with cell size whereas light that is scattered orthogonally correlates with cellular complexity. The FSC and SSC

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detectors are not sensitive to fluorescence and are used to discriminate cells—both unstained and fluorescence-stained—from particulate debris and to measure cell size and granularity. The other four detectors—i.e., FL1, FL2, FL3, and FL4—measure fluorescence-emission wavelength bands ("colors") as determined by optical filters installed in front of the photomultiplier tubes (PMTs).

The fluorochromes that can be used on a FACS Calibur are determined by the excitation wavelength and emission wavelength maxima of the dye, the wavelength of the laser, and the optical filters installed in front of each detector. Table 5.4.1 lists the most commonly used dyes, and the detector that is configured optimally for that dye. The most common combination of fluorochromes comprises FITC (green fluorescence; FL1 detector), PE (orange-red fluorescence; FL2 detector), and PE-Cy5 or PerCP (red fluorescence: FL3 detector). On instruments equipped with the red-diode laser, APC (red fluorescence; FL4 detector) can be used in combination with the above fluorochromes. Electronic circuitry allows the investigator to compensate for partially overlapping emission spectra of different fluorescent dyes.

CELLQuest software operating on the Macintosh computer platform incorporates many features that users of other Macintosh software should be familiar with, such as cut and paste capabilities, pull-down menus, and printing options. Users unfamiliar with Macintosh computers should complete the Macintosh Basics tutorial provided by Apple. It is highly recommended that new users read the CELLQuest Software User's Guide and complete the CELLQuest tutorial located in Chapter 1.

CELLQuest is used for both data acquisition and analysis. When started, CELLQuest opens an untitled window, known as an Experiment Document. The Experiment Document can be saved and recalled for later use to speed setup. In addition, instrument settings can be saved and recalled to facilitate setup for samples that are run on a routine basis.

See Commentary at the end of this unit as well as the overview in *UNIT 5.1* for further details about the underlying theory and operation of flow cytometers. Analysis of data is described in *UNIT 5.2*.

Table 5.4.1 Fluorochromes Used with the FACS Calibur

Fluorochromes[a,b]	Emission peak (nm)	Detector	Filter
Fluorescein isothiocyanate (FITC)	525 (green)	FL1	530/30
R-phycoerythrin (PE)	575 (orange-red)	FL2	585/42
Propidium iodide (PI)	620 (red)	FL2, FL3	
PerCP	675 (red)	FL3	670LP
PE-Cy7[b]	767 (red)	FL3	
PE-Cy5[b]	670 (red)	FL3	
APC	660	FL4	661/16
Cy-5	670	FL4	

[a]Dyes that cannot be used with the FACS Calibur are rhodamine and its derivatives, as these are not excited by the 488-nm argon ion laser. Texas red is very poorly excited at 488 nm and other orange and red fluorescent dyes listed above are much preferred.

[b]PE-Cy5 also known as CyChrome (Pharmingen), TriColor (Caltag), Quantum Red (Sigma), and RED670 (Life Technologies).

SINGLE-COLOR ANALYSIS USING FITC-CONJUGATED ANTIBODIES

In most single-color analyses, fluoresceinated antibodies are used so the FL1 channel detects antibody reactivity. Electronic gates can be set to acquire data from cells with certain characteristics such as a high fluorescence intensity or a particular cell size. Once the instrument is appropriately set, data are acquired, stored, and analyzed by the computer.

Materials

Isotonic saline for FACS Calibur sheath solution reservoir (follow Becton Dickinson's recommendations for source)

Control and test cell populations: 1–2 × 10^6 cells/ml unstained control cells, FITC-stained positive control cells, and FITC-stained test cell samples (*UNIT 5.3*), kept on ice in sample buffer (≥250 µl each)

Sample buffer: 2% (v/v) heat-inactivated FBS *or* 1% (w/v) BSA/0.1% (w/v) sodium azide (either solution prepared in PBS)

10% (v/v) bleach (sodium hypochlorite; Clorox) in deionized water

Flow cytometry system consisting of:
FACS Calibur (Becton Dickinson)
FACStation acquisition interface for Macintosh (Becton Dickinson)
Macintosh Quadra 650 (or higher Quadra series) computer with 16 MB RAM and 1 MB VRAM
Macintosh system software version 7 or higher
Macintosh compatible printer

NOTE: Version 1.0 of CELLQuest does not support PowerMac computers for data acquisition.

Start up FACS Calibur and Macintosh computer

1. Perform the usual FACS Calibur fluidics maintenance and checkout, including filling the sheath-fluid reservoir with isotonic saline and emptying waste. Turn on the power, flush air from the lines, turn fluid control valve to Standby, then turn on computer and printer.

   The FACS Calibur must be powered up before the computer is turned on.

   The user should be familiar with FACS Calibur fluidics, FACS Calibur front panel controls, and the Macintosh computer system.

   Warmup of the FACS Calibur takes ~5 min, after which the FACS Calibur indicator light will change from Not Ready to Standby, indicating that the instrument is ready to accept samples for acquisition and analysis.

2. Perform instrument checkout and sensitivity test using FACSComp software and CaliBRITE beads (see Support Protocol 1).

   Alternatively, fluorescent plastic beads for such testing are available from several different manufacturers. Day-to-day instrument performance is monitored by acquiring and analyzing fluorescent-bead data, using the same amplifier gains with each checkout. Suggested amplifier gains for 4.5-µm yellow-green fluorescent microspheres from Polysciences are given in Table 5.4.2.

3. Exit FACSComp and launch CELLQuest on the Macintosh.

   CELLQuest automatically creates a new Experiment Document named "Untitled-1" each time the program is launched. You may create your own new Experiment Document each time or use Open from the File menu to select an existing Experiment Document. Generic Templates can be created and used again for routine acquisition and analysis by converting an Experiment Document into a Stationary Pad. Refer to the CELLQuest Software User's Guide, Chapter 2, for more details.

Table 5.4.2 Typical Parameter Settings for Two- and Three-Color Analysis on the FACS Calibur[a,b]

Parameter	Fluorescent beads[c]	FITC/PE/PI[d]	FITC/PE/red[d,e]
FSC	E(−1)/log	E(00)/1.53	E(00)/1.53
SSC	log/250	2.90/310 V	2.90/310 V
FL1 (log)	250	626	600
FL2 (log)	250	497	500
FL3 (log)	250	397	590
FL1-FL2	0	0.7	0.5
FL2-FL1	0	24.7	36.5
FL2-FL3	0	0	1.9
FL3-FL2	0	8.9	10.0
Threshold	FL1/52	FSC/52	FSC/52

[a]The given values are merely guidelines and should not be followed precisely in most cases. Settings used on one FACS Calibur may not produce identical results on another FACS Calibur.

[b]Abbreviations: FSC, forward light scattering; SSC, side (90°) light scattering; FL1, fluorescence 1 (530 nm); FL2, fluorescence 2 (585 nm); FL3, fluorescence 3 (650 nm); E(−1), exponential (−1) setting.

[c]4.5-µm-diameter YG Fluoresbrite Microspheres (Polysciences; *APPENDIX 5*). Other beads may require different settings.

[d]Suggested settings are for freshly isolated lymphocytes; cultured cells and long-term cell lines are larger (greater FSC values) and more autofluorescent, requiring a reduction in gain and PMT voltage.

[e]Suggested settings are for TRI-COLOR (Caltag) or CyChrome (Pharmingen). Other red dyes—e.g., RED613 (Life Technologies) and PerCP (Becton Dickinson)—will require different settings (see Table 5.4.1).

4. Expand the Experiment Document to full size by clicking the zoom box in the upper right-hand corner of the window.
5. Choose Dot Plot from the Plots menu. Click and hold the Plot Source box to open the pop-up menu and choose Acquisition. Click OK on the defaults for X and Y parameters (FSC and SSC).
6. Repeat step 5, but choose FL1-H for Y Parameter. Click and drag the frame of the plot to position it away from the other dot plot.
7. Choose Histogram Plot from the Plots menu. Click and hold the Plot Source box to open the pop-up menu and choose Acquisition. Click OK on the default for Parameter (FSC). Click and drag the frame of the plot to position it away from the other plots.
8. Repeat step 7, but choose FL1-H for Parameter. Click and drag the frame of the plot to position it away from the other plots.

   Creating histograms or plots from the Plots menu generates windows of preset size. When histograms or other plots are created using the Tool Palette, the size of the window is determined by clicking and dragging the mouse to obtain the desired size and shape, in which case subsequent windows created using the Plots menu will be of the same size and shape as the original plot window.

9. Choose Connect to Cytometer from the Acquire menu. Choose Detectors/Amps from the Cytometer menu and set the amplifier gain in the Detectors/Amps window that pops up. Choose Threshold from the Cytometer menu and set the FSC threshold in the Threshold window that pops up.

Amplifier gains and FSC threshold may be set approximately either by using Table 5.4.2 as a guide or by restoring the Calib settings file generated by FACSComp (see Support Protocol 1). The Calib setting file is restored by choosing Instrument Settings from the Cytometer menu, opening the Calib file and clicking Set in the Instrument Settings window. The FSC threshold is used to discriminate between cells, small debris, and electronic noise.

Amplifier gains and photomultiplier tube (PMT) voltages can be set from FACS Calibur front-panel controls or from the Detectors/Amps window.

Optimize parameter settings

10. Remove tube of deionized water from FACS Calibur sample-uptake stage and press the Run button. Resuspend unstained control cells by vortexing and place sample tube on FACS Calibur. Make sure that the Setup checkbox is checked and click Acquire in the Acquisition Control window.

    In Setup mode the data are displayed but are not saved in memory or written to disk. This allows for real-time adjustment of gains, compensation, and displays.

11. Increase or decrease FSC gain to position the majority of the cells near the midpoint of the FSC axis as displayed on the histogram. Increase or decrease SSC gain on PMT to position cells near the midpoint of the SSC axis (see Fig. 5.4.1).

12. Use the Polygon Region tool in the Tool Palette to define region 1 on the FSC versus SSC dot plot as that which encloses and defines live cells and excludes dead cells, debris, and red blood cells.

13. Apply region 1 as a gate to the FSC × FL1 dot plots and the FL1 histogram display by selecting the dot plot or histogram (i.e., by clicking on it), and then choosing Format from the Plots menu. In the gate pull down menu, select G1 = R1.

    The region tool can also be used to enclose specific populations of interest defined by FSC and SSC. Figure 5.4.1B shows human PBL with regions defined for lymphocytes, monocytes, and granulocytes.

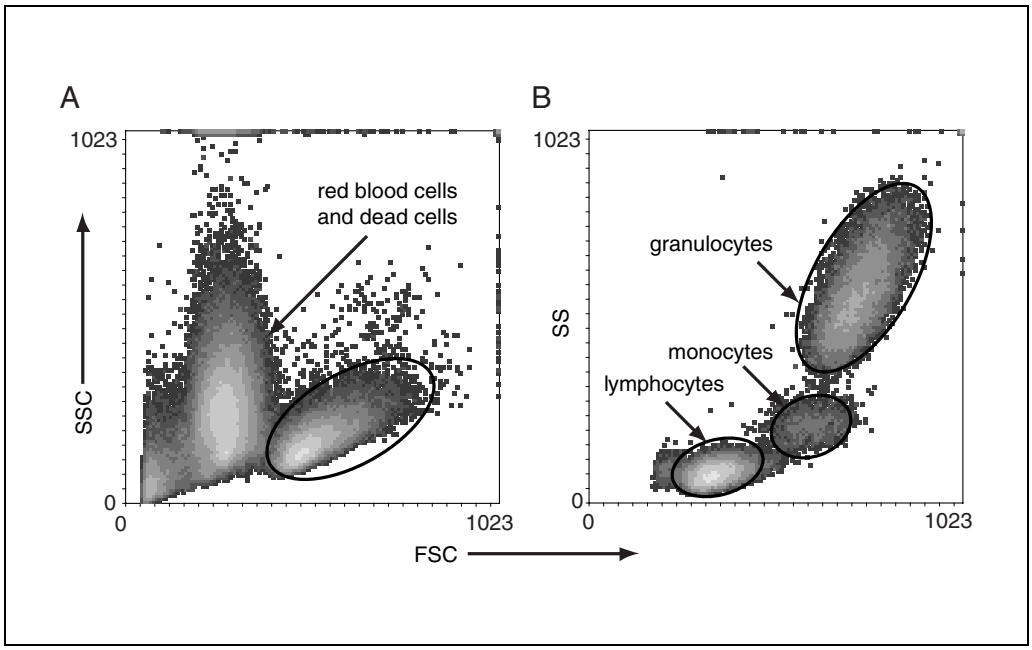

Figure 5.4.1 Dot density plot displays of (**A**) murine spleen cells and (**B**) human peripheral blood leukocytes. (A) Region including leukocytes and excluding red blood cells and dead cells is shown. (B) Regions including lymphocytes, monocytes or granulocytes cell populations.

Defining region 1 on the FSC versus SSC dot plot is especially useful for excluding from acquisition occasional small air bubbles that show spurious light-scattering characteristics. Do not make region 1 so restrictive that it excludes cells of interest. Further "gating" of the data can be done after data acquisition to more rigorously exclude debris and dead cells.

14. Adjust the FL1 PMT voltage so that the majority of unstained cells show between 1 and 10 fluorescence units (U) on the FL1 histogram.

    The modal FL1 value should be ≥1 U. Note that values ≤1 U are automatically set to 1, and values ≥10,000 U are set to 10,000.

    Dead cells and debris often show spurious and heterogenous FL1 levels (Fig. 5.4.1).

Set up CELLQuest for data acquisition and storage

15. Choose Acquisition and Storage from the Acquire menu to activate the Acquisition and Storage window. Do not change the default settings (i.e., select Accept and All) in the Acquisition Gate box.

    This allows all events that are above the FSC threshold to be processed.

    IMPORTANT NOTE: *If a defined gate (e.g., R1 or R2) is selected, then only events within that gate will be seen on histogram displays or stored in the data files.*

16. In the Collection Criteria box, define the number of cells to collect (of all events or events within a defined gate). In the Storage Gate box, define whether All events or events meeting a defined gate are to be stored in the data file. Finally, in the Resolution box, select either 1024 or 256 for the resolution.

    Generally, data are better represented by 1024 resolution, but if storage space is a limiting factor, 256 resolution can be used for immunofluorescence applications. Also, disk space may be saved by deselecting parameters that are not being used in the Parameters Saved box.

17. Choose Parameter Description from the Acquire menu to activate the Parameter Description window. Choose the Folder button and use the Destination Folder pop-up menu that appears to select a folder on the hard disk where the data will be saved. Click the Select button at the bottom of the Destination Folder pop-up menu dialog box.

18. Click the File button in the Parameter Description window to activate the File Name Editor window. In the Custom Prefix field of the File Name Editor window, enter a name for the data file. Click OK to accept choices.

    If the default File Count is accepted in the File Name Suffix pull-down menu, CELLQuest will attach a .001 suffix to the Custom Prefix and automatically increment each file when it is saved as .002, .003, etc.

19. Place the first test cell sample on the FACS Calibur uptake stage. In the Acquisition Control window, deselect the Setup checkbox and click on Acquire.

20. Remove the sample when counting is complete. Replace with a new sample and repeat step 19, proceeding in this manner with all test cell samples.

    If cell density in samples is low, briefly run deionized water or PBS through the FACS Calibur for 10 to 20 sec between samples to flush out the previous sample.

21. When sample acquisition and data storage are complete, place a tube containing 2 to 3 ml of 10% bleach on the sample-uptake stage and run 5 min at HI flow rate. Replace with a tube of 2 to 3 ml deionized water and run 5 min at HI flow rate.

22. Turn fluid-control valve to Standby. Turn FACS Calibur off, then place a tube containing 1 ml deionized water on the uptake stage.
23. Select Quit from the File menu.

    At this point, the Experiment Document may be saved for future use by clicking Save; if this is not desired, click Don't Save to exit the program.

INSTRUMENT CHECKOUT USING CALIBRITE BEADS AND FACSComp SOFTWARE

SUPPORT PROTOCOL 1

Standard fluorescent beads are used to check performance and alignment of the FACS Calibur. The following protocol describes the use of CaliBRITE beads and the accompanying FACSComp software (both from Becton Dickinson); alternatively, other brands of standard fluorescent beads can be used in conjunction with the CELLQuest software.

A complete description of FACSComp is found in the FACS Calibur FACSComp Software User's Guide. FACSComp is an interactive program that performs three functions: automatic adjustment of PMT voltages, automatic adjustment of FL1 and FL2 electronic compensation, and determination of instrument sensitivity with regard to FSC, SSC, FL1, and FL2 parameters.

Additional Materials *(also see Basic Protocol)*

CaliBRITE standard fluorescent beads (Becton Dickinson)
FACSComp software program (Becton Dickinson)

1. Prepare one tube of unlabeled beads and one tube of mixed (unlabeled + FITC- + PE-labeled) beads as described in Chapter 3 of the FACSComp Software User's Guide.

   Diluted beads can be stored 3 to 4 days in the dark, refrigerated, and free of microorganisms. If one or more of the FACSComp sensitivity tests fails, fresh bead samples must be prepared and reanalyzed. If the newly prepared beads also fail, BD should be contacted.
2. Launch FACSComp on the Macintosh. Enter the appropriate information in the Sign Up display and click Accept.
3. Enter the lot numbers of the unlabeled FITC- and PE-labeled CaliBRITE beads in the Set Up display. Click on Accept.
4. Insert the sample of unlabeled beads. Push the Run button.
5. Select the Start button, located at the bottom of the FACSComp window, to initiate automatic adjustment of photomultiplier tubes.

   When test is complete the message PMTs Set Successfully is displayed.
6. Remove sample of unlabeled beads and replace with three-bead mixture.
7. Select Comp from the main menu or Next from the menu at the bottom for automatic fluorescence compensation adjustment.

   When adjustment is complete the message Compensation Set Successfully is displayed.
8. Select the Sens button from the main menu across the top of the screen or Next from the menu in the window at the bottom. Turn on and off FACS Calibur test pulses on and off when prompted by FACSComp.

   Sensitivity results will be shown and will print automatically upon quitting FACSComp if the option to do this was saved in the Preferences menu. The printout should be archived for future comparisons.

   An instrument-settings file containing only the current FACSComp settings is updated after the sensitivity tests. The file is called Calib, and is stored in the BD Preferences folder, located in the Preferences folder in the Systems folder.

DISCRIMINATION OF LIVE/DEAD CELLS IN SINGLE-COLOR ANALYSIS

Dead cells frequently fluoresce nonspecifically as a result of the uptake of fluoresceinated antibodies, thereby creating false positives. This protocol takes advantage of the FL2 detector to recognize dead cells that incorporate the red fluorochrome propidium iodide (PI) into cellular DNA. Electronic "gating" allows the user to exclude from consideration FL2-positive dead cells that may stain nonspecifically with FITC-conjugated antibodies, hence effectively limiting FL1 analysis to viable cells. Because of the intensity of staining and the broad PI emission spectrum, some PI-positive cells will appear to be FL1-positive. More significantly, cells that are brightly stained by FITC-conjugated antibodies can appear as FL2-positive unless electronic compensation is applied. This protocol describes the basic use of FL1-FL2 electronic compensation; however, the principles described apply to any combination of dyes whose emission spectra partially overlap. Red fluorescence of PI-stained dead cells can also be measured with the FL3 detector (see Support Protocol 3).

Additional Materials *(also see Basic Protocol)*

100 µg/ml propidium iodide (PI) in PBS: store at 4°C protected from light

CAUTION: PI is a potential carcinogen and mutagen and should be handled with extreme care.

1. Set up FACS Calibur and optimize settings (see Basic Protocol, steps 1 to 13).
2. After step 13 of the Basic Protocol, while in Setup mode of acquisition, run unstained control cells. Adjust FL1 and FL2 PMT voltages to place the majority of events in the lower-left corner of the FL1 versus FL2 dot plot (Fig. 5.4.2A).

   Screen displays in Setup should be (a) FSC versus SSC dot plot, (b) FL1 versus FL2 dot plot, (c) FL1 histogram, and (d) FL2 histogram. Set up displays using Dot Plot from the Plots menu (see Basic Protocol, step 5).

3. Choose Compensation from the Cytometer menu. Run FITC-stained positive control cells. Increase the FL2-%FL1 compensation so that the FL1-positive cells have the same low background level of FL2 signals as the unstained control (Fig. 5.4.2B).

   To properly adjust the compensation, it is useful to run a sample that contains both FL1-negative and FL1-positive cells. If the sample does not contain negative and positive cells, mix small aliquots of unstained control cells and FITC-positive control cells. An FL2-%FL1 compensation of 5% to 30% is typically required.

4. Add 5 to 10 µl of 100 µg/ml PI per milliliter unstained control cells, then run sample (a minority population of FL2-positive cells should be detectable).

   Many but not all PI-positive dead cells are excluded from region 1 (defined as live cells on the FSC versus SSC dot plot). To visualize all PI-positive cells, make sure the dot plot is not gated by selecting the plot and choosing Format Dot Plot from the Plots menu. Check to see that No Gate appears in the Gate pull-down option. Change the Gate selection back to region 1.

5. Using the Polygon Region tool in the Tool Palette, define region 2 to include the PI-negative live cells on the FL2 histogram or the FL1 versus FL2 dot plot (Fig. 5.4.2D).
6. Select Gate List from the Gates menu. Define gate 3 (G3) to be R1 and R2.

   To pass through gate 3 a cell must lie within region 1 (FSC versus SSC) and within region 2 (FL2-negative live cells that exclude PI).

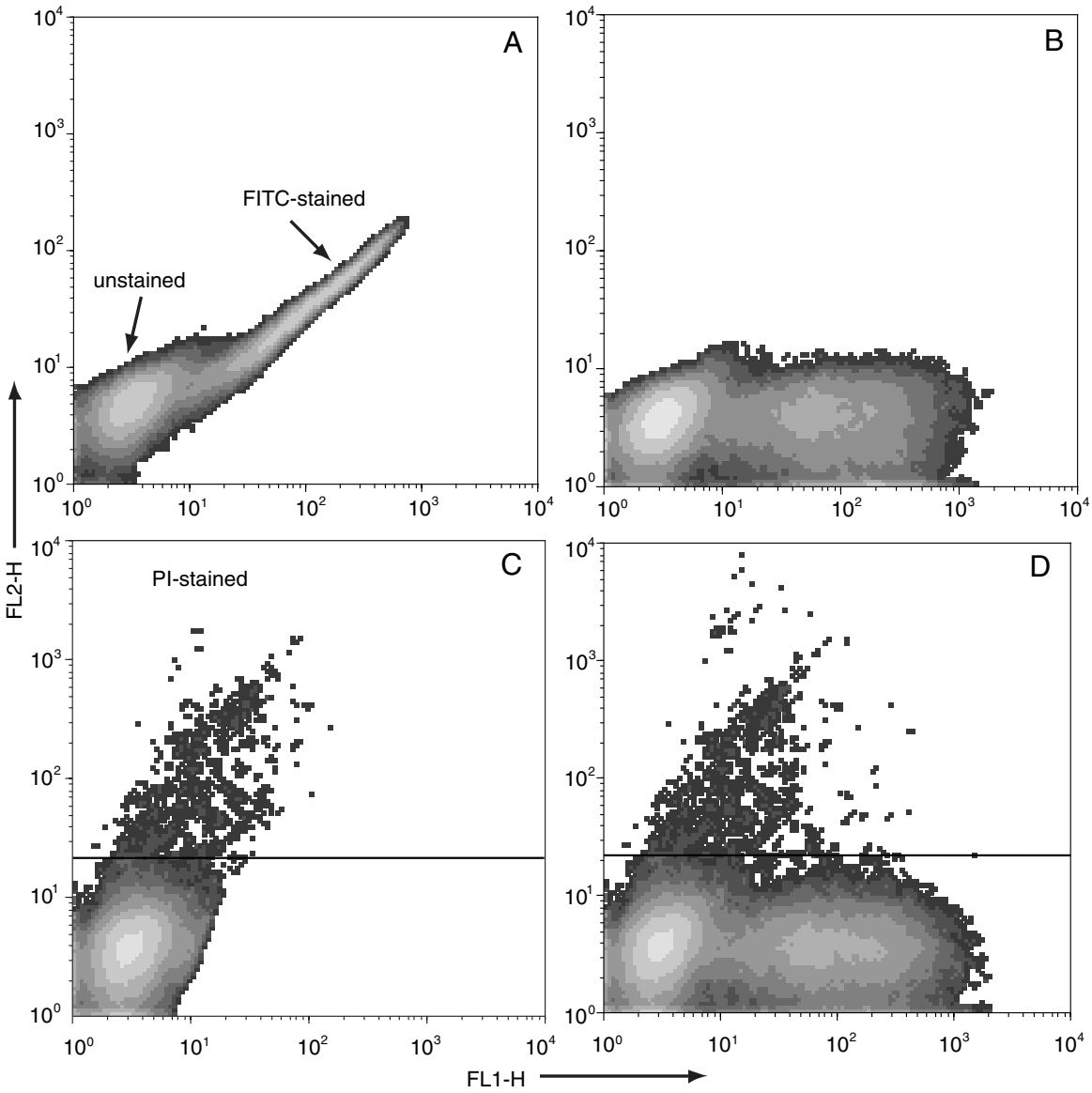

Figure 5.4.2 Two-color density-plot displays and setting of FITC/PI compensation. (**A**) Unstained and FITC-stained cells without electronic fluorescence compensation. (**B**) Same cells after the FL2 – %FL1 compensation has been set. (**C**) Unstained cells incubated with PI. Live cells appear below the horizontal line, while dead cells appear above. (**D**) FITC-stained cells and unstained cells in the presence of PI. Live cells appear below the horizontal line, while dead cells that are both $FITC^+$ and $FITC^-$ appear above; therefore, a region defined below the horizontal line includes live cells and excludes dead ones.

7. From the Acquire menu, choose Acquisition and Storage and define Collection Criteria and Storage Gate to be gate 3 (i.e., G3 = R1 and R2).

   Only events that fall within G3 (live cells, PI-negative) are counted and written to the disk-storage file; however, the live displays will show all events (gated and ungated) unless the Gate box is changed to G3 for each display or if Accept G3 is selected in the Acquisition Gate pull-down menu in the Acquisition and Storage window.

8. Acquire and store data for each sample (see Basic Protocol, steps 15 to 23).

   Acquire ≥10,000 events per sample for statistical significance.

   PI is toxic to some cells and may be taken up slowly even by live cells; therefore, unless it has been predetermined that cells can remain in PI solution for long periods of time, it is best to add PI to each sample immediately before acquiring data for that sample.

   To reduce data-file size, FL2 can be turned Off in the Parameters Saved box, but will still be used to discriminate between live and dead cells. Alternatively, all cells can be acquired regardless of PI staining by defining Storage Gate to be R1. In this case, live/dead discrimination can be done during post-acquisition analysis; however, the number of PI-negative events will vary from one sample to the next.

ALTERNATE PROTOCOL 1

TWO-COLOR ANALYSIS USING FITC- AND PE-CONJUGATED ANTIBODIES

The basic FACS Calibur is equipped with standard filters to detect emitted light simultaneously at 530 nm (FL1, FITC) and at 585 nm (FL2, PE) as each cell passes the excitation beam. Thus, cells can be identified that express determinants recognized by an antibody selectively conjugated with FITC, an antibody labeled with PE, both of these reagents, or neither. Because a small amount of FITC fluorescence is collected by the FL2 detector and a small amount of PE fluorescence is collected by the FL1 detector, electronic subtraction (compensation) of signals must be applied to avoid false results. As described below, the user adjusts the compensation circuitry with the aid of control samples stained with FITC- or PE-conjugated antibody. Because use of PE precludes determination of PI-stained (dead) cells with the FL2 channel, FL3 must be used for simultaneous analysis of live versus dead cells. The use of PI (FL3) for discrimination of live and dead cells in two-color analysis is described in the accompanying Support Protocol 3.

Additional Materials *(also see Basic Protocol)*

Control and test cell populations: 1–2 × 10^6 cells/ml unstained control cells, FITC- and PE-stained positive control cells, and test cell samples stained with FITC and/or PE-conjugated antibodies (*UNIT 5.3*), kept on ice in sample buffer (≥250 µl each; see Basic Protocol)

1. Adjust detector gains and voltages according to the guidelines in Table 5.4.2.
2. Start up CELLQuest software and optimize parameters (see Basic Protocol, steps 1 to 13). Create an FL1 versus FL2 dot plot (see Basic Protocol, step 5).
3. Place unstained control cells on uptake stage of FACS Calibur. Adjust FL1 and FL2 PMT voltages so that unstained cells appear in the lower-left corner of the histogram (Fig. 5.4.3A).
4. Run FITC-stained positive control cells and observe the FL1 versus FL2 dot plot. Adjust FL2-%FL1 compensation so that FL1-bright and FL1-negative cells have the same low level of FL2 (Fig. 5.4.3B).

   The FL2 median of the FL1-bright population should align with the FL2 median of the negative population.

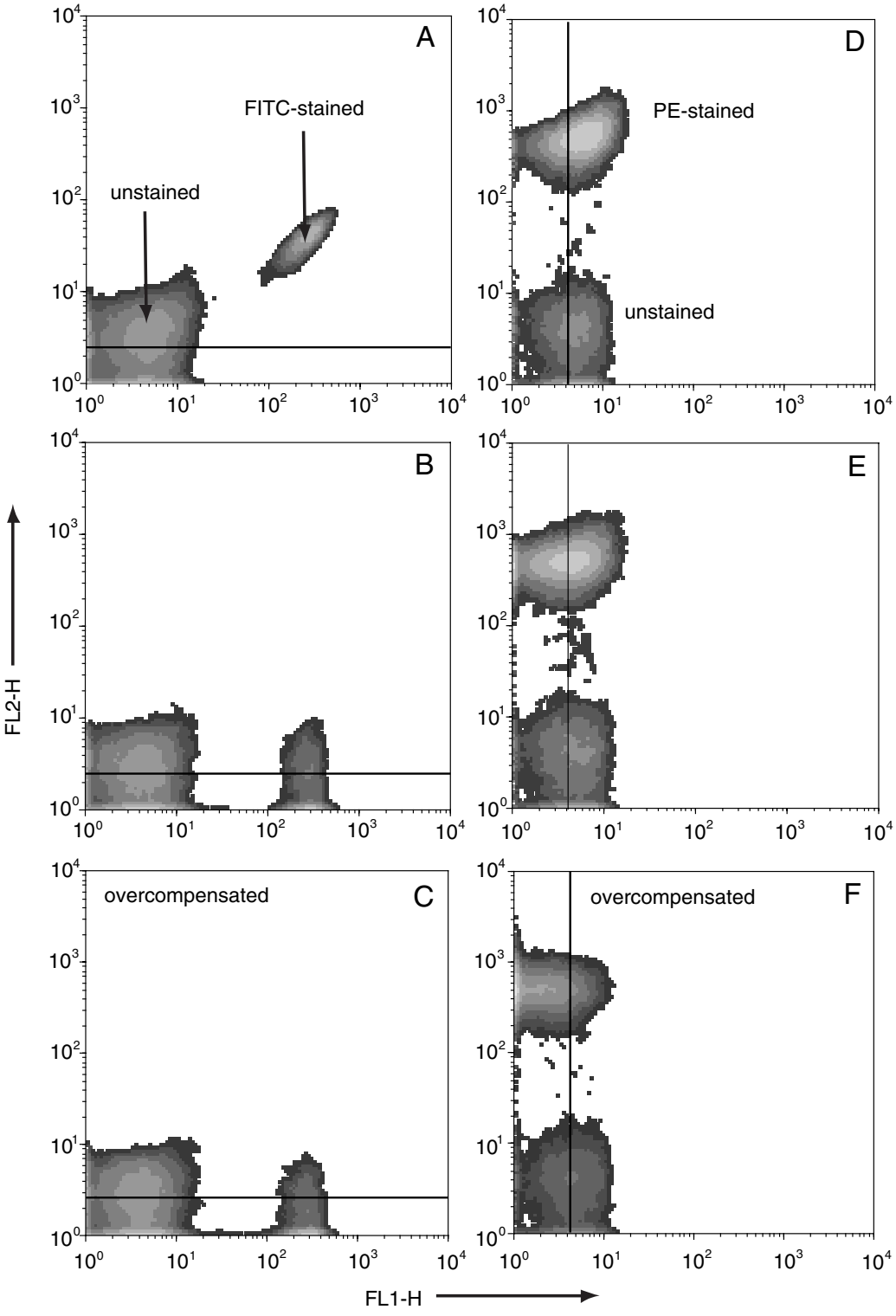

Figure 5.4.3 FL1 versus FL2 density plot displays used to optimize fluorescence compensation for the analysis of double- (i.e., FITC/PE-) stained cells. (**A**) Unstained and FITC-stained cells without compensation. (**B**) Same cells as (A) after FL2 – %FL1 compensation. (**C**) Same cells as (B) with overcompensation. (**D**) Unstained and PE-stained cells without compensation. (**E**) Same cells as (D) after FL1 – %FL2 compensation. (**F**) Same cells as (E) with overcompensation.

5. Run PE-stained positive control cells and observe the FL1 versus FL2 dot plot (Fig. 5.4.3D). Adjust FL1-%FL2 compensation so that FL2-bright and FL2-negative cells have the same low level of FL1 (Fig. 5.4.3E).

   The FL1 median of the FL2-bright population should align with the FL2-median of the negative population.

6. Run a mixture of unstained cells, FITC-stained positive control cells, and PE-stained positive control cells. Make fine adjustments to compensation if necessary.

   Do not change FL1 and FL2 PMT voltages after final adjustment to compensation circuitry. Any voltage changes require resetting compensation values. Do not overcompensate (Fig. 5.4.3C and F). With highly autofluorescent cells or very brightly stained cells it may be invalid to assume that fluorescence emission spectra are the same for all cells. Hence, it may be difficult to obtain perfect compensation.

7. Run and acquire data for control and test samples as described for single-color analysis (see Basic Protocol, steps 15 to 23).

   Acquire ≥ 30,000 events for statistical significance. Be sure that FL1-H and FL2-H are checked in the Parameters Saved box (Acquisition and Storage window).

SUPPORT PROTOCOL 3

DISCRIMINATION OF LIVE/DEAD CELLS IN TWO-COLOR ANALYSIS

The availability of the additional PMT (FL3), can be used to detect PI-stained cells in a channel other than the channel in which PE is read. This procedure uses the two-color analysis methodology of Alternate Protocol 1, but employs PI staining to detect dead cells.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

100 µg/ml propidium iodide (PI) in PBS: store at 4°C protected from light

CAUTION: PI is a potential carcinogen and mutagen; handle with extreme care.

1. Adjust FL1 and FL2 voltages and compensation for analysis of FITC- and PE-stained cells (see Alternate Protocol 1, steps 1 to 5).
2. Set up a display as an FL2 versus FL3 dot plot. Set FL3 voltage so that unstained cells apear in the lower left corner of the dot plot.
3. Run unstained control cells, then PE-stained positive control cells. Adjust the FL3-%FL2 compensation so that both populations have the same low level of FL3 signal.

   The FL3 median of the PE positive populations should align with the FL3 median of the negative population.

4. Add 5 to 10 µl of 100 µg/ml PI solution per milliliter unstained control cells. On the FL2 versus FL3 dot display, define region 2 (rectangular) around the PI-negative (live) cell population.

   It is not necessary to adjust the FL2-%FL3 compensation because PI-positive cells will be excluded by gating.

5. In the Gate List window, define G3 (gate 3) as R1 and R2.

   This defines G3 to include cells (R1, as defined on the FSC versus SSC dot plot) that are live (R2, FL3/PI-negative).

6. From the Acquire menu, choose Acquisition and Storage and define Collection Criteria and Storage Gate to be gate 3 (G3 = R1 and R2).
7. Acquire and store data for each sample (see Basic Protocol, steps 15 to 23).

Acquire 30,000 to 100,000 events for statistical significance. Be sure that both FL1 and FL2 are checked in the Parameters Saved box (Acquisition and Storage window). Note that FL3 can be deselected in the Parameters Saved box but will still be used to discriminate between live and dead cells. Alternatively, data can be acquired for all cells regardless of PI staining (check FL3 in the Parameters Saved box) and live/dead discrimination can be performed during analysis; however, the number of PI-negative events will vary from one sample to the next.

PI is toxic to some cells and may be taken up slowly even by live cells. Therefore, unless it has been predetermined that cells can remain in PI solution for long periods of time, it is best to add PI to each sample immediately prior to acquiring data for that sample.

THREE-COLOR ANALYSIS

ALTERNATE PROTOCOL 2

Three-color analysis allows for simultaneous detection of three cell markers on a single cell. Usually, two of the staining reagents are conjugated to FITC or PE whereas the third reagent fluoresces in the red or far-red spectrum. Heterogeneous cell populations can be examined for reactivity with three separate reagents, each individually tagged with a different fluorochrome. This permits discrimination of cells reacting with one, two, or all three of the reagents. Because rare subpopulations can be detected, statistical significance may necessitate acquisition of many events (100,000 to 500,000), which requires large amounts of disk storage space. Three-color analysis precludes the use of PI for live/dead discrimination; it is therefore critical that stained cell populations be of high viability. Isotype-matched, nonstaining "negative control" antibodies can be used to monitor nonspecific staining.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Red-stained positive control cells (see commercially available red fluorchromes in Table 5.4.1)

Test cell samples stained with one to three fluorochrome-conjugated antibody combinations (FITC, PE, red)

1. Discriminate live cells from dead cells and debris using region 1 (FSC versus SSC dot plot).
2. Perform two-color analysis to adjust FL1 and FL2 voltages and compensation for analysis of FITC- and PE-stained cells (see Alternate Protocol 1, steps 1 to 5).
3. Set up a display as an FL2 versus FL3 dot plot.
4. Set the FL3 PMT voltage so that unstained cells appear in the lower left corner of the dot plot.
5. Run unstained, then PE-stained control cells. Adjust the FL3-%FL2 compensation so that unstained and PE-stained cells have the same (background) level of FL3.
6. Run unstained, then red-stained control cells. Adjust the FL2-%FL3 compensation so that unstained and red-stained cells have the same (background) level of FL2.
7. Run a mixed sample containing unstained control cells and FITC-, PE-, and red-stained control cells. By observing the live displays and using Figure 5.4.3 as a guide, verify that compensation has been set properly for each dye pair (FITC/PE, PE/red). Alter electronic compensation if necessary.
8. Run and acquire data for control and test samples as described for single-color analysis (see Basic Protocol, steps 15 to 23).

   Acquire a minimum of 50,000 events for statistical significance. Collecting 100,000 to 500,000 events gives better statistical certainty for rare subpopulations; however, acquisition time constraints, disk storage capacity, and computer memory may preclude the creation of such large data files.

ALTERNATE PROTOCOL 3

FOUR-COLOR ANALYSIS

The FACS Calibur can be purchased with an optional red-diode laser (635 nm) plus FL4 detector that enables measurement of up to four fluorochromes simultaneously. The diode laser beam is focused on the sample core stream so that the cells are illuminated first by the diode laser, followed 15 to 20 μsec later by the argon laser. This spatial separation is maintained within the optical pathway and enables the detection of dyes that share similar emission spectra but different excitation spectra; however, because of the similarity of filters installed in front of FL3 and FL4 detectors, any excitation of fluorochromes by the laser not intended as the primary excitation source causes inappropriate emission within the FL3 and FL4 detectors. This is rectified through the use of interlaser compensation circuitry, which is necessary when dyes are excitable by both lasers—i.e., if the dyes have either a broad excitation spectrum (e.g., APC) or have separate excitation spectra for each component of a tandem conjugate dye (e.g., PE and Cy5 of PE-Cy5). Interlaser compensation is accomplished using an adjustable electronic time delay module to exactly match the pulses generated by the red laser with those generated by the argon. The adjustment of the time delay is accomplished using software control within CellQuest or FACSComp and needs to be performed whenever the FL4 detector is used.

Additional Materials *(also see Basic Protocol and Alternate Protocol 2)*

APC-labeled beads (Becton Dickinson)
APC (or Cy-5) -stained positive control cells and PE-Cy5 (or PerCP) -stained positive control cells
Test cell samples stained with one to four fluorochrome-conjugated antibody combinations (i.e., FITC, PE, PE-CY5 and/or APC)

Carry out time delay calibration procedure

1. Launch CellQuest, connect to cytometer, and open the document template entitled Time Delay Calibration.

   This template contains two histograms with statistics markers and instructions for performing time delay calibration.

2. Turn on the red laser by clicking on the Four Color selection box at the bottom of the Detectors/Amps window.
3. Select Log Mode on FL4 and make sure that all compensations settings are set to zero.
4. Make sure FSC is selected as the threshold parameter in the Threshold window and set the threshold to a value of 200.
5. Put on a tube containing APC-labeled beads and select the Run button.
6. Adjust the FSC amplifier gain until the mean of the peak on the FSC histogram is at channel 400 ± 5.
7. Adjust the FL4 PMT until the mean of the peak on the FL4 histogram is at the channel corresponding to the target channel listed on the APC beads package insert.
8. Press the High sample pressure button, and select Time Delay Calibration from the Cytometer menu. Click on Calibrate, and wait for the procedure to finish.

   Successful calibration is indicated by an audible tone.

Perform four-color analysis

9. Perform Time Delay Calibration.

10. Discriminate live cells from dead cells and debris using region 1 (FSC versus SSC dot plot; see Basic Protocol, step 5). Apply region 1 as a gate as outlined (see Basic Protocol, steps 12 and 13).

11. Perform three-color analysis to adjust FL1, FL2, and FL3 voltages and compensation for analysis of FITC-, PE-, and red-stained cells (see Alternate Protocol 1, steps 1 to 5, followed by Alternate Protocol 2, step 3).

12. Set up the display as an FL3 versus FL4 dot plot.

13. Set the FL4 PMT voltage so that unstained cells appear in the lower left corner of the histogram.

14. Run red-stained control cells. Adjust the FL4 – %FL3 compensation so that unstained and red-stained cells have the same (i.e., background) level of FL4.

15. Run APC-stained control cells. Adjust the FL3 – %FL4 compensation so that unstained and APC-stained cells have the same (i.e., background) level of FL3.

16. Run a mixed sample containing unstained and FITC-, PE-, red-, and APC-stained control cells. By observing the live displays and using Fig. 5.4.3 as a guide, verify that compensation has been set properly for each dye pair (i.e., FITC/PE, PE/red, and red/APC). Alter electronic compensation if necessary.

17. Run and acquire data for control and test samples as described for single-color analysis (see Basic Protocol, steps 15 to 23).

COMMENTARY

Background Information

Flow cytometry can be used to detect molecules expressed by cells. Other applications—e.g., detection of Ca^{+2} flux (*UNIT 5.5*), measurement of intercellular conjugates (*UNIT 5.6*), or DNA analysis (*UNIT 5.7*)—are beyond the scope of this discussion. Flow cytometry can be preferable to other methods used to analyze antigen expression, such as immunoprecipitation (*UNIT 8.3*) or radioimmunoassay (Cooper and Paterson, 1993), because individual cells can be analyzed and no radioactivity is involved. Cells can be analyzed simultaneously for several different characteristics or parameters, such as size or the expression of another cell membrane determinant. Moreover, the method is semiquantitative, fast (10,000 cells can be analyzed in less than a minute), reliable, and reproducible.

Antibodies, particularly monoclonal antibodies, are frequently used in flow cytometry because of their specificity and ease of preparation, but other reagents specific for the molecules of interest can be used. The antibodies are covalently conjugated to a fluorochrome such as FITC and excess free fluorochrome is discarded (*UNIT 5.3*). The FITC-labeled antibody is then incubated with the cells of interest, and the cells are washed to get rid of excess unbound antibody.

When the stained cells are placed on the instrument, a stream of cells is directed through an argon laser beam that excites the fluorochrome to emit light. This emitted light is detected by a photomultiplier tube (PMT) specific for the emission wavelength of the fluorochrome by virtue of a set of optical filters. The signal detected by the PMT is amplified (logarithmically or linearly) in its own channel and displayed by a computer in a variety of different forms—e.g., a histogram, dot display, or contour display. Thus, fluorescent cells express a molecule that is reactive with the fluorochrome-labeled reagent, whereas nonfluorescent cells do not express this molecule. The flow cytometer is also semiquantitative in that it displays the amount of fluorescence (fluorescence intensity) expressed by the cell. This correlates, in a relative sense, to the number of the molecules expressed by the cell.

Flow cytometers are equipped to measure nonfluorescent parameters such as cell volume or light scattered by the cell as it passes through the excitation beam. Cell volume is usually a direct measurement. The light-scatter PMTs detect light at the excitation wavelength, scattered by the cell either in a forward angle

(forward scatter; FSC) or at a right angle (side scatter; SSC). FSC is usually an index of size whereas SSC is an index of cellular complexity; however, both parameters can be influenced by other factors.

Many flow cytometers are equipped with more than one PMT emission detector. The additional PMT may detect another emission wavelength, allowing simultaneous detection of more than one fluorochrome, each in individual separate channels. Computers allow the analysis of each channel or the correlation of each parameter with another (see *UNIT 5.2*).

The Becton Dickinson FACS Calibur is an air-cooled argon-laser-based flow cytometer that measures five parameters (FSC, SSC, FL1, FL2, and FL3) on each cell as it passes through the 488-nm excitation beam. The FL1 channel detects light emitted at 530 nm (FITC), the FL2 channel detects light at 585 nm (PE or PI), and the FL3 channel detects light at >650 nm (PI or other dyes; see Table 5.4.1). With the red laser option, an additional detector (FL4) is added which detects light with emission of 661 nm. The instrument's major advantages are that it is easy to operate, compact, and requires no special plumbing or electrical connections.

Principles of operation

The FACS Calibur consists of two basic parts: (1) the cytometer containing the light source, fluidics system, and light detectors; and (2) the computer. FACS Calibur setup requires the computer to be on-line before any data is displayed. The computer acquires the data and with the menu-driven software, produces histogram or two-parameter displays (dot plot or contour plot) of the light scattered by the cells or of their fluorescent emissions. Statistics, such as mean fluorescence intensity and percent positive cells, may also be derived (*UNIT 5.2*; Shapiro, 1995). Discussion of statistical analysis is beyond the scope of this unit.

To analyze samples on the FACS Calibur or any other flow cytometer, the nonfluorescent parameters of light scatter or volume are adjusted so that the instrument detects only the events having the scatter or volume characteristics of cells. Once determined, scatter settings for a particular type of cell often will be appropriate for similar cells, although slight adjustments may be necessary. Volume determinations based solely on scatter parameters are inaccurate, as these parameters are also influenced by other factors—e.g., cell granularity, surface texture, and refractive index—which may vary between samples.

Once the scatter is appropriately adjusted to detect only the events (cells) of interest, the fluorescence channels are adjusted so that positive and negative cells can be discriminated. This latter adjustment is done so that unstained cells form a negative peak near the origin for either of the fluorescence channels. It is usually preferable to show the entire negative peak on the histogram or contour map, because a more accurate mean fluorescence intensity can be calculated for negative cells. If some cells fall below the lowest value on the histogram, the program assigns this value to the cells, leading to a falsely high mean fluorescence intensity.

Fluorescence of the unstained sample (i.e., background fluorescence) is due to several factors, including electronic and optical noise and intrinsic autofluorescence (caused by fluorescence of internal compounds such as riboflavin). The total background fluorescence signal of a cell increases, together with the fluorescence of the positive cells, as the voltage of the PMT for that fluorescent parameter increases. Although small increases in PMT voltage do not affect the fluorescence in the other channels, larger increases will significantly affect the degree of compensation required to eliminate the overlap of fluorescence signals that may be seen with particularly bright positive samples. Thus, alterations in PMT voltages should be coupled with resetting of compensation.

Although only unstained and positive control samples (FITC and/or PE) are required to prepare the instrument for data acquisition, other controls may be necessary for correct interpretation of a typical flow cytometry experiment. Additional controls include the second-step fluorochrome-conjugated reagent alone and cells stained with an isotype-matched irrelevant antibody. The specificity of the antibody preparations should be verified by independent means such as staining known positive and negative tissues or cell lines (*UNIT 5.3*), immunoprecipitation (*UNIT 8.3*), or antibody-blocking experiments (*UNIT 5.3*).

Excluding nonviable cells

Nonviable cells can be read as false positives because their permeable membranes allow the passage of the fluorescent reagents, which become trapped intracellularly, causing nonspecific fluorescence. Although only highly viable cell suspensions should be used in flow cytometry experiments, the staining procedure itself generates a few dead cells. Thus, optimal analysis, especially of rare subpopulations, requires the exclusion of nonviable cells.

The fluorescent polar tracer propidium iodide (PI) is used to identify nonviable cells. It is normally excluded from intact cells, but is able to pass through the permeable membrane of a dead cell and bind to nucleic acids. It is excited by the 488-nm light source and fluoresces brightly in the red spectrum. Thus, bright red cells are readily detectable and can be excluded from analysis by setting a gate that does not include any cells that are stained bright red. Appropriate electronic compensation is required so that bright FITC-stained viable cells are not excluded from analysis.

Setting gates

The FACS Calibur permits three different methods for selecting (gating) the population of cells (events) to be analyzed: (1) small debris can be eliminated from acquisition by using the scatter threshold, (2) data-acquisition gates can be set to allow only data for cells that satisfy the gating parameters to be acquired by the computer, and (3) data-analysis gates can be placed on the data during analysis. Of these options, the first two are performed during data acquisition and storage; these are preferred because the same number of events will be acquired for each sample. Gating the data after acquisition will result in a different number of events analyzed for each sample, but will allow the option of data manipulation long after it has been acquired. The data must be stored in list mode (all parameters correlated) if data-analysis gates are used. Storage of list data for each sample requires large amounts of memory.

The optimal threshold setting is best determined in accordance with the sample composition. Because red blood cells (RBCs) and dead cells scatter less light than viable lymphocytes, a sample containing all of these cell types should have two populations discernable by FSC—a low FSC population (RBCs and dead cells) and a high FSC population (viable lymphocytes). Adjusting the FSC threshold to a low value of 52 permits all populations to be visible but exclude debris. Changes in the FSC versus SSC pattern during collection may indicate partial obstruction of the flow cell. Setting a high threshold may mask these changes; however, if a high cells per second rate is desired, it may be necessary to raise the threshold on FSC to exclude RBCs and doublets.

Multicolor analysis

Several different fluorochromes (fluorescent dyes) with different excitation and emission spectra can be coupled covalently to antibodies or other ligands (e.g., cytokines, hormones, or soluble isoforms of cell-adhesion molecules). A number of these fluorochromes are efficiently excited by the 488-nm light produced by the argon-ion laser of the FACS Calibur; however, some of these fluorochromes have sufficiently different emission spectra (see Table 5.4.1) that spectrally selective bandpass optical filters can be used to select or reject a significant portion of the emission spectrum of a given fluorochrome. The basic FACS Calibur contains three separate fluorescence detectors (FL1, FL2, FL3), each of which consists of a set of bandpass filters along with a PMT that functions as the detector proper. Because fluorescence emission occurs over a broad range of wavelengths, the spectrally selective optical filters of the FACS Calibur are not sufficient to discriminate fluorochromes; therefore adjustable electronic-signal subtraction "compensation" circuitry is used to eliminate residual spectral overlap between fluorochromes.

The FACS Calibur with the second (i.e., 635 nm) laser option contains an additional detector (FL4) for dyes that have a higher red excitation. Electronic compensation circuitry and time delay calibration circuitry is necessary to correct for excitation of dyes by the inappropriate laser.

Quantitation of fluorescence and volume

Measurement of the fluorescence intensity of cells provides semiquantitative data on the number of molecules expressed by the cells that are reactive with the fluorescent reagents. The rules governing typical ligand-receptor binding assays cannot be applied because it is impractical to perform the analysis under equilibrium conditions. Significant time elapses between the analysis of the first sample and the last (even between the first and last cells of a given sample), and the free unbound ligand cannot be directly measured in the same way as the bound ligand. Thus, a precise measurement of the number of molecules expressed by the cells cannot be made. Nevertheless, a reasonable estimate of the number of molecules of a given antigen can be obtained.

If the fluorescence signals have been logarithmically amplified, then the histograms and dot plots are displayed with logarithmic axes. Each axis is divided into four equal intervals or "decades." It should be noted that the axis values and the mean values determined by the CELLQuest statistical subroutines are not necessarily accurate. Not all logarithmic amplifiers (log amps) have the same gain characteristics, and the division of the fluorescence axes into

four decades is true only for the average log amp. A quick way to determine the characteristics of a given fluorescence log amp is to analyze fluorescence bead standards. If the modal/mean values for single beads and "doublets" are not different by a factor of two, then the amplifier may not be precisely a four-decade log amp. In addition, log amp gain characteristics may vary with input signal level; hence, the true scale may not be linear across the entire axis. For these reasons, calculated fluorescence values should be taken as approximate.

Critical Parameters

Routine maintenance

The FACS Calibur is very reliable and can be used daily for months to years without significant maintenance. Routine maintenance includes discarding fluid waste and refilling the sheath-fluid reservoir with saline. Commercially available preparations of saline for particle counters often contain small particles that are detectable by the FACS Calibur unless the fluid has been filtered. If these particles are smaller than the cells being analyzed, they can be excluded by adjusting the scatter threshold. Routine flushing of the sheath fluid system and replacement of the sheath fluid filter, according to the manufacturer's instructions and schedule, will help to minimize a similar problem caused by bacteria in the fluidics system. Fluid waste from potentially infectious materials and all human-derived samples should be treated appropriately. The waste container should contain a disinfectant such as bleach.

Immunofluorescently-stained fixed cells can greatly aid in establishing instrument parameter settings and in troubleshooting. Unstained or stained lymphocytes can be fixed in 2% paraformaldehyde/PBS. Fixation appears not to affect either fluorescence or light-scattering profiles to any significant extent. If protected from light and refrigerated, such samples will remain virtually unchanged for 1 week; however, fixed cells do not exclude PI, so live/dead discrimination with PI is not possible.

Sample flow rate

The sample flow rate is adjusted by changing the differential pressure between the two concentric streams of fluid (i.e., the sample stream and the surrounding sheath fluid) that pass in front of the light source. Increasing the differential pressure (i.e., sample fluid pressure minus sheath fluid pressure) increases the width of the sample stream and could allow cells to pass outside the focal plane of the focused light beam, thereby diminishing their fluorescent output. Thus, too high a differential pressure increases the coefficient of variation and also increases the likelihood of counting multiple cells as a single event. The FACS Calibur minimizes these effects by allowing only two pressure settings to be chosen. It is usually best to adjust the cell concentration to ~1×10^6 cells/ml so that the high setting on the FACS Calibur will result in a flow rate of ~1000 cells/sec.

Parameter settings

It is critical to know the general volume, scatter, and fluorescence parameters of the cells being analyzed so as not to collect data on artifacts or debris. Table 5.4.2 can be used as an initial guide. Flow cytometers with sorting capabilities—e.g., the BD FACS Vantage and the Coulter Elite—can sort cells having a particular scatter or fluorescent profile for verification by morphologic analysis under a fluorescence microscope; however, the FACS Calibur does not provide this option. Comparison of the profiles from other instruments run by experienced operators may be helpful. The FACS Calibur can be automatically set with a program from BD (FACSComp) and a standard set of beads (CaliBRITE) that mimic unstained, FITC- and PE-labeled cells (see Support Protocol 1). Once the instrument has been appropriately adjusted by either of these methods, settings for a given cell can be used in other experiments using the same or similar cells.

Setting gates

There may be a tendency to set too many gates on the data, with the result that the collected data may not accurately present the entire population of cells in the sample. When in doubt, it is best to collect the data with as few gates as possible and to use the gates in the data-analysis software on stored data; these gates can be changed later if necessary.

Multicolor analysis

Positive and negative populations should be clearly separable for optimal analysis. The optimal concentrations of antibody should be determined by staining cells with several serial dilutions of the antibody. Preparations with the highest specific activity should be used. Directly labeled or biotinylated reagents are recommended to avoid cross-reactivity of second-step reagents that may bind to both first-step

antibodies. Because direct PE conjugation is difficult, most two-color analyses are performed with an antibody that has been directly labeled with FITC and with a biotinylated antibody that is developed with avidin-PE. If possible, the less-reactive antibody should be used with biotin/avidin-PE conjugate, because PE has a higher specific fluorescence than FITC.

Three-color analysis is facilitated by the use of directly conjugated reagents, which can be added simultaneously to the cells during the staining procedure. When performing three-color analysis, it is also possible to use one biotin-labeled antibody (in which case the avidin- or streptavidin-conjugated fluorochrome should be added in a second step).

It is critical to set compensation appropriately. Too little compensation will result in false double-positive cells, especially if there is high expression of the antigen identified by the FITC-labeled antibody.

Use of PI to detect (and electronically eliminate) dead cells is critical to accurate analysis; however, when performing three-color analysis as described in Alternate Protocol 3—i.e., when a third staining reagent other than PI is being employed—it is not possible to detect dead cells. Thus, samples of very high viability are required to avoid compromising accurate detection of rare subpopulations.

However, three-color analysis together with PI gating can be performed on a FACS Calibur with a second-laser option. In this case, the most common reagents are FITC, PE, PI, and APC.

Troubleshooting

See Table 5.4.3 for a troubleshooting guide to operation of the FACS Calibur.

Time Delay Calibration fails

Failure of Time Delay Calibration is indicated by an error message display and lack of audible tone after activation of the Calibrate button. Two types of errors are indicated. Too-few-events errors are due to either an insufficient concentration of APC beads or a failure to run the beads on the High sample pressure setting. Both are easily corrected. The other error type is a sample-out-of-range error. This error can have a variety of causes, but primarily involves a change in the sheath pressure. A list of causes and solutions of Time Delay Calibration out-of-range errors is given in Table 5.4.3.

Standard bead analysis

Daily analysis of a standard preparation of fluorescent beads under standard conditions is the best check of instrument variability and sensitivity. The instruments are quite reliable, with the peak channels often varying by no more than 2 to 3 channels from day to day. The most common causes of low peak channels are an old, faded bead preparation or a dirty flow cell. Inadvertent use of the wrong instrument settings or incorrect measurement of peak channels must also be considered. If the peak channels remain significantly lower than expected after correction of these problems, a service call may be warranted. Following realignment of the instrument, the service person should be asked to use the same lot of standard beads normally used in the laboratory to produce a printout of the peak channels. This will be the new standard for daily comparison. At any time, if there is any question of the accuracy or integrity of the instrument's performance, the beads should be rechecked for peak channels in each parameter, which can then be compared to the observed peak channels in the log book.

Orifice obstruction

Partial obstruction of the sample orifice produces false parameter measurements, but this is an uncommon problem on the FACS Calibur because the orifice is very large (250 μm). A clogged orifice will cause sudden slowing or stoppage of sample uptake when the sample is on the instrument, and/or no dripping from the sample intake when the sample is off the instrument. This blockage is most frequently caused by an air bubble in the sample intake line; clearing can be achieved by sample removal followed by several cycles in which the fluidics switch is turned first to drain, then to fill, then to back-flush. On rare occasions large cells, or more likely clumps of cells, will clog the orifice and be impossible to dislodge. This may require a service call.

Anticipated Results

Nearly all cell samples contain a few nonviable cells after the staining procedure. Unstained cells should appear in the lower left corner of the FL1 (horizontal) versus FL2 (vertical) dot display (Fig. 5.4.2A). When PI is added, the nonviable cells should be displaced toward the top of the dot display (Figs. 5.4.2C and D). FL1-positive cells should not be affected by PI gating unless they are dead. This is achieved by placing the FITC-stained cells on

Table 5.4.3 Troubleshooting the FACS Calibur

Problem	Possible cause	Solution
No events in acquisition display		
If the Status window reads "READY"	Threshold parameter gain set too high or low	Increase or decrease gain settings
	Threshold not set to correct parameter (usually FSC)	Set to correct parameter (usually FSC)
	Sample injection tube clogged	Remove sample tube to allow back flushing. Clean sample injection tube using Contrad or bleach by running as a sample.
	BAL seal worn	Replace BAL seal
	Communication failure between computer and FACS Calibur instrument	Turn off computer and instrument. Turn on instrument, followed by computer.
	GPIO error, cannot read instrument status	Turn off computer and instrument. Reseat GPIO cable, located next to power cord in back of cytometer. Turn on computer.
If the Status window reads "STNDBY"	The RUN fluid control button is not activated	Press the RUN fluid control button
	Sample tube cracked	Replace sample tube
	Sheath reservoir cap not tightened	Tighten sheath reservoir cap
	Vent valve toggle switch pushed away from experimenter (i.e., sheath reservoir is vented)	Flip toggle switch towards experimenter to pressurize the reservoir
	BAL seal worn	Replace BAL seal
If the Status Window reads "NOT READY"	Laser warming up	Wait 5 min
	Laser not functioning	Check laser power in the Status window. If power is 0 mW, turn off the FACS Calibur instrument and computer, then turn on the instrument, followed by the computer. If power is still 0 mW, contact Becton Dickinson.
	Leak at sheath area	Check Status window with test tube off sample injection port and instrument in RUN mode. Sample voltage should be 10.2 V (approximate). If under 10.0 V, replace sheath reservoir, replace cap, and finally, replace gasket.
	Sheath reservoir empty or waste reservoir full	Check reservoir, fill sheath, and empty waste if necessary

continued

Table 5.4.3 Troubleshooting the FACS Calibur, continued

Problem	Possible cause	Solution
High sample event rate	Air bubble in flow cell	Press PRIME to drain and fill the flow cell
	Air in sheath	Vent air from sheath filter
	Threshold level too low	Increase Threshold level
	Sample too concentrated	Dilute sample. Cell concentration should be 1×10^5 to 1×10^7 cells/ml for optimal event rates.
Low sample event rate	Threshold level set too high	Lower Threshold level
	Sample not adequately mixed	Mix sample to suspend cells
	Sample too dilute	Concentrate sample
	Clog in injection tube	Run 10% bleach or Contrad as a sample for 20 min followed by deionized water for 10 min
Scatter parameters appear distorted	Air bubble in flow cell	Press PRIME to drain and fill the flow cell
	Air in sheath filter	Vent air from sheath filter
	Flow cell dirty	Perform monthly cleaning procedure
Time Delay Calibration out-of-range errors	Bubbles in sheath filter	Purge filter of bubbles
	Obstructed sheath filter	Replace filter
	Dirty fluidics	Clean using Contrad detergent
	Sheath cap is loose	Tighten cap
	Crack in sheath tank seams	Replace tank
	Laser alignment has drifted	Call service

the instrument and compensating for overlap of green fluorescence into red fluorescence (Fig. 5.4.2B).

In two-color analysis, at least four different cell populations can be identified: negative for both antibodies, positive for either, and positive for both. In addition, the semiquantitative nature of the flow cytometer makes it possible to distinguish bright and dull subpopulations. A marker can be set for each color, above which a cell may be considered positive. Thus, a two-parameter dot plot or contour plot can be divided into four quadrants and the data analyzed by determining the percentage of cells in each quadrant.

Time Considerations

FACS Calibur single-color analysis requires 15 min to prepare and warm up the instrument and 1 to 2 min to acquire and save data on each sample, where 10,000 events are acquired. Three FACS Calibur flow rates are HI (60 µl/min), MEDIUM (35 µl/min), and LO (12 µl/min). For cell samples at 1×10^6 cells/ml, event rates are 1000/sec at HI and 200/sec at LO. PI gating requires an additional 5 to 10 min of instrument preparation, and slightly increases the sample-acquisition time. Acquisition time is significantly increased if the samples contain many dead cells.

Two- and three-color analyses require substantially more time. A ten-tube experiment may require an hour to complete because of increased instrument-setup time (required for additional checks on compensation) and increased data acquisition (30,000 to 500,000 events). Data analysis is usually performed later and this time is not included.

Four color analysis requires additional time to perform Time Delay Calibration (~5 min) and adjustment of compensation between FL3 and FL4 (5 to 10 min).

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Provides detailed descriptions of a wide variety of flow cytometry protocols and techniques, with extensive references.

FACS Calibur User's Guide, CELLQuest Software User's Guide, and FACS Calibur FACSComp User's Guide. Becton Dickinson, San Jose, Calif. (see *APPENDIX 5*).

These manuals offer a complete description and guide to use of the hardware and software detailed in this unit.

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Shapiro, H.M. 1995. See above.

Details all aspects of flow cytometry.

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