Technical Resources

Standard Operation Tips and Recommendations for the BD FACSAria Flow Cytometer

The following is a series of helpful hints and recommendations for standard operation of the BD FACSAria flow cytometer. Refer to the *BD FACSAria User's Guide* for specific instructions, details, and guidelines.

Daily Startup

1. Open the flow cell access door to engage the laser shutter.
2. Turn on the instrument main power and lasers.
   NOTICE: Allow the lasers to warm up for 30 minutes.
3. Start up the BD FACSAria™ workstation; launch BD FACSDiva™ software.
4. Verify fluidics levels in the Instrument frame; replenish fluids or empty the waste, if needed.
5. Choose Instrument > Fluidics Startup; click OK when the system is ready.
6. Verify the nozzle size.
   As a general guideline, the nozzle opening should be at least three times the diameter of the particle to be sorted. If necessary, change the nozzle before starting the stream.
   - Click the Sorting button in the Workspace toolbar to display the Breakoff and Side Stream windows.
   - Click the Stream button in the Breakoff window to turn on the stream.
7. Use software controls to start the stream.
8. Check the stream position in the waste aspirator and inspect the stream image in the Breakoff window.
   Open the flow cell access and sort block door to view the stream position in the aspirator.

   If you see any dripping or spraying or the stream appears abnormal, turn off the stream and go to Troubleshooting the Stream in Chapter 7.
9. Choose Sort Setup from the Sort menu.

Sorting Setup

1. Start up the instrument as described in the previous section.
2. Adjust the Amplitude to set the drop breakoff.
   Adjust the Drop 1 target, if needed, to match the actual value.
3. Turn on the Sweet Spot when the drop pattern is stable.
4. Check the laser delay and the area scaling factor for your sheath pressure.
5. Perform sample optimization for the sample to be sorted.
6. Install the required collection device and set up the side streams.
7. Use BD FACS™ Accudrop to calculate the drop delay.
8. Run the sample to be sorted; use gating tools and subsetting methods to define the population(s) of interest.
   NOTICE: Snap-To gates cannot be used for sort gates.
9. Define a Sort Layout for the Tube or Acquisition template containing the defined sort populations.

Technical Resources

Standard Operation Tips and Recommendations for the BD FACSAria Flow Cytometer *(continued)*

Sorting

1. Install the collection tubes, plate, or slide.
2. Install the sample tube on the loading port and click Load.
3. Turn on the deflection plates and open the aspirator drawer.
4. Verify that the green Acquisition pointer is indicating the appropriate Tube in the Browser; click Sort.
5. Sorting continues until the required number of cells has been sorted. If the number of Target Events is set to Continuous, sorting continues until you manually stop sorting by clicking the Acquire or Sort button.

   TIP: You can click Record to save data for the Tube. Acquisition and sorting continue when the required number of events has been recorded.

Daily Shutdown

1. Turn off the lasers.
2. Turn off the stream.

   NOTICE: Make sure the current sample has been unloaded before you turn off the stream.
3. Choose Instrument > Fluidics Shutdown.
   Follow the prompts on the screen to shut down the fluidics system.
4. Turn off the instrument main power; quit BD FACSDiva software and shut down the computer.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD LSR II Flow Cytometer

The following is a series of helpful hints and recommendations for standard operation and maintenance of the BD LSR II flow cytometer. Refer to the *BD LSR II User's Guide* for specific instructions, details, and guidelines.

Daily Startup

1. Turn on the cytometer; turn on the computer.
2. Fill the sheath tank and verify the following.
   - Tank lid is properly sealed.
   - Tank is pressurized.
   - Tubing is not kinked.
3. Empty the waste tank and verify the following.
   - Tank contains sufficient bleach.
   - Tubing is not kinked.
4. Purge air bubbles from the sheath filter and sheath line.
   - Press Prime twice.
5. Allow the cytometer to warm up.
   - 60 minutes for UV applications using 325-nm lasers
   - 30 minutes for UV applications using 355-nm lasers
   - 30 minutes for all other applications

Daily Shutdown

1. Install a 12 x 75-mm tube containing 3 mL of a 10% bleach solution on the sample injection port (SIP). Use undiluted BD FACS cleaning solution or bleach diluted in deionized water (DI).
2. Leave the support arm to the side for 1 minute.
3. Place the support arm under the tube, make sure the cytometer is in RUN, and let it run at high flow rate for 5 minutes.
4. Repeat steps 1 through 3 using BD FACS rinsing solution.
5. Repeat steps 1 through 3 using DI water.
6. Leave a tube containing 1 mL of DI water on the SIP with the support arm under the tube.
7. Set the cytometer to standby; turn off cytometer.
8. Shut down the computer.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD LSR II Flow Cytometer *(continued)*

Periodic Maintenance

To be performed every two weeks for regular use, more often if sticky dyes, adherent cells, or viscous samples are run frequently.

1. Empty the sheath in the sheath tank and rinse it with deionized water; fill it with a 10% bleach solution. Use undiluted BD FACS cleaning solution or bleach diluted in deionized water (DI).
2. Bypass the sheath filter.
3. Remove the tube of DI water from the sample injection port (SIP); prime twice.
4. Install a 12 x 75-mm tube containing 3 mL of 10% bleach solution on the SIP.
5. Run for 30 minutes at high flow rate.
6. Repeat steps 1 through 5 using 10% BD FACS rinsing solution.
7. Repeat steps 1 through 5 using DI water.
8. Perform instrument startup procedure before running samples.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD FACSCalibur Flow Cytometer

The following is a series of helpful hints and recommendations for standard operation and maintenance procedures for the BD FACSCalibur™ flow cytometer. Refer to the appropriate user's guides for specific instructions, details, and guidelines (*BD FACSCalibur User's Guide* or *BD FACS Loader User's Guide* or both).

BD FACSCalibur Daily Startup

1. Turn on the cytometer.
2. Turn on the BD FACStation™ system.
3. Fill the sheath tank and verify the following.
   - Tank is full (~3 L).
   - Cap is tight.
   - Tank is pressurized.
   - Tubing is not kinked.
4. Empty the waste tank and verify the following.
   - Tank contains 400 mL of bleach.
   - Tubing is not kinked.
5. Purge bubbles from the sheath filter.
6. Allow the cytometer to warm up for 5 minutes.

BD FACSCalibur Daily Shutdown

1. Install a 12 x 75-mm tube containing 3 mL of 10% bleach on the sample injection port (SIP).
2. Leave the support arm to the side for 1 minute.
3. Place the support arm under the tube, make sure the cytometer is in RUN, and let it run at HI flow rate for 5 minutes.
4. Repeat steps 1 through 3 using distilled water.
5. Leave a tube containing 1 mL of distilled water on the SIP with the support arm under the tube.
6. Set the cytometer to STANDBY mode.
7. Shut down the BD FACStation system.
8. Turn off the cytometer.

Daily Shutdown for BD FACSCalibur with BD FACS™ Loader

If cleaning was already performed with the last sample run of the day, begin shutdown with step 7.

1. Install a 12 x 75-mm tube containing 3 mL of 10% bleach in position 39 and a tube containing 3 mL of distilled water in position 40 on a rack.
2. Install the rack on the cytometer and install the BD FACS Loader cover.
3. Press the RUN button on the cytometer.
4. Launch BD™ Loader Manager software from the Apple menu.
5. Click the Maintenance button and choose Long Clean.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD FACSCalibur Flow Cytometer *(continued)*

Daily Shutdown for BD FACSCalibur with BD FACS Loader *(continued)*

6. Click Run. Click Run again.
7. When the cleaning is finished, remove the rack and install a 12 x 75-mm tube containing 1 mL of distilled water on the SIP with the support arm directly under the tube.
8. Place the instrument in STANDBY mode.
9. Shut down the BD FACStation System.
10. Turn off the cytometer and the BD FACS Loader.

BD FACSCalibur Monthly Maintenance

1. Remove the sheath reservoir.
2. Bypass the sheath filter to avoid damage.
3. Install a reservoir containing 10% bleach.
4. Install a 12 x 75-mm tube containing 3 mL of 10% bleach on the SIP.
5. Run for 20 to 30 minutes at HI flow rate.
6. Remove the tube of bleach and the bleach reservoir.
7. Repeat steps 2 through 5 using distilled water instead of bleach.
8. Replace the original sheath reservoir and reconnect the sheath filter.
9. Perform instrument startup procedure before running samples.

BD FACSCalibur Sorting

1. Check the following.
   - Sheath tank contains ~3 L of phosphate-buffered saline (PBS).
   - Sheath cap is tight.
   - Waste tank contains only 400 mL of bleach.
   - Tubing is not kinked.
2. Prime the sort lines and ensure that each collection station port is dripping.
3. Optimize instrument settings for cells of interest.
4. Create a sort gate.
5. Collect a pre-sort data file.
6. Remove your sample tube from the SIP.
7. Make selections in the Sort Setup and Sort Counters windows.
8. Install bovine serum albumin (BSA)–coated tubes in the collection stations.
9. Set the cytometer to LO flow rate and then sort the population of interest.
10. Concentrate the sorted cells.
11. Clean the sort lines.
12. Collect a post-sort data file.
13. Verify sort purity.

Technical Resources

Non-Sterile Sorting Tips and Recommendations for the BD FACSCalibur Sort Option

Several factors should be considered to obtain the best sorting performance from the BD FACSCalibur Sort Option. This document compiles many of these factors for easy reference. For detailed instructions on sorting procedures, refer to the *BD FACSCalibur User's Guide*.

Sheath Fluid

- Use clean, filtered phosphate-buffered saline (PBS) (pH 7.2 to 7.4) as sheath fluid when sorting.
- Begin sorts with 3 L in the sheath tank to avoid refilling during a sort.

Cell Recovery and Viability

- Coat collection tubes with 4% bovine serum albumin (BSA) in PBS (with azide) for at least an hour at 2° to 8°C before using them in order to decrease loss of cells and maintain their viability.
- At the end of the sort, remove the sample tube, install a tube of sheath fluid on the sample injection port (SIP), and restart or continue sorting for 20 seconds so that all sorted cells reach the collection tube.

Thresholding

- The instrument will not see events below the threshold level. Events that do not trigger the system cannot be excluded by the sort logic. For example, if erythrocytes and platelets are in your sample and must be rejected from the sorted fraction, the scatter threshold level must be low enough to allow them to be detected.
- If the threshold level is too low, the instrument will detect debris. The coincidence of debris with the target cell could prevent the cell from being sorted.

Flow Rate

The sorting specifications have been validated at the LO flow rate. Purity using the HI and MED flow rates might not be equivalent.

Technical Resources

Non-Sterile Sorting Tips and Recommendations for the BD FACSCalibur Sort Option

Sample Concentration

- Figure 1 illustrates the relationship between sort rate and event rate when sorting in Single Cell mode. Note that the maximum sort rate usually occurs when the event rate is approximately 2,000 events/sec. This requires an input concentration of approximately 10^7 cells/mL when using the LO flow rate.

  If your target population is below 30% to 35%, you cannot reach the 300 sorts/sec maximum sorting rate in Single Cell mode. For instance, a 10% target population sorted at 2,000 events/sec should give a sort rate of 50 to 60 sorts/sec. Any increase or decrease in sample concentration will decrease the sort rate.

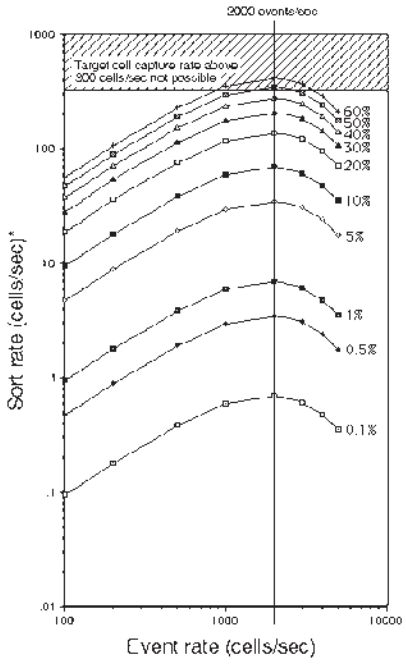


Figure 1. Sort rates at various event rates and target cell percentages
** Multiply by 12 to get cells sorted (cells/mL).*

- Unlike the Single Cell and Exclusion modes, the sort rate in the Recovery mode will continue to increase as the total rate increases above 2,000 events/sec (up to at least 25,000 events/sec), provided the 300 sorts/sec rate has not been exceeded. Since coincidence is ignored in the Recovery mode, the percent purity will decrease as the total sample rate increases because of the increasing probability of nontarget cells in the sort envelope.
- When an input concentration of 10^7 cells/mL is used, the sample concentration might need to be readjusted to achieve an event rate of 2,000 events/sec. This can happen because the LO flow rate might not be exactly 12 µL/min (it is specified as 12 ± 2 µL/min), or because a hemacytometer count rarely accounts for the erythrocytes, platelets, and debris that the flow cytometer detects.
- If you are starting with a particularly low number of cells, it might be best to use an event rate of <2,000 events/sec to decrease coincidence and maximize recovery in Single Cell mode. Use Recovery mode when recovery of target cells is the primary concern.
- Check your sort lines and the station electronics to ensure that the collection station is dripping when sorting.
- Vortex your sample occasionally during long sorts to avoid sample settling.
- After you have filled all three collection tubes, install three new empty BSA-coated tubes on the collection stations.

Technical Resources

Non-Sterile Sorting Tips and Recommendations for the BD FACSCalibur Sort Option *(continued)*

Reanalysis

- To improve the accuracy of purity assessment when reanalyzing by flow cytometry, eliminate carryover before acquiring the sorted sample.
- Verify sample purity by examining a cytospin preparation or by reanalyzing using flow cytometry.
- Spike your sorted cells with beads when assessing recovery using reanalysis by flow cytometry.

One method of estimating the number of sorted cells (and therefore the recovery) is to add an aliquot of beads of a known concentration to the tube of sorted cells and reanalyze by flow cytometry.

$$\frac{\text{Number of beads acquired}}{\text{Total number of beads added}} = \text{Fraction of total volume reanalyzed}$$

$$\frac{\text{Number of cells acquired}}{\text{Fraction of total volume reanalyzed}} = \text{Total number of cells recovered}$$

Cleaning

- Purge the sort lines to prevent salt crystal formation and ensure clean lines before and after sorting.
- Refer to the B*D FACSCalibur User's Guide* for instructions on recommended periodic maintenance.

Technical Resources

Aseptic Sorting for the BD FACSCalibur Sort Option

The BD FACSCalibur system can sort cells to be used for culture or functional studies. To meet the needs of this application, sorting requires a clean environment to keep the sorted sample free from contaminants when put into culture. Perform all steps of your preparation procedure using aseptic technique.

Setting Up for Ethanol Rinse

1. Prepare the following solutions:
   - 3 L of 70% ethanol (EtOH). Dilute 95% ethanol in sterile, distilled water.
   - 5 L of sterile 1X PBS
2. Fill a clean sheath reservoir with 3 L of 70% EtOH.
3. Cap and shake the reservoir to ensure that the entire inner surface of the reservoir is washed with EtOH.
4. Install the reservoir in the instrument.
5. Using a squirt bottle filled with EtOH, rinse off the collection station ports.
6. Place three collection tubes in the collection station.
7. Install a tube of 70% EtOH on the sample injection port (SIP).

Ethanol Rinse

1. Set a Sort Gate.
2. From the Acquire menu, choose Sort Setup.
3. From the Sort Gate pop-up menu, choose the gate drawn in step 1.
4. Press the RUN fluid control button.
5. Click Acquire in the Acquisition Control window.
   NOTE: Make sure the Setup box is checked.
6. Run the EtOH on the BD FACSCalibur instrument until all three collection tubes are filled.
7. Click Pause, then Abort.
8. Disconnect the reservoir.
9. Working under a hood, empty the remaining EtOH.

Technical Resources

Aseptic Sorting for the BD FACSCalibur Sort Option *(continued)*

PBS Rinse

1. Pour approximately 500 mL of sterile 1X PBS into the reservoir.
   Swirl to wash out any remaining EtOH.
   Empty the reservoir and repeat.
2. Fill the reservoir with 3 L of sterile 1X PBS.
   NOTE: Cap the reservoir before removing it from the hood.
3. Install the reservoir in the instrument.
4. Place three new collection tubes in the collection station.
5. Install a tube of sterile PBS on the SIP.
6. Click Acquire in the Acquisition Control window.
7. Run the sterile PBS for approximately 10 minutes to wash residual EtOH out of the lines.
   Allow approximately 15 mL of PBS to run into each collection tube.
8. Click Pause, then Abort.

Aseptic Sort

1. Using aseptic technique, coat the appropriate number of 50-mL conical tubes with sterile PBS/4% BSA buffer.
2. Place the prepared conical tubes in the collection station.
3. Sort the sample.
   Refer to Sorting the Sample in the *BD FACSCalibur User's Guide* for detailed instructions.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD FACSVantage SE Flow Cytometer

The following is a series of helpful hints and recommendations for standard operation and maintenance of the BD FACSVantage™ SE flow cytometer. Please remember these are tips and recommendations. Refer to the *BD FACSVantage SE User's Guide* for specific instructions, details, and guidelines.

BD FACSVantage SE Daily Startup

1. Turn on laser water.
2. Turn on laser(s).
3. Turn on air and vacuum sources.
4. Fill sheath container. Empty waste container.
5. Turn on the main pressure toggle.
6. Turn the fluidics control knob to FILL for 10 to 20 seconds.
7. Remove the tube from the sample injection port (SIP).
8. Set the fluidics control knob to RUN.
9. Turn on main instrument power.
10. Turn on the BD FACStation™ system.

BD FACSVantage SE Daily Shutdown

1. Turn off laser(s).
2. Shut down the BD FACStation system.
3. Run a 12 x 75-mm tube of 10% bleach in deionized water for 10 minutes.
4. Run a 12 x 75-mm tube of deionized water for 10 minutes. Leave this tube installed.
5. Turn the fluidics control knob to OFF.
6. Turn off in the following order:
   - main pressure toggle
   - air and vacuum
   - main instrument power
   - laser water

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD FACSVantage SE Flow Cytometer *(continued)*

BD FACSVantage SE Daily Alignment

1. Load the instrument settings and appropriate Experiment document(s).
2. Set the distance from the laser intercept to the nozzle and adjust stream.
   - Adjust the camera to place the viewing mark at the laser intercept.
   - Adjust the Z knob to position the nozzle at the reference mark.
   - Adjust A and Q control knobs, if necessary.
3. Maximize the FSC signal.
   - Maximize the FSC signal with the Y adjust knob.
   - Adjust the Excitation Beam Focus to obtain the highest signal intensity with the most narrow width of the FSC signal pulse.
4. Maximize the FL1 signal.
   - Adjust the X control knob.
   - Adjust the fluorescence focus control knob.
   - Adjust the fluorescence channel height adjustment wheel.
5. Close the FL1 iris.
   - Close the iris so the FL1 signal decreases by half.
   - Repeat the fluorescence adjustments (step 4) to maximize the FL1 signal.
6. Repeat step 5 until the iris is completely closed.
   The FL1 signal intensity with the iris open should decrease by less than half its maximum when the iris is closed.
7. Peak Y and laser focus again.
   The Excitation Beam Focus should be adjusted for maximum FL1 intensity and minimum width of the signal pulses.
8. Open the FL1 iris completely and adjust the beam splitters for maximum signal intensity for SSC, FL3, and FL2.
9. Adjust the obscuration bars vertically.
   - SSC—With the SSC amp in LOG, adjust for minimum noise on the SSC pulse display.
   - FSC—Adjust for best FSC (size) resolution, with minimum noise.
10. Check the trajectory of the fluid stream.
    - Verify that the stream is still entering the front third of the stream aspirator.
    - If you have to make adjustments using either A or Q knob, repeat the alignment (steps 1 through 9).
11. Record the primary laser quality control results.

Technical Resources

Standard Operation and Maintenance Tips and Recommendations for the BD FACSVantage SE Flow Cytometer *(continued)*

BD FACSVantage SE Sorting

Instrument Setup

1. Turn on in the following order:
   - drop drive
   - deflection plates
   - left and right stream control buttons
2. Allow the drop drive and deflection plates to warm up at least 20 minutes.
3. Check the nozzle diameter for determining the drop-drive frequency range.
4. Set the amplitude level to approximately 3 V.
5. Adjust the Frequency to find visually the shortest droplet breakoff distance.
6. Adjust FSC and SSC obscuration bars to minimize laser scatter.
7. Turn on Test Mode and Test Sort.
8. Adjust the Phase to see single side streams.
9. Count and set the Drop Delay.
10. Select the desired Sort Mode (usually Normal-R).
11. Turn off Test Mode.
12. Run the sample to be sorted.
13. Set and load the desired sort windows.
14. Turn on Test Mode.
15. Recheck the Phase.
    If the Phase needs to be adjusted, the drop delay also needs to be recounted and reset.
16. Turn off Test Mode and Test Sort.

Sorting

1. Set the counters as desired to monitor the sort.
2. Press Reset to zero the counters.
3. Place recovery tubes containing nutrient medium into the holder.
   Be sure to push in the holder all the way.
4. Close the stream door.
   The instrument is now sorting. Continue to sort until the required number of cells have been sorted.
5. To stop the sort, open the stream door.
6. Be sure to rinse the sample tubing thoroughly before rerunning cells from the recovery tubes.

Cytometer Daily Maintenance Log

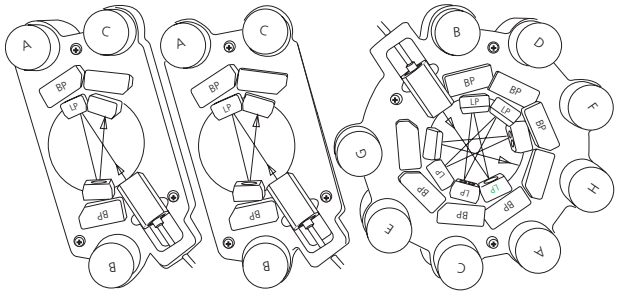
Instrument Serial Number: _____ Month: _____

Date																														
Time On/ Operator Initials																														
Startup Procedure																														
BD FACSComp™ Pass/Fail																														
Lyse/Wash																														
Lyse/No-Wash																														
HLA-B27 Calibration																														
DNA QC																														
Shutdown Procedure																														
Time Off/ Operator Initials																														
Max Laser Hours (=Time off–Time on)																														
Monthly Maintenance/Initials																														

Date	Comments	Date	Comments
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Technical Resources

BD LSR II Instrument and BD FACSAria Instrument Optical Layouts and Components



Default BD LSR II flow cytometer filter configuration

DETECTOR ARRAY (LASER)	PMT (DETECTOR)	LONGPASS DICHROIC MIRROR	BANDPASS FILTER	INTENDED DYE
Octagon (488-nm blue laser)	A	735 lp	780/60	PE-Cy7
	B	685 lp	695/40	PerCP-Cy5.5
	C	550 lp	575/26	PE, PI
	D	505 lp	530/30	FITC, GFP
	E	blank	488/10	SSC
	F	blank	blank	N/A
	G	blank	blank	N/A
	H	—	blank	N/A
Violet Trigon (405-nm violet laser)	A	505 LP	525/50	Cyan fluorescent protein (CFP), Alexa Fluor® 430
	B	blank	440/40	Pacific Blue™, Marina Blue®, Alexa Fluor® 405
	C	—	blank	N/A
UV Trigon (325-nm or 355-nm UV laser)	A	505 LP	530/30	indo-1 (blue)
	B	blank	440/40	DAPI, Alexa Fluor® 350
	C	—	blank	N/A
Red Trigon (633-nm red laser)	A	735 LP	780/60	APC-Cy7
	B	blank	660/20	APC
	C	—	blank	N/A

blank *indicates that a blank optical holder should be used instead of a mirror or filter. A dash (—) indicates that no slot exists for a filter or mirror in the PMT position.*

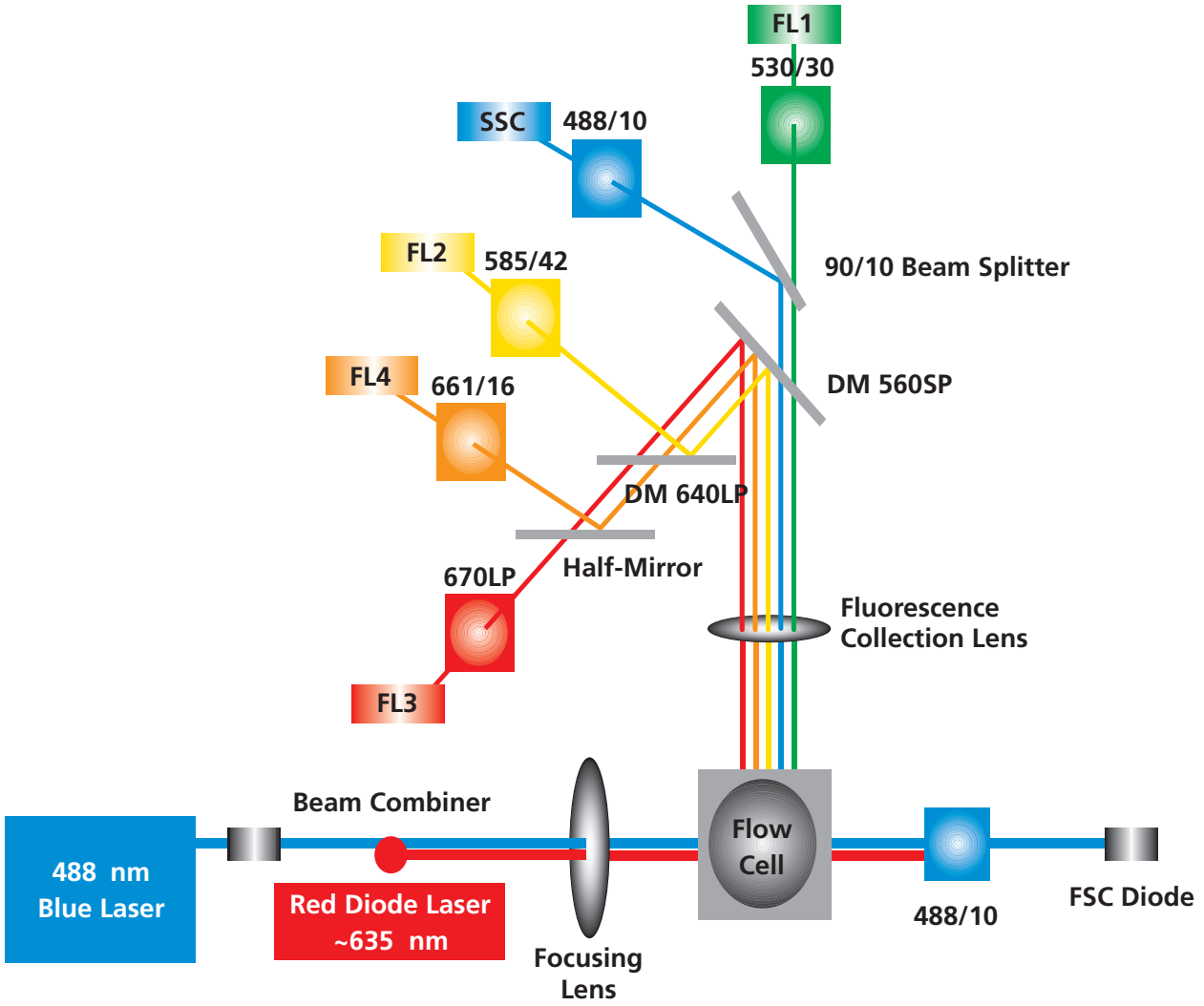
Default BD FACSAria flow cytometer filter configuration (three-laser system)

DETECTOR ARRAY (LASER)	PMT (DETECTOR)	LONGPASS DICHROIC MIRROR	BANDPASS FILTER	INTENDED DYE
Octagon (488-nm blue laser)	A	735	780/60	PE-Cy7
	B	655	695/40	PerCP-Cy5.5 or PI
			675/20	PerCP
	C	595	610/20	PE-Texas Red®
	D	556	575/26	PE or PI
			585/42	Alternative for PE/PI when not using PE-Texas Red®
	E	502	530/30	FITC
	F		488/10	SSC
	G	blank	blank	blank
	H	—		—
Trigon (633-nm red laser)	A	735	780/60	APC-Cy7
	B	blank	660/20	APC
	C	—	blank	blank
Trigon (407-nm violet laser)	A	502	530/30	Alexa Fluor® 430
	B	blank	450/40	Cascade Blue®, Pacific Blue™, Hoechst, DAPI, Alexa Fluor® 405
	C	—	blank	blank

blank *indicates that a blank optical holder should be used instead of a mirror or filter. A dash (—) indicates that no slot exists for a filter or mirror in the PMT position.*

Technical Resources

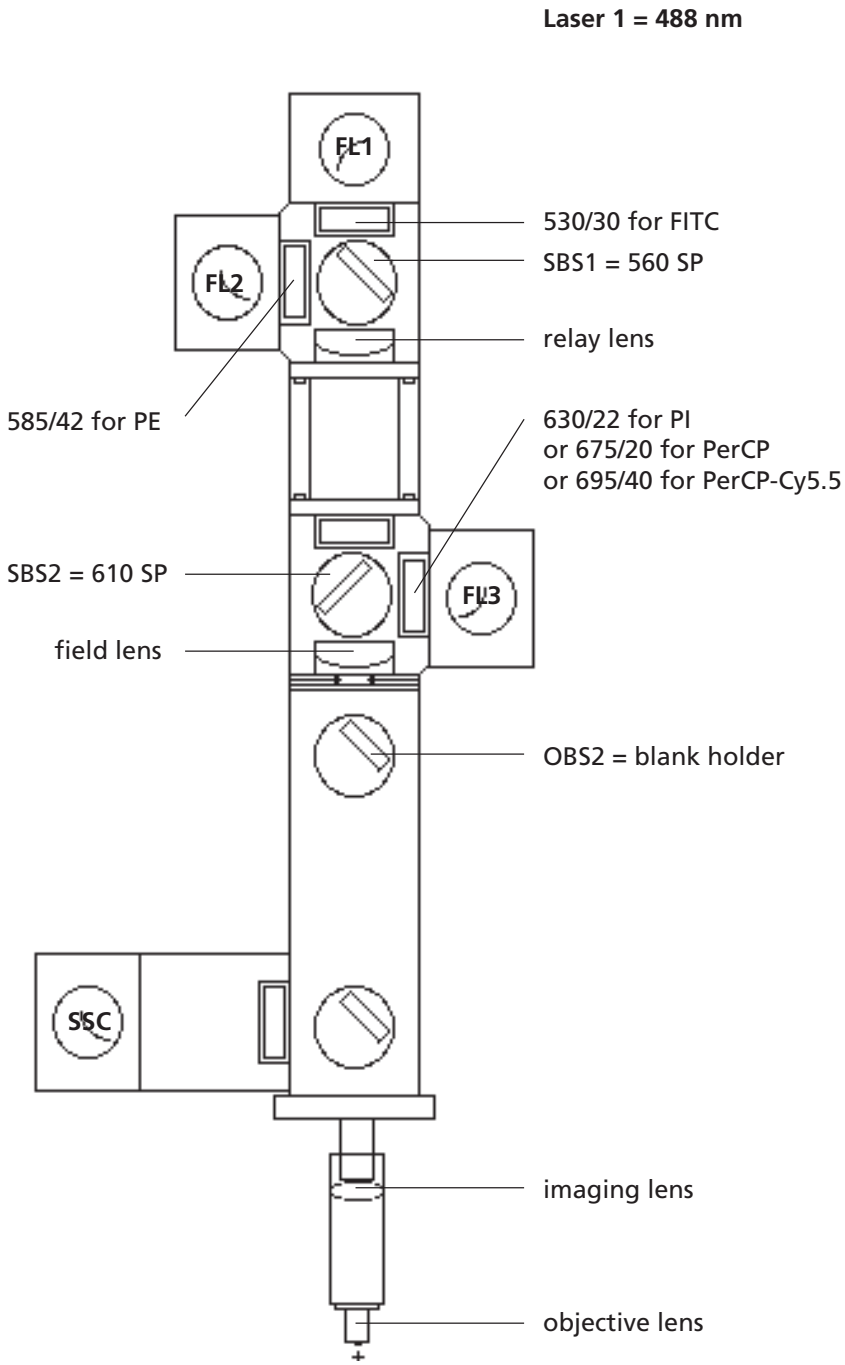
BD FACSCalibur™ Optical Layout



Technical Resources

BD FACSVantage™ SE Configuration

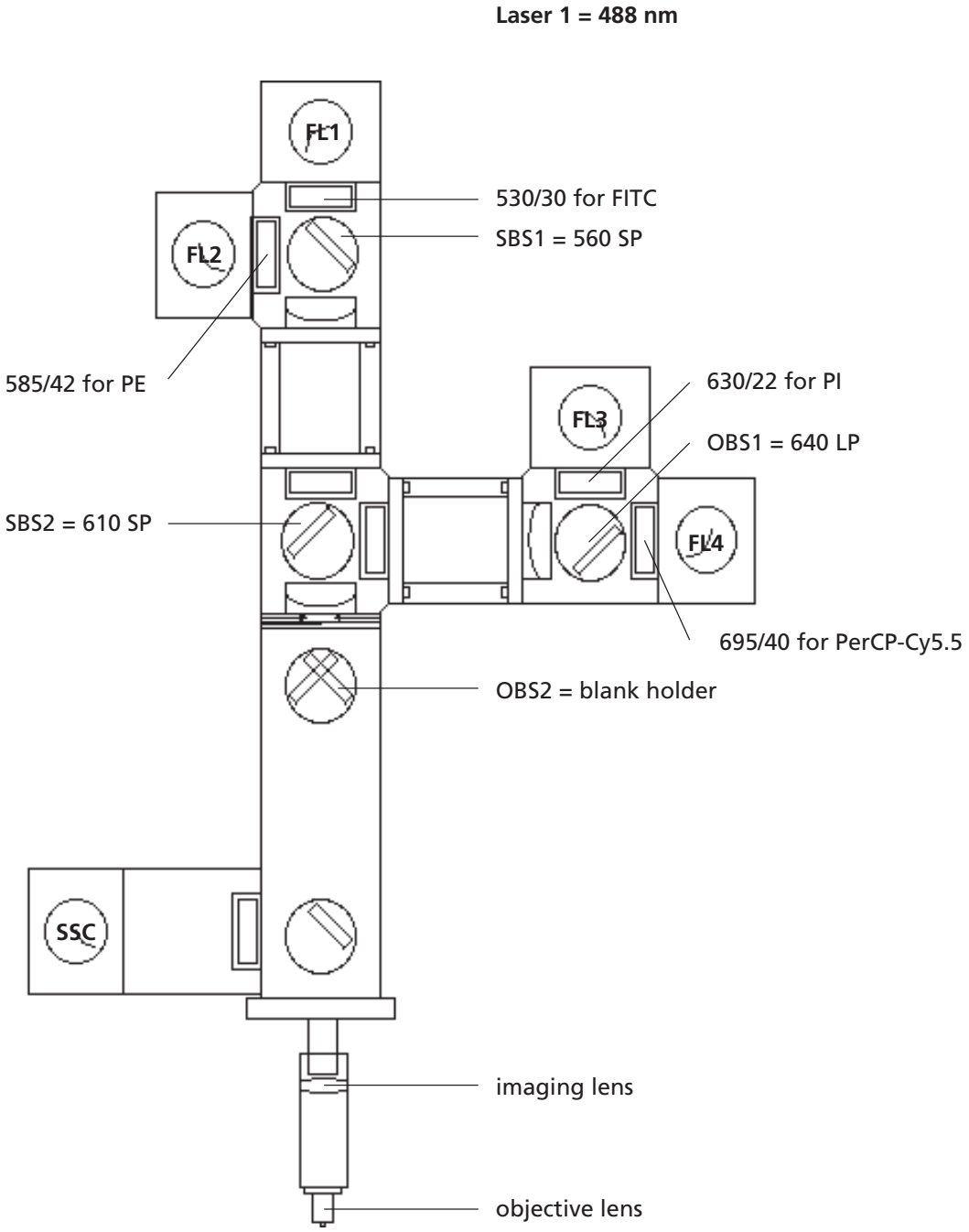
Example 1: Base Configuration—3 Colors/1 Laser



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

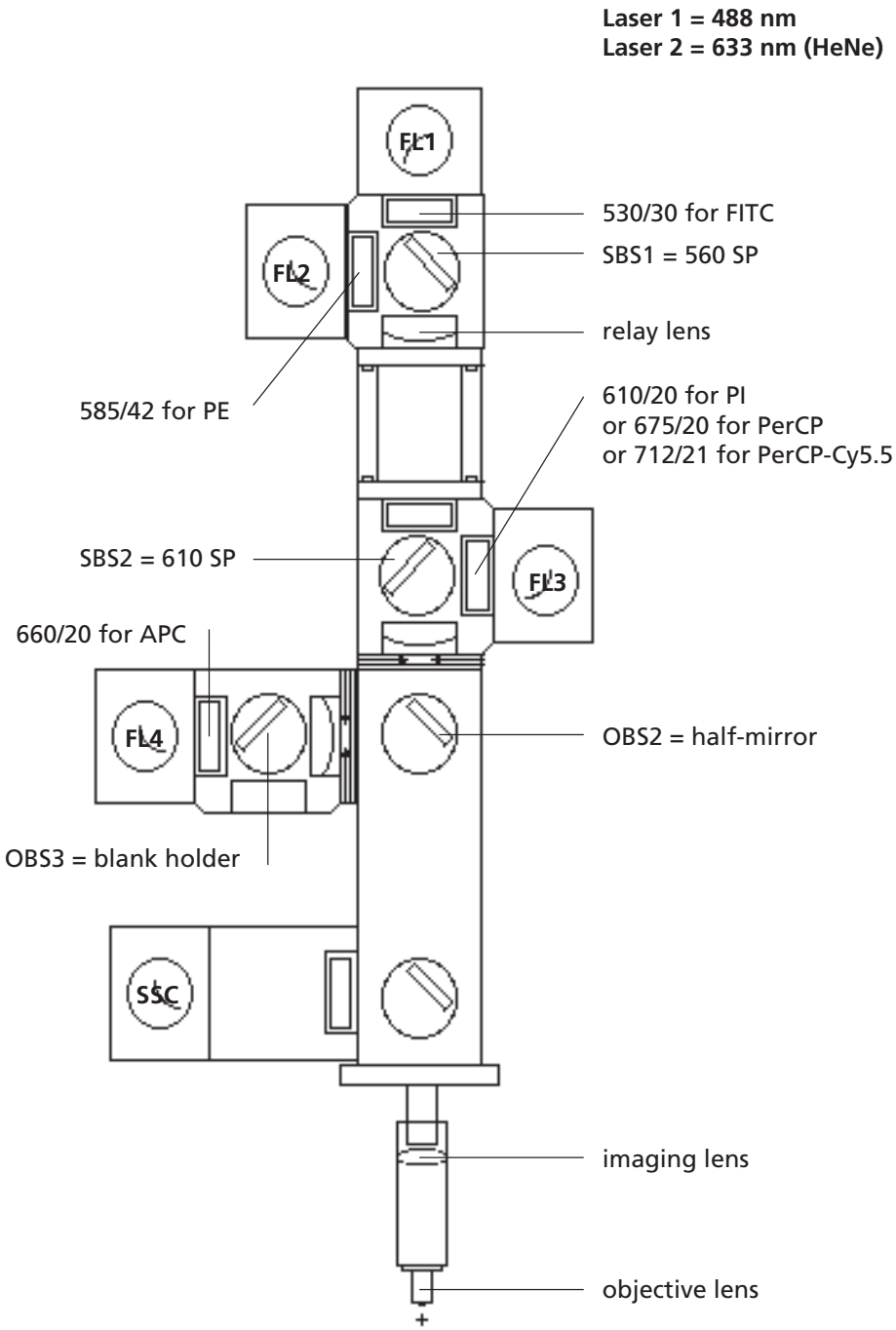
Example 2: Base Configuration + 1 Optional Detector— 4 Colors/1 Laser



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

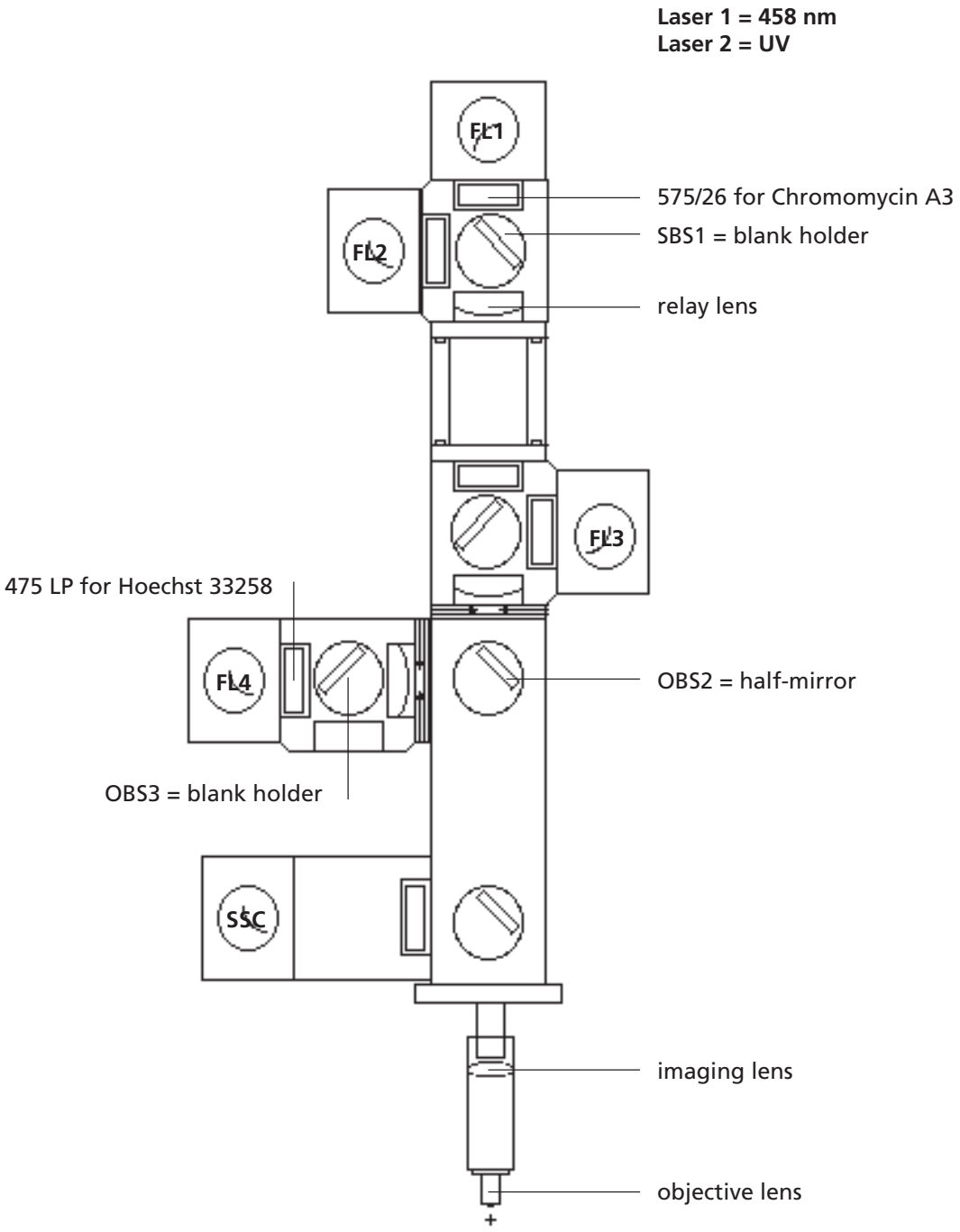
Example 3: Base Configuration + 1 Optional Detector— 4 Colors/2 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

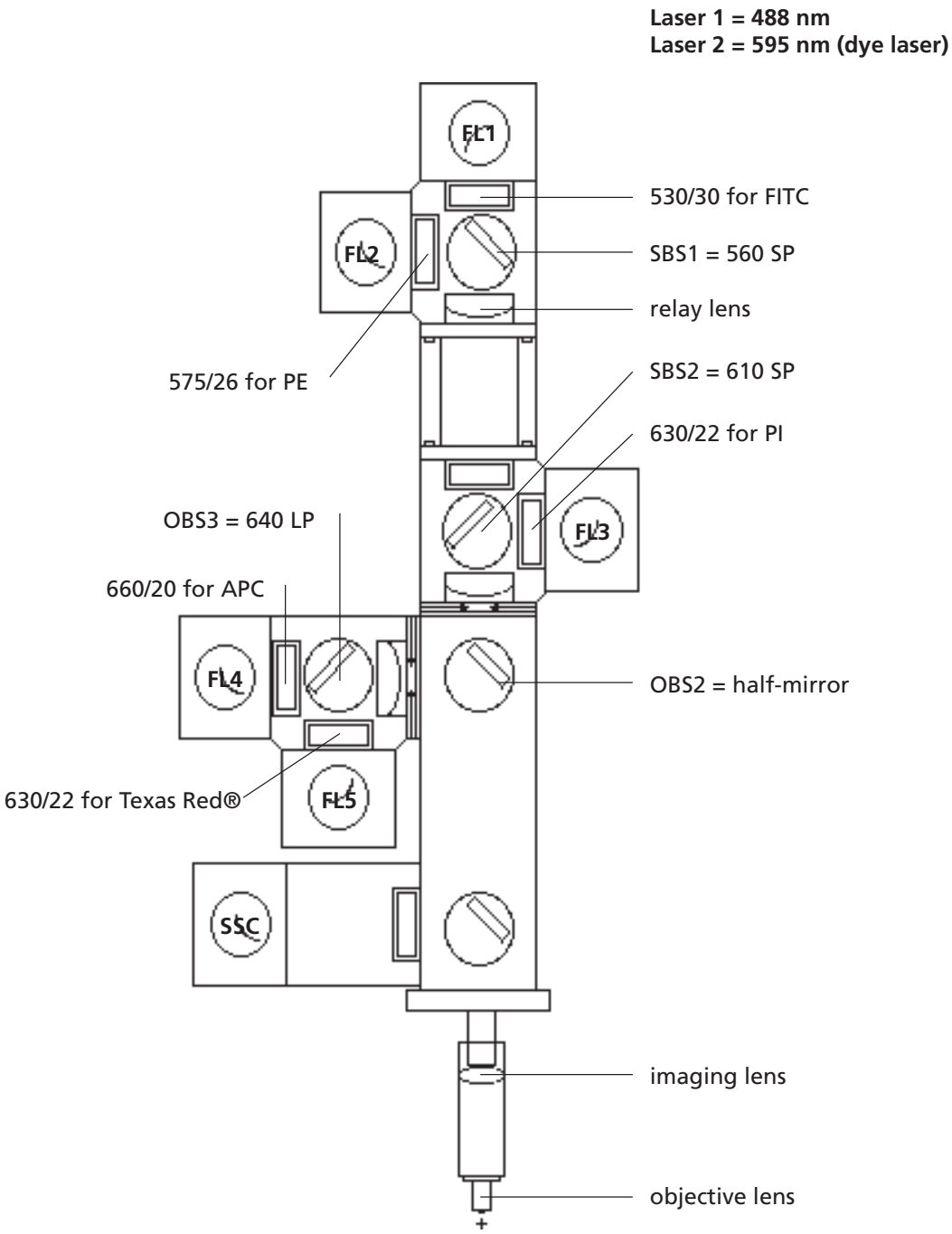
Example 4: Base Configuration + 1 Optional Detector—Chromosome Application/2 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

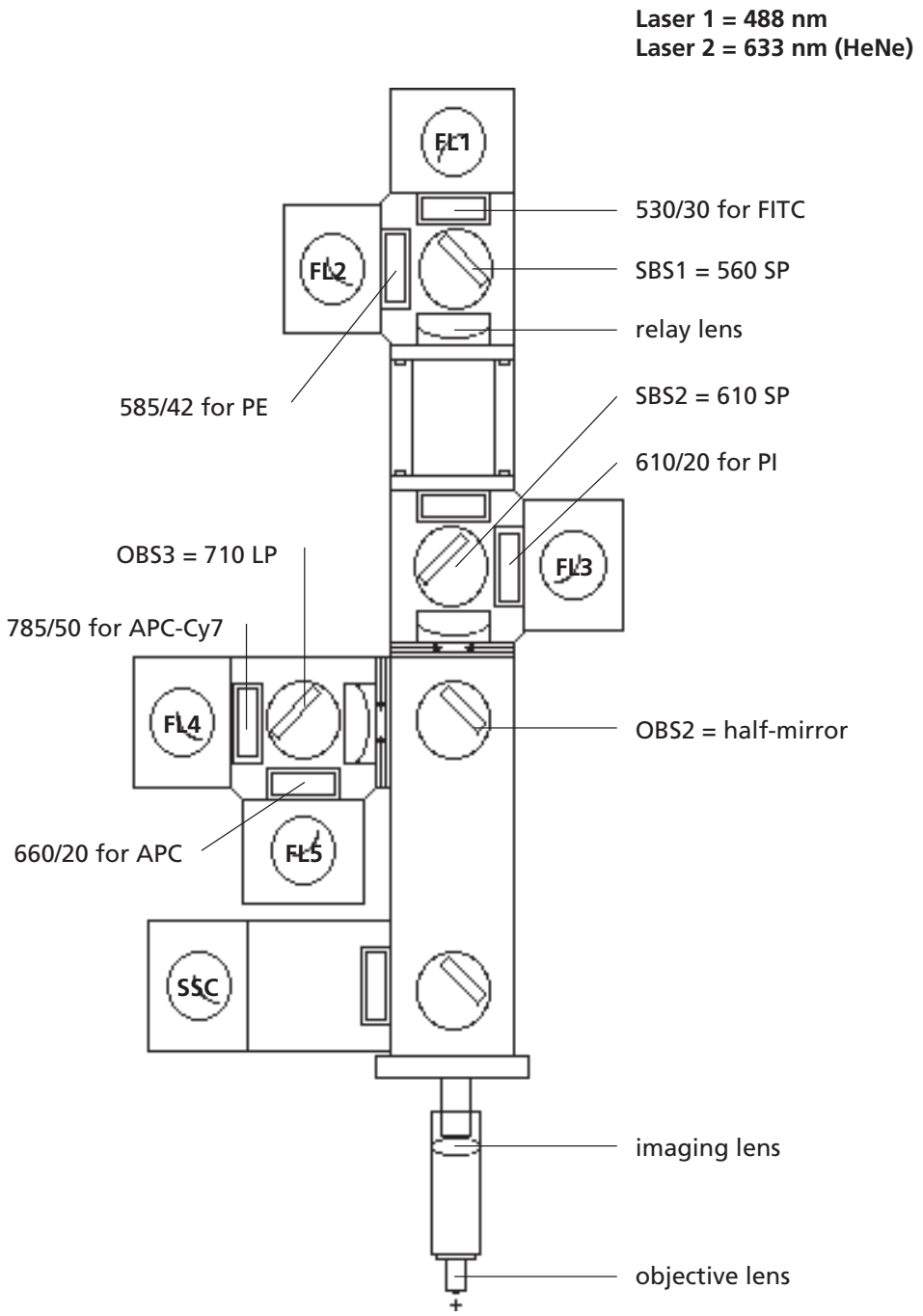
Example 5: Base Configuration + 2 Optional Detectors— 5 Colors/2 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

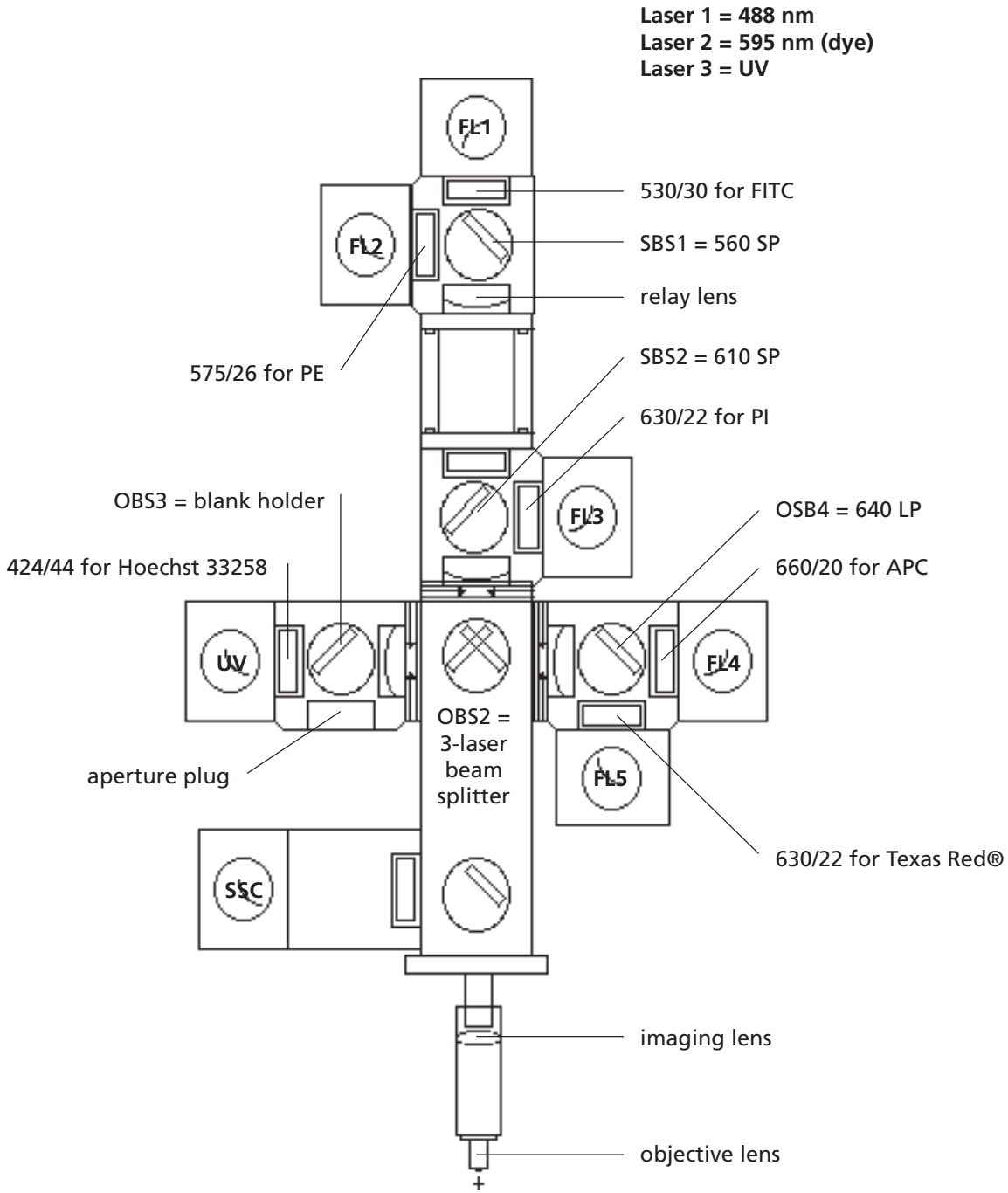
Example 6: Base Configuration + 2 Optional Detectors— 5 Colors/2 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

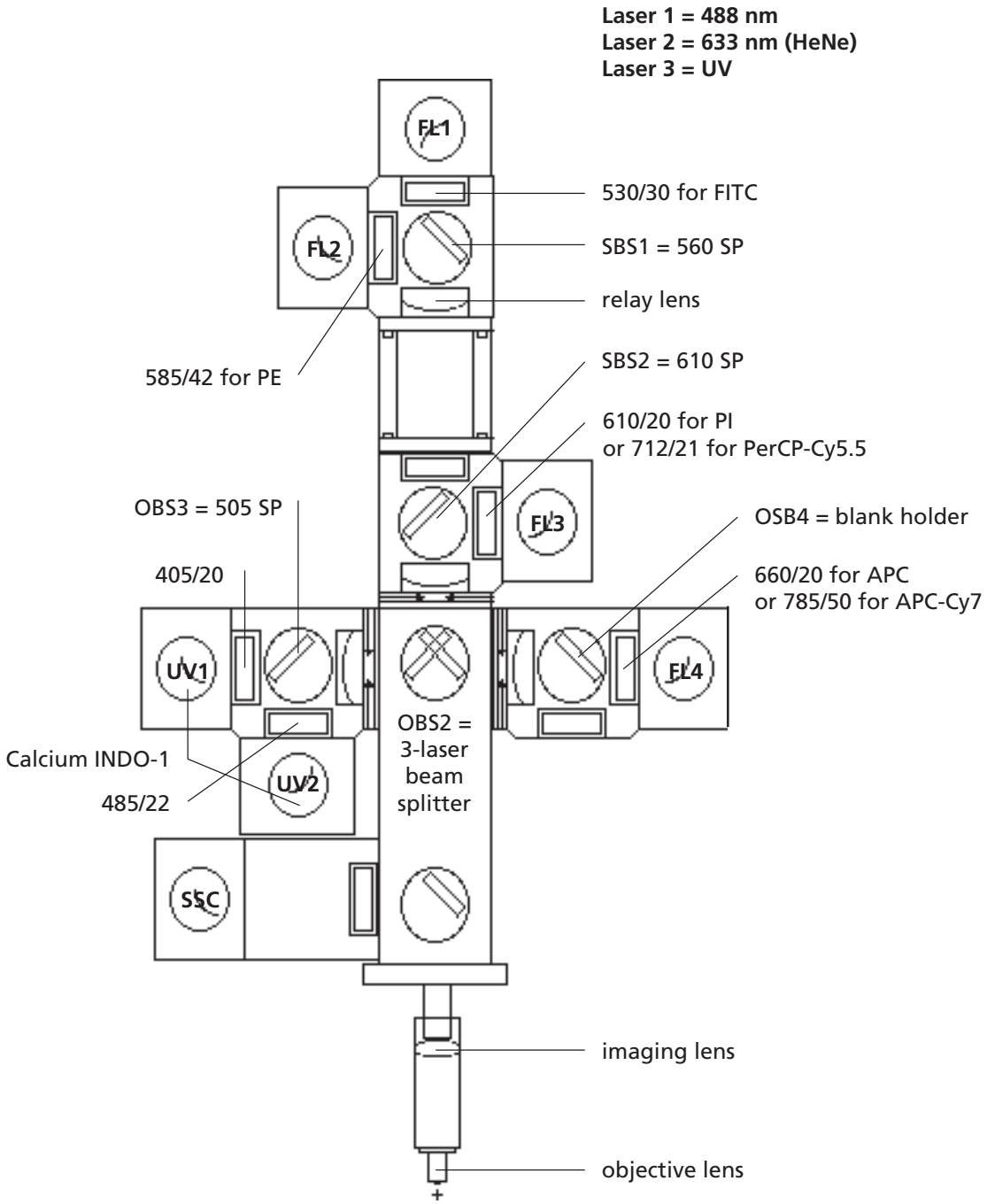
Example 7: Base Configuration + 3 Optional Detectors— 5 Colors + DNA/3 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

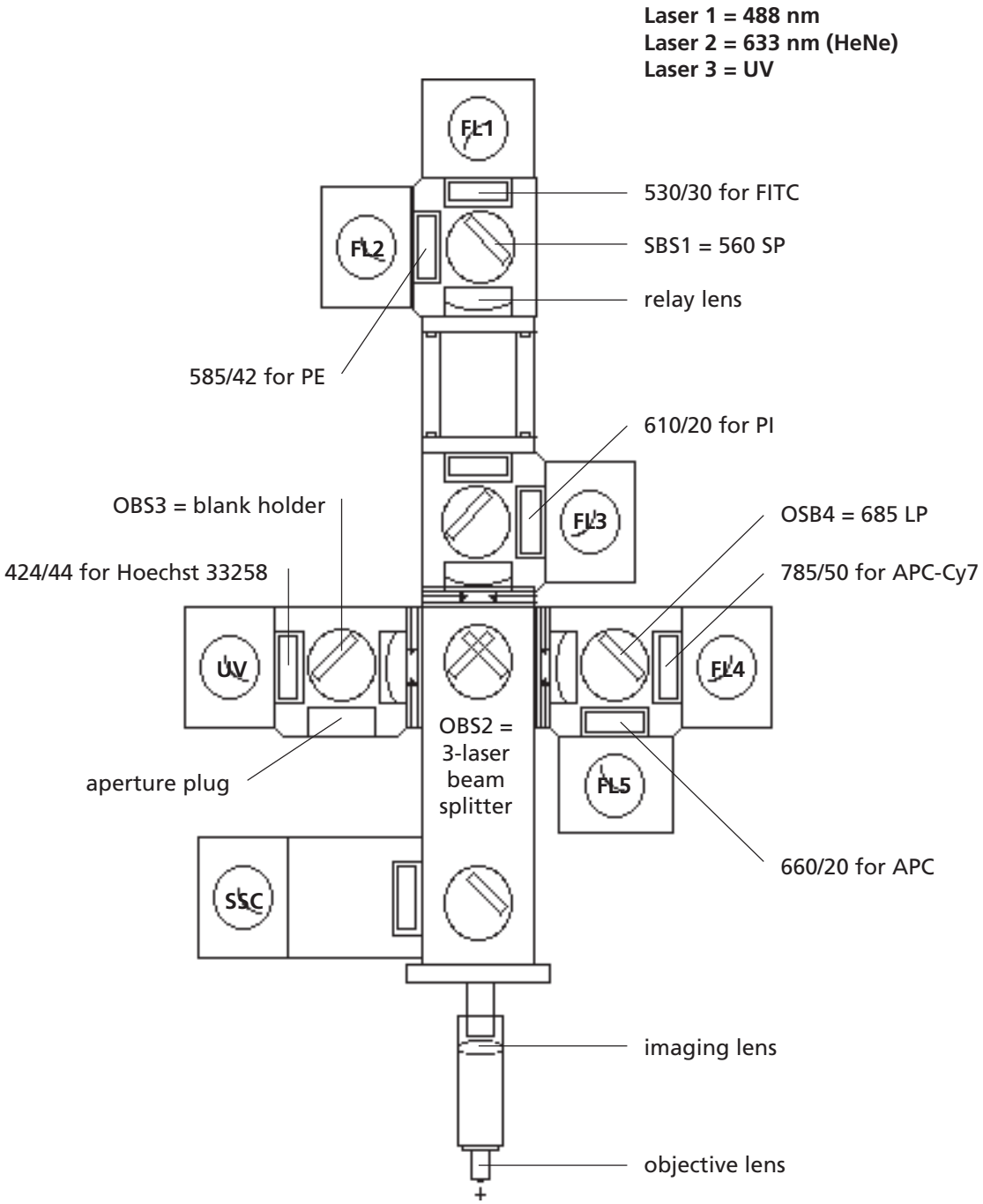
Example 8: Base Configuration + 3 Optional Detectors— 4 Colors + Calcium/3 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

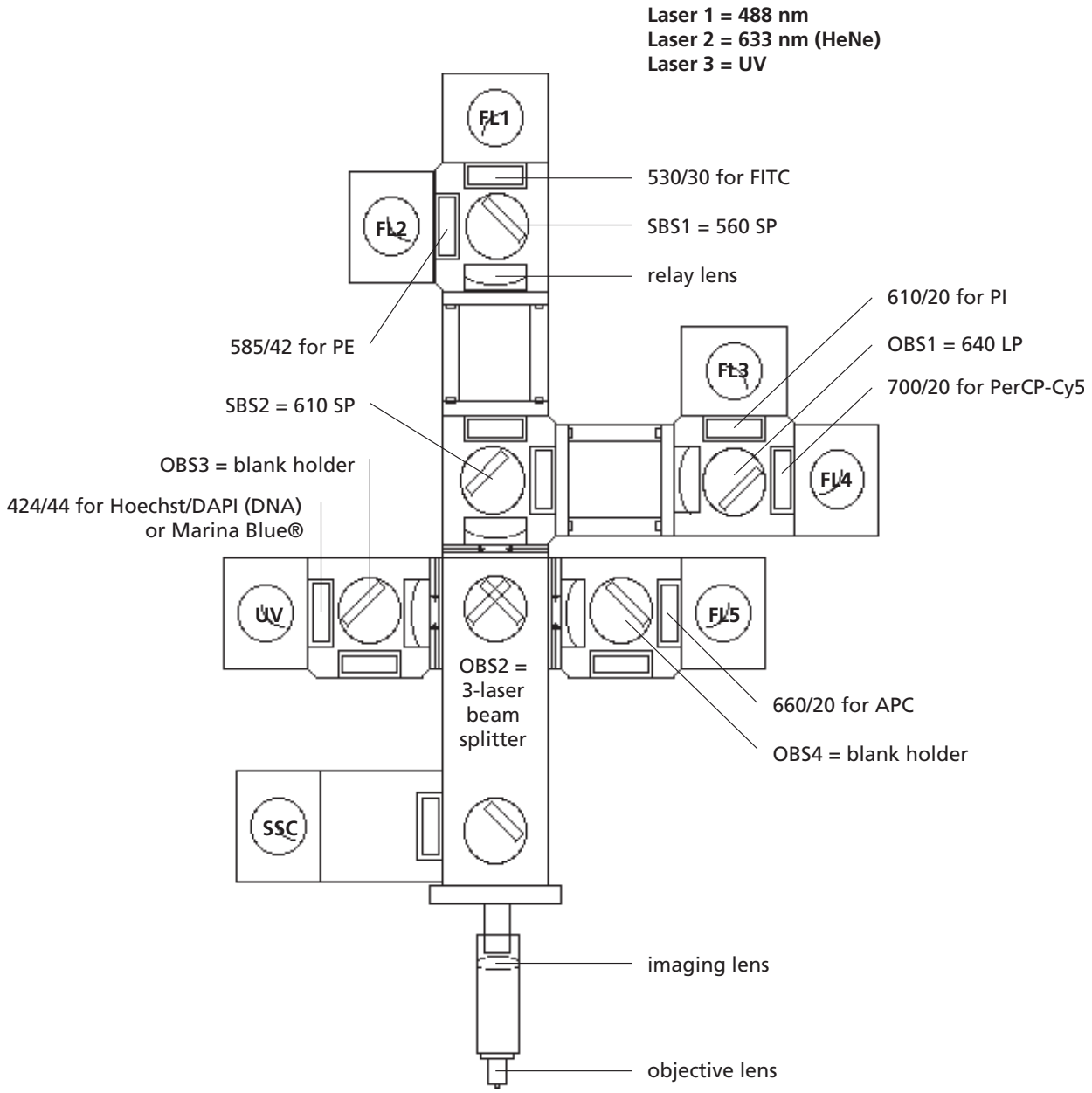
Example 9: Base Configuration + 3 Optional Detectors— 6 Colors/3 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

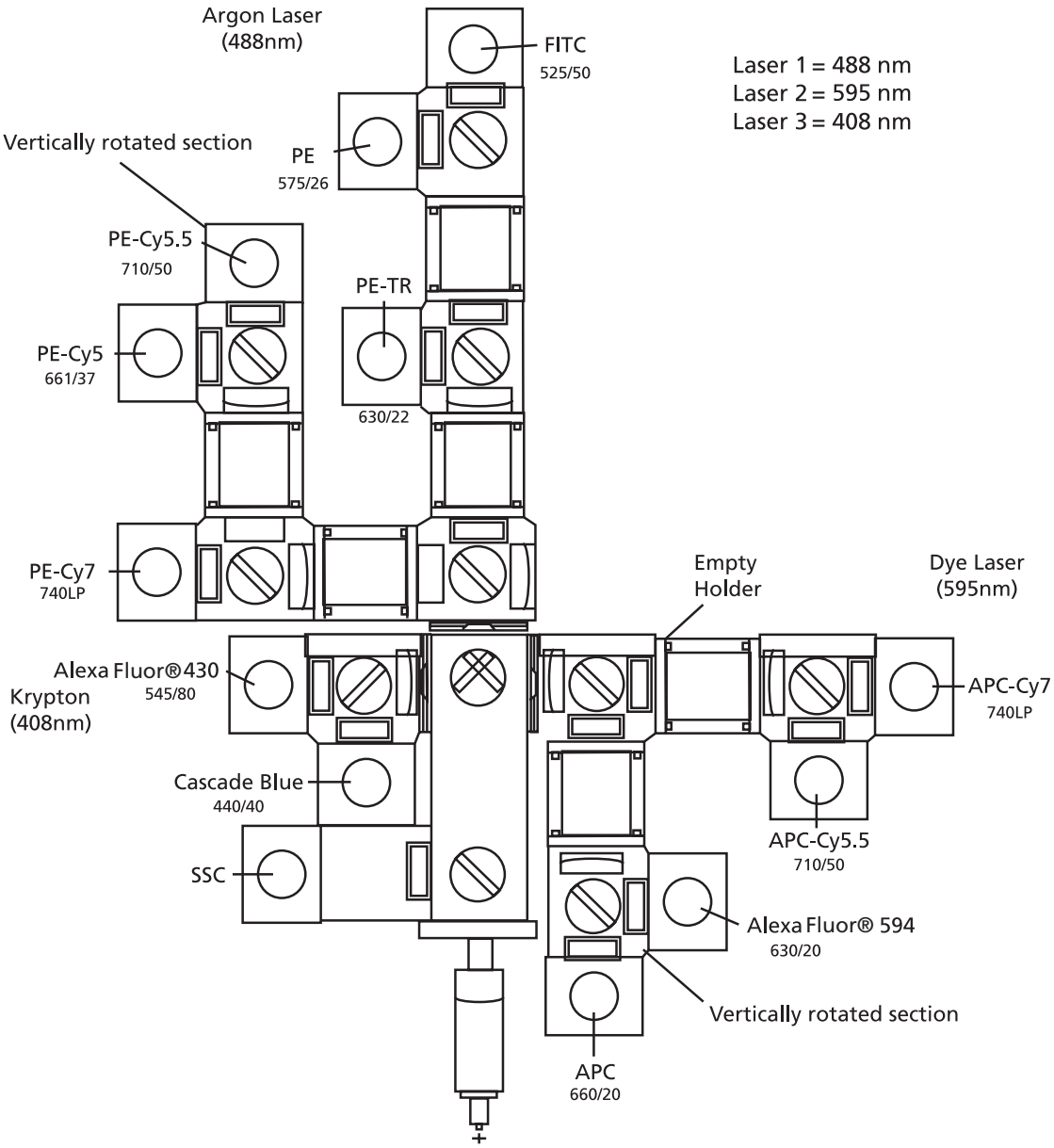
Example 10: Base Configuration + 3 Optional Detectors— 6 Colors/3 Lasers



Technical Resources

BD FACSVantage™ SE Configuration *(continued)*

Example 11: Base Configuration + 9 Optional Detectors—12 Colors/3 Lasers



Lasers and Fluorochromes by Application

Spectrum view: **www.bdbiosciences.com/spectra/**

	BD LSR II			BD FACSAria			BD FACSCalibur			BD FACSArray	BD FACSVantage SE								
	Lightwave Solid State UV	Kimmon HeCd	Coherent Violflame	Sapphire 20 mW	JDS Uniphase HeNe	Point Source Violet	Uniphase Argon-Ion	Hitachi diode	JDS Uniphase 532 nm	Omnichrome HeCd	Innova 70C-Spectrum	Innova 302C Krypton	Innova 90C-A4 Innova 90C-A5 Innova 304C-UV Innova 305C-UV Innova 306C-UV	Enterprise IIC	Innova 70C-4	Spectra-Physics 177G	Sapphire 200 mW	Spectra-Physics HeNe
Immunophenotyping																		
AMCA 350	445									445	445	445	445	445				
Marina Blue®	460									460	460	460	460	460				
Cascade Blue®			419			419					419	419	419	419				
Alexa Fluor® 405			422			422					422	422	422	422	422			
Pacific Blue®			455			455					455	455	455	455	455			
Alexa Fluor® 430			540			540					540		540	540	540			
FITC				525			525				525		525	525	525	525	525	
Alexa Fluor® 488				525			525				525		525	525	525	525	525	
PE				575			575		575		575		575	575	575	575	575	
PE-Texas Red®				613			613				613		613	613	613	613	613	
PE-Cy5				670			670				670		670	670	670	670	670	
PerCP				670			670				670					670		
PerCP-Cy5.5				695			695		695		695					695		
PE-Cy7				760			760		760		760		760	760	760	760	760	
Cascade Yellow™											570	570	570		570			
Rhodamine											580							
TR											630							
APC					670			670			670							670
Alexa Fluor® 647					675			675*			675	675						675
APC-Cy7					760			760			760							760
Alexa Fluor® 700												719						
DNA, RNA, Viability																		
Hoechst 33342	475	475	475			475				475	475	475	475	475				
DAPI	480	480	480			480				480	480	480	480	480				
Chromomycin													575	575				
Thiazole Orange				525			525				525		525	525	525	525	525	
AO (RNA)				530			530				530		530	530	530	530	530	
AO (DNA)				650			650				650		650	650	650	650	650	
PI				620			620				620		620	620	620	620	620	
7-AAD				660			660				660		660	660	660	660	660	
Ethidium Monoazide				625			625				625		625	625	625	625	625	
Pyronin Y				575			575				575		575	575	575	575	575	
TO-PRO®-3											660	660						
Cell Function																		
Indo-1 (bound)	405	405								405	405		405	405				
Indo-1 (free)	485	485								485	485		485	485				
Fluo-3				525			525				525		525	525	525	525	525	
DiOCn				525			525				525		525	525	525	525	525	
Rhodamine 123				525			525				525		525	525	525	525	525	
SNARF® (low pH)				575			575				575		575	575	575	575	575	
SNARF® (high pH)				660			660				660		660	660	660	660	660	

** Validation in progress*

All applications are either tested in-house or reported in the literature. See Technical Data Sheets for details. Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.

Technical Resources

Laser Output Table

	BD LSR II				BD FACSAria			BD FACSCalibur		BD FACSArray	BD FACSVantage SE												
	Lightwave Solid State UV	Kimmon HeCd	Coherent Violflame	Sapphire 20 mW	JDS Uniphase HeNe	Point Source Violet	Uniphase Argon-Ion	Hitachi diode	JDS Uniphase 532 nm	Omnichrome HeCd	Innova 70C-Spectrum	Innova 302C Krypton	Innova 90C-A4	Innova 90C-A5	Innova 304C-UV	Innova 305C-UV	Innova 306C-UV	Enterprise IIC	Innova 70C-4	Spectra-Physics 177G	Sapphire 200 mW	Spectra-Physics HeNe
Multiline Visible											2.500W		4.000W	5.000W	4.000W	5.000W	6.000W		4.00W			
UV (MM) 325 nm	0.020W	0.008W								0.035W	0.050W											
Multiline UV (Ar-Kr) 350.7-363.8 nm																						
Multiline UV (Kr) 337.5-356.4 nm												0.500w										
Multiline UV (Ar) 351.1-363.8 nm													0.200W	0.300W	0.20W	0.400W	0.500W	0.05W				
Multiline Violet 406.7-415.4 nm												0.600w										
Violet 406.7 nm			0.025W			0.017W						0.200w										
Violet 413.1 nm												0.300w										
Blue 457.9 nm											0.030W		0.300W	0.350W	0.250W	0.350W	0.420W		0.300W			
Blue 476.2 nm													0.500W	0.600W					0.500W			
Blue 482.5 nm																						
Blue 488 nm				0.020W			0.020W				0.250W		1.300W	1.500W	1.300W	1.500W	1.800W	0.180W	1.300W	0.100W	0.200W	
Blue 514.5 nm											0.250W		1.700W	2.000W	1.700W	2.000W	2.400W		1.700W			
Green 520.8 nm											0.130W	0.070W										
Green 528.7 nm													0.300W	0.350W	0.200W	0.350W	0.420W		0.300W			
Green 530.9 nm											0.130W											
Green 532 nm									?													
Yellow 588.3 nm											0.150W											
Red 633 nm					0.018W			0.005W														0.035W
Red 647 nm											0.250W	0.800W										
Far Red 676 nm												0.150W										

Technical Resources

Choosing Antibody-Fluorochrome Combinations

When performing multicolor flow cytometric analysis, a major factor in the success of the analysis is the choice of which antibody to use with which fluorochrome. There are often many *correct* combinations possible. A number of factors needs to be considered in making these choices.

Intensity (Brightness) of the Fluorochrome/mAb Conjugate

Each fluorochrome differs in its relative fluorescence intensity. The ability of a given antibody to resolve a positive from a negative result depends on which fluorochrome conjugate is used. Figure 1 shows an example of the staining pattern using the same monoclonal antibody (mAb) conjugated to eight different fluorochromes.

Note the following.

- For a given mAb the S/N ratio of positive and negative cells can differ four- to six-fold depending on the fluorochrome used.
- The relative fluorochrome intensity depends on the instrument (not shown). This is because of differences in the laser and filter combinations used on the different instruments.

A general guideline for the relative intensities of the various fluorochromes is shown in the following table. Note that this is a general pattern. Some differences are seen for individual mAbs.

INSTRUMENT	RELATIVE FLUOROCHROME INTENSITY (BRIGHTER→DIMMER)
BD FACSVantage SE instrument equipped with an Enterprise IIC laser at 180 mW and a HeNe laser at 35 mW	PE, PE-Cy7, PE-Cy5, APC > PerCP-Cy5.5, APC-Cy7 > PerCP, FITC

F/P Ratio

The number of fluorochromes present on the antibody (F/P ratio) can also affect the relative brightness. FITC and PerCP conjugates are made with several (2 to 9 depending on the antibody) fluorochromes per antibody, while APC and PE conjugates are made at approximately one fluorochrome per antibody. Tandem conjugates of Cy7 with PE and APC have multiple Cy7 molecules bound per PE or APC protein, whereas approximately one PE or APC molecule is conjugated to each antibody. In contrast, tandem conjugates of Cy5.5 with PerCP contain approximately one Cy5.5 molecule per PerCP molecule. Because of the conjugation chemistry required, IgM antibodies are conjugated only to small-molecule fluorochromes, such as FITC, Texas Red®, Cy3, and Cy5.

BD FACSVantage™ SE Flow Cytometer

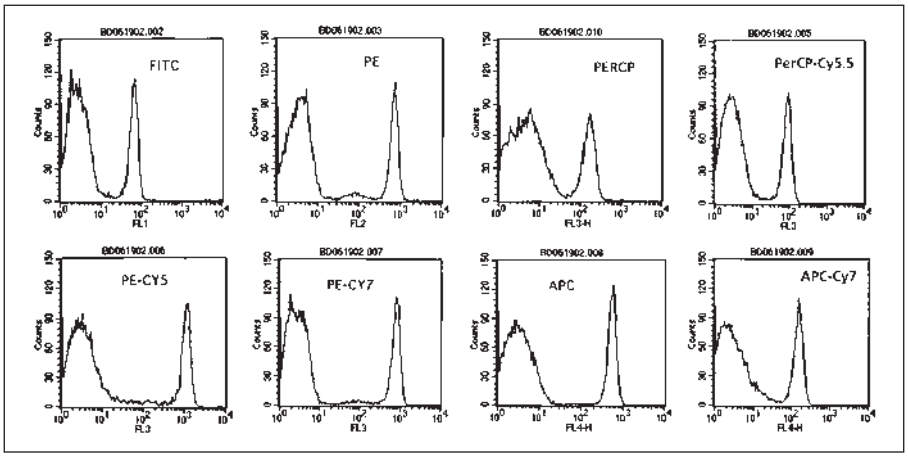


Figure 1. Signal-to-noise (S/N) ratios on the BD FACSVantage SE instrument for various fluorochromes conjugated to the same mAb

Technical Resources

Choosing Antibody-Fluorochrome Combinations *(continued)*

Antigen Density

A highly expressed antigen will be resolved with almost any fluorophore. An antigen expressed at lower density might require the higher S/N ratio provided by a PE or APC conjugate to separate the positive cells adequately from the unstained cells.

Autofluorescence

Individual cell populations have characteristic levels of autofluorescence (fluorescent signals generated by the cells themselves). While autofluorescence is observed in all fluorescence channels, it decreases dramatically at longer wavelengths (>600 nm).

- For cell types that are very autofluorescent, using a dye with a longer emission wavelength (eg, APC) can give a better S/N ratio.
- For cell types that are not very autofluorescent, the improved separation seen with long-wavelength excitation is less apparent. FITC conjugates can be used.

Non-Specific Binding

A number of antibody conjugates exhibit low-level non-specific binding that can increase the fluorescence of negative cells to levels above autofluorescence. This non-specific binding is typically caused by the following.

- Isotype of the mAb

  Some IgG isotypes are more likely to bind to Fc receptors on some cell types.

- Fluorochrome used

  Carbocyanin (Cy3, Cy5, Cy5.5, and Cy7) and Texas Red® direct conjugates and certain tandem conjugates can sometimes show a tendency to increased binding to some cell subsets. In the case of Cy5 this has been shown to be caused by a very low affinity interaction of the dye with the low affinity Fc Receptors. This is also a property of PE-Cy5 tandem conjugates.

NOTE: In certain situations, such as in the identification of monocytes using our BD QuantiBRITE Anti–HLA-DR PE/Anti-Monocyte PerCP-Cy5.5 product, this property can be exploited by intentionally putting more carbocyanin dye on the conjugate. This helps to ensure detecting of all monocytes regardless of CD14 antigen expression levels.

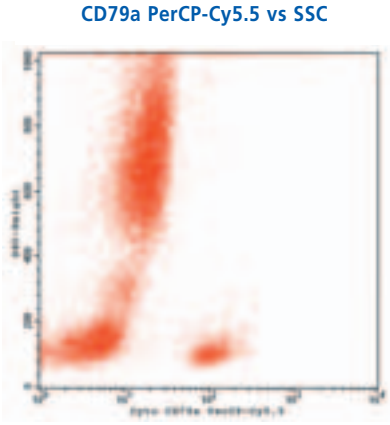
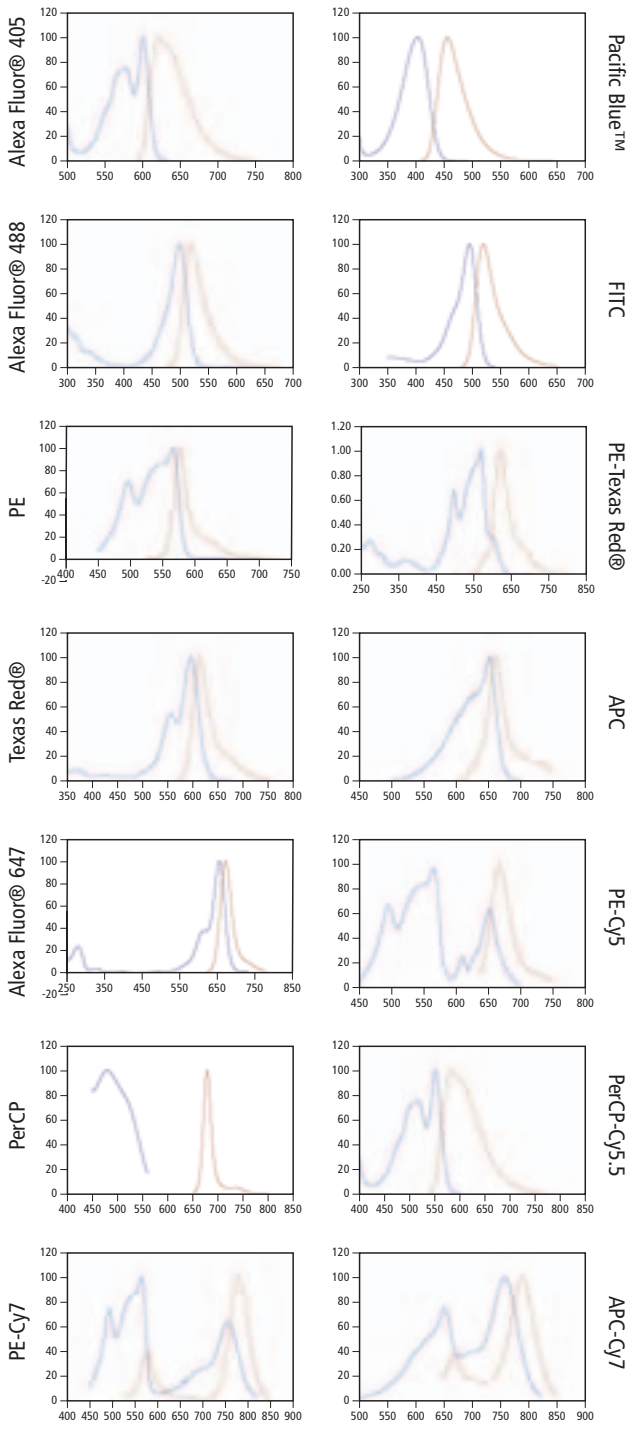


Figure 2. *PerCP-Cy5.5 tandem conjugates differ from PE-Cy5 tandem conjugates in that substantially less of the carbocyanin component is required to achieve more intense emission. As a result, the degree of monocyte staining of these reagents can be virtually eliminated as demonstrated in the CD79a PerCP-Cy5.5 vs. SSC plot.*

Technical Resources

Absorption and Emission Spectra of BD Biosciences Fluorochromes



Absorption: Blue Emission: Red

Direct Immunofluorescence Staining of Whole Blood Using a Lyse/No-Wash Procedure

Scope

Use this method to detect cells bearing specific membrane antigens. Begin by adding whole blood to fluorochrome-conjugated monoclonal antibodies that bind specifically to cell surface antigens. Next, treat the stained sample with FACS Lysing Solution to lyse erythrocytes under gentle hypotonic conditions while preserving the leucocytes. Finally, analyze the cells by flow cytometry.

This protocol is optimized for use with MultiTEST and TriTEST reagents and with TruCOUNT Tubes to perform absolute counts.

Reagents and Equipment Required

Refer to the appropriate product labeling for intended use and precautions.

1. K_3 EDTA VACUTAINER blood collection tubes (BD Cat. No. **6457**) or equivalent
2. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent
3. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246** or equivalent)
4. BD fluorochrome-conjugated monoclonal antibodies to human cell surface antigens (for example, MultiTEST or TriTEST reagents). Refer to the appropriate reagent package insert for more information.
5. Vortex mixer
6. FACS Lysing Solution (10X) (BD Cat. No. **349202**). For dilution instructions and warnings, refer to the *FACS Lysing Solution* package insert.
7. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.

Procedure

Specimen Collection and Preparation

Collect blood aseptically by venipuncture[1,2] into a sterile K_3 EDTA VACUTAINER blood collection tube. Follow the collection tube manufacturer's guidelines for the minimum volume of blood to be collected. Store anticoagulated blood at room temperature (20° to 25°C) until ready for staining and lysing. Refer to the appropriate package insert for storage restrictions prior to staining.

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

Lysing and Staining

1. Add 20 µL of fluorochrome-conjugated monoclonal antibody to 50 µL of whole blood in a 12 x 75-mm tube.
2. Vortex gently and incubate for 15 minutes in the dark at room temperature (20° to 25°C).
3. Add 450 µL of 1X FACS Lysing Solution.
4. Vortex gently and incubate for 15 to 30 minutes in the dark at room temperature.
5. Analyze on a FACS brand flow cytometer. Mix samples thoroughly before acquisition. Refer to the appropriate package insert for storage restrictions prior to analysis.

Direct Immunofluorescence Staining of Whole Blood Using a Lyse/No-Wash Procedure

Recommendations

1. Use EDTA as the anticoagulant. BD has limited information concerning use of other anticoagulants such as heparin.
2. FACS Lysing Solution is specifically formulated for use with BD FACS brand flow cytometers.
3. Samples with nucleated red blood cells can show incomplete lysis of red blood cells because FACS Lysing Solution does not lyse nucleated erythrocytes. This can also occur when assaying blood samples from patients with certain hematologic disorders in which red cells are difficult to lyse, as in myelofibrosis, sickle-cell anemia, thalassemia, and spherocytosis.[3]
4. When using monoclonal antibodies that react with serum immunoglobulins, blood samples should be washed with 1X PBS or physiological saline prior to staining and lysing.[4]
5. A monoclonal antibody against a cell surface antigen or receptor that is shed into plasma (for example, IL-2 receptor) or occupied by plasma components (for example, complement receptors) can have reduced staining intensity when analyzed with lysed whole blood methodology.

References

1. *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes; Tentative Guideline.* Villanova, PA: National Committee for Clinical Laboratory Standards; 1992. NCCLS document H42-T.
2. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard*. Villanova, PA: National Committee for Clinical Laboratory Standards; 1991. NCCLS document H3-A3.
3. Landay AL, Muirhead KA. Procedural guidelines for performing immunophenotyping by flow cytometry. *Clin Immunol Immunopath*. 1989;52:48-60.
4. Nicholson JKA, Rao PE, Calvelli T, et al. Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. *Cytometry*. 1994;18:140-146.

Direct Immunofluorescence Staining of Whole Blood Using a Lyse/Wash Procedure

Scope

Use this method to detect cells bearing specific membrane antigens. Begin by adding whole blood to fluorochrome-conjugated monoclonal antibodies that bind specifically to cell surface antigens. Next, treat the stained sample with FACS Lysing Solution to lyse erythrocytes under gentle hypotonic conditions while preserving the leucocytes; then wash the sample to remove excess antibody and debris. Finally, analyze the cells by flow cytometry.

Reagents and Equipment Required

Refer to the appropriate product labeling for intended use and precautions.

1. K_3 EDTA VACUTAINER blood collection tubes (BD Cat. No. **6457**) or equivalent.
2. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent.
3. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246** or equivalent).
4. BD fluorochrome-conjugated monoclonal antibodies to human cell surface antigens (for example, Simultest reagents). Refer to the appropriate reagent package insert for more information.
5. Vortex mixer
6. FACS Lysing Solution (10X) (BD Cat. No. **349202**). For dilution instructions and warnings, refer to the *FACS Lysing Solution* package insert.
7. Centrifuge
8. Wash buffer: phosphate-buffered saline (PBS) with 0.1% sodium azide (Dulbecco's PBS without calcium, magnesium, or phenol red, pH 7.2 ± 0.2). Filter the PBS through a 0.2-µm filter prior to use; store at 2° to 8°C.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show the container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
9. 1% paraformaldehyde solution prepared in PBS with 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week.

   WARNING: Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. Can cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show the container or label. Dispose of according to federal, state, and local regulations.
10. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.

Procedure

Specimen Collection and Preparation

Collect blood aseptically by venipuncture[1,2] into a sterile K_3 EDTA VACUTAINER blood collection tube. Follow the collection tube manufacturer's guidelines for the minimum volume of blood to be collected. Store anticoagulated blood at room temperature (20° to 25°C) until ready for staining and lysing. Refer to the appropriate package insert for storage restrictions prior to staining.

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

Lysing and Staining

1. Add appropriate volume of fluorochrome-conjugated monoclonal antibody to 100 µL of whole blood in a 12 x 75-mm tube.
2. Vortex gently and incubate 15 to 30 minutes in the dark at room temperature (20° to 25°C).
3. Add 2 mL of 1X FACS Lysing Solution.
4. Vortex gently and incubate for 10 minutes in the dark at room temperature.
5. Centrifuge at 500 x *g* for 5 minutes. Remove the supernatant.

Direct Immunofluorescence Staining of Whole Blood Using a Lyse/Wash Procedure

6. Add 2 to 3 mL of wash buffer and centrifuge at 500 x *g* for 5 minutes. Remove the supernatant.
7. Add 0.5 mL of 1% paraformaldehyde solution and mix thoroughly. Store at 2° to 8°C until analyzed.
8. Analyze on a FACS brand flow cytometer. Mix samples thoroughly before acquisition. Refer to the appropriate package insert for storage restrictions prior to analysis.

Recommendations

1. Use EDTA as the anticoagulant. BD has limited information concerning use of other anticoagulants such as heparin.
2. FACS Lysing Solution is specifically formulated for use with BD FACS brand flow cytometers.
3. Samples with nucleated red blood cells can show incomplete lysis of red blood cells because FACS Lysing Solution does not lyse nucleated erythrocytes. This can also occur when assaying blood samples from patients with certain hematologic disorders in which red cells are difficult to lyse, as in myelofibrosis, sickle-cell anemia, thalassemia, and spherocytosis.[3]
4. When using monoclonal antibodies that react with serum immunoglobulins, blood samples should be washed with 1X PBS or physiological saline prior to staining and lysing.[4]
5. A monoclonal antibody against a cell surface antigen or receptor that is shed into plasma (for example, IL-2 receptor) or occupied by plasma components (for example, complement receptors) can have reduced staining intensity when analyzed with lysed whole blood methodology.

References

1. *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes; Tentative Guideline*. Villanova, PA: National Committee for Clinical Laboratory Standards; 1992. NCCLS document H42-T.
2. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard*. Villanova, PA: National Committee for Clinical Laboratory Standards; 1991. NCCLS document H3-A3.
3. Landay AL, Muirhead KA. Procedural guidelines for performing immunophenotyping by flow cytometry. *Clin Immunol Immunopath*. 1989;52:48-60.
4. Nicholson JKA, Rao PE, Calvelli T, et al. Artifactual staining of monoclonal antibodies in two-color combinations is due to an immunoglobulin in the serum and plasma. *Cytometry*. 1994;18:140-146.

Direct Immunofluorescence Staining of Whole Blood Using Ammonium Chloride Lysing Solution

Scope

Use this method to detect cells bearing specific membrane antigens. Begin by treating whole blood with ammonium chloride lysing solution to lyse erythrocytes. Next, wash the sample and add to fluorochrome-conjugated monoclonal antibodies that bind specifically to cell surface antigens. Finally, wash again to remove excess antibody and debris. Analyze the cells by flow cytometry.

NOTE: The possibility of cellular aggregates exists with this procedure. Refer to The Escapee Phenomenon *technical update.*

Reagents and Equipment Required

1. K_3 EDTA VACUTAINER blood collection tubes (BD Cat. No. **6457**) or equivalent.
2. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246**) or equivalent.
3. Falcon 15-mL conical tubes (BD Cat. No. **2099**) or equivalent.
4. Ammonium chloride lysing solution

*NOTE: This is **not** FACS Lysing Solution.*

To prepare 10X ammonium chloride lysing solution, dissolve the following in 1 liter of distilled water:

89.9 g NH_4Cl

10.0 g $KHCO_3$

370.0 mg tetrasodium EDTA

Adjust to pH 7.3. Store at 2° to 8°C in a tightly closed bottle.

To prepare 1X ammonium chloride lysing solution: add 10 mL of 10X ammonium chloride lysing solution to 90 mL of distilled water, and mix well. Store at room temperature (20° to 25°C). Discard after one week.

5. Centrifuge
6. Vortex mixer
7. Wash buffer: phosphate-buffered saline (PBS) containing 0.1% sodium azide (Dulbecco's PBS without calcium, magnesium, or phenol red, pH 7.2 ± 0.2). Filter the PBS through a 0.2-µm filter prior to use; store at 2° to 8°C.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show the container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
8. Staining buffer: PBS containing 0.1% sodium azide and 2% fetal bovine serum (FBS). Store at 2° to 8°C.
9. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent
10. BD fluorochrome-conjugated monoclonal antibodies to human cell surface antigens. Refer to the appropriate reagent package insert for more information.
11. 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week.

    WARNING: Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. Can cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show the container or label. Dispose of according to federal, state, and local regulations.
12. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.

Direct Immunofluorescence Staining of Whole Blood Using Ammonium Chloride Lysing Solution

Procedure

Specimen Collection and Preparation

Collect blood aseptically by venipuncture[1,2] into a sterile K_3 EDTA VACUTAINER blood collection tube. Follow the collection tube manufacturer's guidelines for the minimum volume of blood to be collected. Store anticoagulated blood at room temperature (20° to 25°C) until ready for staining and lysing. Refer to the appropriate package insert for storage restrictions prior to staining.

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

Lysing

1. Add 1 mL of blood to a 15-mL tube containing 14 mL of room-temperature (20° to 25°C) 1X ammonium chloride lysing solution. Cap tube and mix immediately by inverting.

   Do **not** use FACS Lysing Solution for this procedure. See the *FACS Lysing Solution* package insert for directions on using this reagent.
2. Let mixture stand at room temperature for 3 to 5 minutes. Do **not** extend incubation beyond this time.
3. Centrifuge at 300 x *g* for 5 minutes at room temperature.
4. Remove the supernatant.
5. Add 5 mL of cold wash buffer. Vortex gently and centrifuge at 300 x *g* for 5 minutes at 2° to 8°C.
6. Remove the supernatant. Resuspend pellet in 1 mL of staining buffer.
7. Test for viability. The specimen should contain >90% viable cells. The cytoplasm of dead cells stains nonspecifically, especially with FITC-conjugated antibodies.

Staining Procedure

1. Add 50 µL of the cell preparation to each 12 x 75-mm tube. If the white cell count is above or below normal, the volume can be adjusted. Total volume should not exceed 100 µL.
2. Add appropriate volume of fluorochrome-conjugated monoclonal antibody.
3. Incubate on ice for 30 minutes in the dark.
4. Add 2 to 3 mL of wash buffer and mix gently.
5. Centrifuge at 200 x *g* for 5 minutes.
6. Remove the supernatant.
7. Add 0.5 mL of 1% paraformaldehyde solution and mix thoroughly. Store at 2° to 8°C until analyzed.
8. Analyze on a FACS brand flow cytometer. Mix samples thoroughly before acquisition.

Recommendations

1. Each research laboratory should establish normal ranges using its own test conditions.
2. Avoid excessive centrifuge speeds and vigorous mixing of cells. Cell damage can cause non-specific staining, cell clumping, and excessive debris.
3. The amounts of antibodies recommended for use are based on studies of normal human blood.
4. Monoclonal reagents that react with granulocytes (for example, CD11b or CD16) can require additional amounts of antibody to saturate all binding sites.
5. Donors with nucleated red blood cells can show incomplete lysis of the red blood cell population.

References

1. *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes; Tentative Guideline.* Villanova, PA: National Committee for Clinical Laboratory Standards; 1992. NCCLS document H42-T.
2. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard.* Villanova, PA: National Committee for Clinical Laboratory Standards; 1991. NCCLS document H3-A3.

Direct Immunofluorescence Staining of Mononuclear Cells

Scope

Use this method to detect cells bearing specific membrane antigens. Begin by adding peripheral blood mononuclear cells (PBMCs) to fluorochrome-conjugated monoclonal antibodies that bind specifically to cell surface antigens. Next, wash the stained sample to remove excess antibody and debris. Analyze the cells by flow cytometry.

Reagents and Equipment Required

1. VACUTAINER Cell Preparation Tubes (CPTs) (BD Cat. No. **362753**) or Ficoll-Paque separation medium. Refer to the Ficoll-Paque package insert for materials and reagents required.
2. Falcon 15-mL conical tubes (BD Cat. No. **2099**) or equivalent.
3. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent.
4. Optional: Falcon 96-well U-bottom microtiter plates (BD Cat. No. **353918**) or equivalent.
5. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246**) or equivalent.
6. BD fluorochrome-conjugated monoclonal antibodies to human cell surface antigens. Refer to the appropriate reagent package insert for more information.
7. Centrifuge
8. Wash buffer: phosphate-buffered saline (PBS) containing 0.1% sodium azide (Dulbecco's PBS without calcium, magnesium, or phenol red,pH 7.2 ± 0.2). Filter the PBS through a 0.2-µm filter prior to use; store at 2° to 8°C.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show the container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
9. Staining buffer: PBS containing 0.1% sodium azide and 2% fetal bovine serum (FBS). Store at 2° to 8°C.
10. 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week.

    WARNING: Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. Can cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show the container or label. Dispose of according to federal, state, and local regulations.
11. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.

Direct Immunofluorescence Staining of Mononuclear Cells

Procedure

Specimen Collection and Preparation

Collect blood aseptically by venipuncture[1,2] into a VACUTAINER CPT containing sodium heparin. Follow the manufacturer's collection guidelines for the minimum volume of blood to be collected. Before storage, centrifuge CPTs and resuspend PBMCs in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature (20° to 25°C) on its side. Use within 24 hours of collection. PBMCs can also be separated from whole blood by Ficoll-Paque density-gradient centrifugation.

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

1. Transfer cell suspension to 15-mL conical tubes. Wash with wash buffer.
2. Resuspend cells in staining buffer and adjust concentration of cell suspension to 2 x 10^7 cells/mL. Cells should be >90% viable.
3. Add 50 µL of the cell suspension (1 x 10^6 cells) to each microtiter plate well or to each 12 x 75-mm tube.
4. Add appropriate volume of fluorochrome-conjugated monoclonal antibody.
5. For staining in microtiter plates: Incubate the mixture for 30 to 45 minutes on ice. Centrifuge at 200 x *g* for 3 minutes at 2° to 8°C. Remove the supernatant. Wash two times with 100 µL of cold wash buffer. Centrifuge after each washing at 200 x *g* for 3 minutes. Remove the supernatant. Resuspend cells in 200 µL of 1% paraformaldehyde solution and transfer samples to 12 x 75-mm tubes containing 300 µL of 1% paraformaldehyde solution.

   For staining in tubes: Incubate the mixture for 30 to 45 minutes on ice. Add 2 mL of cold wash buffer. Centrifuge at 300 x *g* for 5 minutes at 2° to 8°C. Remove the supernatant. Resuspend cells in 0.5 mL of 1% paraformaldehyde solution to approximately 1 x 10^6 cells/mL.
6. Store at 2° to 8°C until analyzed.
7. Analyze on a FACS brand flow cytometer. Mix samples thoroughly before acquisition.

References

1. *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes; Tentative Guideline*. Villanova, PA: National Committee for Clinical Laboratory Standards; 1992. NCCLS document H42-T.
2. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard*. Villanova, PA: National Committee for Clinical Laboratory Standards; 1991. NCCLS document H3-A3.

Indirect Immunofluorescence Staining of Mononuclear Cells

Scope

The indirect method is used to enhance the fluorescence signal and also to facilitate multicolor staining of human cells when direct conjugated reagents are not available.[1,2] BD offers two methods for indirect staining:

- biotin-avidin (or streptavidin)
- conventional second antibody

In the biotin-avidin method, cells are incubated first with biotin-conjugated monoclonal antibody and then subsequently incubated with fluorochrome-conjugated avidin. In the second antibody method, cells are incubated with an unconjugated monoclonal antibody followed by fluorochrome-conjugated goat anti-mouse Ig.

These sandwich techniques provide increased fluorescence intensity, which is valuable for microscopy. However, indirect methods can result in the formation of complexes that will artifactually stain Fc receptor-binding cells. The biotin-avidin method gives the least amount of artifactual staining particularly when streptavidin conjugates are used. Indirect methods will alter the proportionality between the amount of antigen and the fluorescence intensity per cell, therefore, these methods are not recommended for assessing the absolute number of antigenic determinants per cell. However, indirect methods can be used to determine the relative brightness differences between cell populations.

Reagents and Equipment

1. VACUTAINER Cell Preparation Tubes (CPTs) (BD Cat. No. **362753**) or Ficoll-Paque separation medium. Refer to the Ficoll-Paque package insert for materials and reagents required.
2. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent.
3. Optional: Falcon 96-well U-bottom microtiter plates (BD Cat. No. **353918**) or equivalent.
4. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246** or equivalent).
5. BD biotin-conjugated monoclonal antibody to human surface antigen; unconjugated monoclonal antibody.
6. Wash buffer: phosphate-buffered saline (PBS) containing 0.1% sodium azide and 2% fetal bovine serum (FBS). Store at 2° to 8°C. Do not use PBS containing biotin with the biotin-avidin indirect method.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show the container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
7. BD fluorochrome-conjugated avidin or fluorochrome- conjugated streptavidin monoclonal antibody; fluorochrome-conjugated goat anti-mouse Ig monoclonal antibody.
8. Centrifuge
9. 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week.

   WARNING: Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. Can cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show the container or label. Dispose of according to federal, state, and local regulations.
10. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.

Indirect Immunofluorescence Staining of Mononuclear Cells

Procedure

Specimen Collection and Preparation

Collect blood aseptically by venipuncture[1,2] into VACUTAINER CPTs containing sodium heparin. Follow the manufacturer's collection guidelines for the minimum volume of blood to be collected. Before storage, centrifuge CPTs and resuspend peripheral blood mononuclear cells (PBMCs) in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature (20° to 25°C) on its side. Use within 24 hours of collection. PBMCs can also be separated from whole blood by Ficoll-Paque density-gradient centrifugation.

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

1. Dilute unconjugated or biotin-conjugated monoclonal antibody by adding 20 of µL antibody to 30 µL of wash buffer for each test. Add the 50 µL of diluted antibody to microtiter wells or 12 x 75-mm tubes.
2. Adjust concentration of the cell suspension to 2 x 10^7 cells/mL of wash buffer. Cells should be >90% viable.
3. Add 50 µL of the cell suspension (1 x 10^6 cells) to each microtiter plate well or to each 12 x 75-mm tube.
4. For staining in microtiter plates: Incubate the mixture for 30 to 45 minutes on ice. Centrifuge at 200 x *g* for 3 minutes at 2° to 8°C. Remove the supernatant. Wash two times with 100 µL of cold wash buffer. Centrifuge after each washing at 200 x *g* for 3 minutes. Remove the supernatant.

   For staining in tubes: Incubate the mixture for 30 to 45 minutes on ice. Add 2 mL of cold wash buffer, and centrifuge at 300 x *g* for 5 minutes at 2° to 8°C. Remove the supernatant.
5. Dilute the appropriate second-step reagent (fluorochrome-conjugated avidin or goat anti-mouse Ig) in wash buffer according to the instructions provided on the appropriate second-step reagent data sheet.
6. For staining in microtiter plates: Add the second-step mixture to the wells and incubate for 30 to 45 minutes on ice. Centrifuge at 200 x *g* for 3 minutes at 2° to 8°C. Remove the supernatant. Wash two times with 100 µL of cold wash buffer. Centrifuge after each washing at 200 x *g* for 3 minutes. Remove the supernatant. Resuspend cells in 200 µL of 1% paraformaldehyde solution and transfer samples to 12 x 75-mm tubes containing 300 µL of 1% paraformaldehyde solution.

   For staining in tubes: Add the second-step mixture to the tubes and incubate for 30 to 45 minutes on ice. Add 2 mL of cold wash buffer. Centrifuge at 300 x *g* for 5 minutes at 2° to 8°C. Remove the supernatant. Resuspend cells in 0.5 mL of 1% paraformaldehyde solution to approximately 1 x 10^6 cells/mL.
7. Store at 2° to 8°C until analyzed.
8. Analyze on a FACS brand flow cytometer. Mix samples thoroughly before acquisition.

References

1. Parks DR, Lanier LL, Herzenberg LA. Flow cytometry and fluorescence activated cell sorting (FACS). In: Weir DM, Herzenberg LA, Blackwell C, eds. *Handbook of Experimental Immunology*. Oxford: Blackwell Scientific Publications; 1986:chap 29.
2. Jackson AL, Warner NL. Preparation, staining, and analysis by flow cytometry of peripheral blood leukocytes. In: Rose NR, Friedman H, Fahey JL, eds. *Manual of Clinical Laboratory Immunology*. 3rd ed. Washington, DC: American Society for Microbiology; 1986:226-235.

Flow Cytometric Procedure for Assessing Lymphocyte Activation

Scope

In vitro lymphocyte activation represents a standard approach for evaluating cell-mediated responses to a variety of stimuli, including polyclonal mitogens, antibodies, specific antigens, and cytokines. However, most methods for quantifying lymphocyte activation are time-consuming assays that result in no information about specific lymphocyte subsets responding to particular stimuli.

The FastImmune assay system monitors the expression of an early activation marker (CD69) in whole blood after stimulation with various mitogenic or antigenic stimuli. Lymphocyte response is measured after only a 4-hour incubation with the stimulus in a three-color lyse/no-wash flow cytometric assay. CD69 is expressed on all activated lymphocytes, so it represents a generic marker to monitor individual subset responses to provocative stimuli.[1] This assay allows the investigator to study activation at the level of specific T-, B-, and natural killer (NK)- ($CD56^+$) lymphocyte subsets.[2]

Cells

Whole blood, collected in a VACUTAINER blood collection tube containing sodium heparin.

Reagents

T-Lymphocyte System

1. Positive activation control CD2/CD2R (BD Cat. No. **340366**), a comitogenic pair of monoclonal antibodies
2. FastImmune reagent cocktails for immunofluorescent staining:
   - γ_1 FITC/γ_1 PE/CD3 PerCP (BD Cat. No. **340369**)
   - CD4 FITC/CD69 PE/CD3 PerCP (BD Cat. No. **340365**)
   - CD8 FITC/CD69 PE/CD3 PerCP (BD Cat. No. **340367**)
   - CD69 PE/CD3 PerCP (BD Cat. No. **340368**)

B-Lymphocyte System

1. γ_1 PE/CD45 PerCP Isotype Control (BD Cat. No. **340416**)*
2. FastImmune reagent cocktail for immunofluorescent staining:
   - CD19 FITC/CD69 PE/CD45 PerCP (BD Cat. No. **340418**)

NK- ($CD56^+$) Lymphocyte System

1. γ_1 PE/CD45 PerCP Isotype Control (BD Cat. No. **340416**)†
2. FastImmune reagent cocktail for immunofluorescent staining:
   - CD56 FITC/CD69 PE/CD45 PerCP (BD Cat. No. **340417**)

*NOTE: FastImmune γ_1/CD45 is an open reagent for the FastImmune system that provides a customized negative control for the B- and NK-lymphocyte reagents. Nonspecific staining of leucocyte populations can be evaluated by adding appropriate FITC-labeled antibodies to this two-color reagent. For the FastImmune B-lymphocyte reagent (CD19/CD69/CD45, Cat. No. **340418**), the corresponding FITC conjugate concentration is 0.06 µg/test; for the FastImmune NK-lymphocyte reagent (CD56/CD69/CD45, Cat. No. **340417**), the corresponding FITC conjugate concentration is 0.03 µg/test.*

Flow Cytometric Procedure for Assessing Lymphocyte Activation

All Systems

1. FACS Lysing Solution (10X) (BD Cat. No. **349202**). For dilution instructions and warnings, refer to the *FACS Lysing Solution* package insert.
2. Suggested Mitogens

   For the T-lymphocyte system:
   - pokeweed mitogen (PWM)
   - phytohemagglutinin (PHA)
   - staphylococcal enterotoxin B (SEB)
   - *Candida albicans*

*NOTE: For the T-lymphocyte system, BD recommends using CD2/CD2R (BD Cat. No. **340366**) as the positive control for activation.*

   For the B-lymphocyte system: PWM

   For the NK-lymphocyte system: IL-2

3. CaliBRITE beads (BD Cat. No. **349502**). For detailed information on use, refer to the *CaliBRITE Beads* package insert.

* *Use either γ_1 FITC (BD Cat. No. **349041**) or CD19 FITC (BD Cat. No. **340409**) with γ_1 PE/CD45 PerCP.*

† *Use either γ_{2a} FITC (BD Cat. No. **349051**) or CD19 FITC (BD Cat. No. **340409**) with γ_1 PE/CD45 PerCP.*

Equipment

1. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent.
2. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246**) or equivalent.
3. 37°C water bath or incubator
4. Vortex mixer
5. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for information.
6. FACSComp software for instrument setup and CellQuest software for acquisition and analysis. Refer to the appropriate software user's guide for detailed information.

Flow Cytometric Procedure for Assessing Lymphocyte Activation

Procedure

WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

For Activating Whole Blood

1. Determine the number of samples to be stained. Add the required amount of blood (based on 50 µL per test) to 12 x 75-mm tubes (with caps).

NOTE: It is recommended that no less than 200 µL and no more than 1 mL of blood be activated per tube.

2. Add desired stimulus. Titrate mitogens and antigens to determine appropriate stimulating concentration. For the T-lymphocyte system, use CD2/CD2R (BD Cat. No. **340366**) as the positive activation control at 20 µL/mL of blood. Include an unstimulated control.
3. Cap tubes and vortex.
4. Incubate tubes at 37°C for 4 hours.

For Staining

1. Add 20 µL of reagent cocktail to 12 x 75-mm tubes.

*NOTE: For either the B- or NK-lymphocyte system, add a FITC reagent of choice when using γ_1 PE/CD45 PerCP Isotype Control (BD Cat. No. **340416**). For the T-lymphocyte system, add a FITC reagent of choice when using CD69 PE/CD3 PerCP (BD Cat. No. **340368**).*

2. Aliquot 50 µL of each blood sample to each tube and vortex.
3. Incubate 15 to 30 minutes in the dark at room temperature (20° to 25°C).
4. Add 450 µL 1X FACS Lysing Solution to each tube. Vortex gently and incubate for at least 15 minutes at room temperature.

If samples are not to be analyzed immediately after preparation, store them in the dark at 2° to 8°C for up to 24 hours. Mix thoroughly before analysis.

For Data Acquisition and Analysis

1. Use CaliBRITE beads and the appropriate software, such as FACSComp, version 1.1 or later, or AutoCOMP, version 3.0.2, for setting the photomultiplier tube (PMT) voltages and the fluorescence compensation, and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Please contact your BD representative if you have an older version of either FACSComp or AutoCOMP.

2. Analyze prepared samples on a FACS brand flow cytometer. Acquire data with CellQuest or LYSYS II software, using fluorescence triggering in the FL3 channel (T-lymphocyte system: CD3 PerCP; B- or NK-lymphocyte system: CD45 PerCP) to gate on $CD3^+$ or $CD45^+$ lymphocyte populations.

NOTE: It is possible that upon activation, debris can interfere with the CD3 resolution. You might have to adjust gate markers or set a live gate to determine that an adequate number of $CD3^+$ events is collected.

3. Display data as two-color dot plots (FL1 versus FL2) to determine the proportion of activated lymphocyte subsets expressing CD69. Data can be analyzed using CellQuest, LYSYS II, or Attractors software.

References

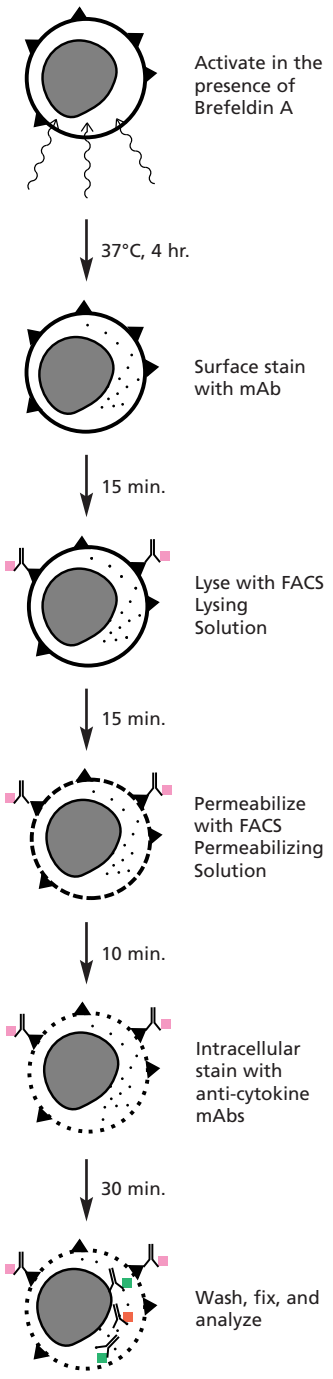
1. Testi R, D'Ambrosio D, De Maria R, Santoni A. The CD69 receptor: A multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today*. 1994;15:479-483.
2. Maino VC, Suni MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry*. 1995;20:127-133.

FastImmune Intracellular Cytokine Staining Procedures

BD has developed protocols for the detection of intracellular cytokines in activated lymphocytes and in activated monocytes. The procedures have been optimized for use with BD anti-cytokine monoclonal antibodies.

The FastImmune cytokine assay begins with activation of whole blood or peripheral blood mononuclear cells (PBMCs). Brefeldin A (BFA) is included during the last 4 hours of activation to inhibit intracellular transport. Thus antigens and cytokines produced during activation are retained inside the cell. The cells are stained with fluorochrome-conjugated monoclonal antibodies to human cell surface markers. Cells are lysed, permeabilized, and stained with fluorochrome-conjugated monoclonal antibodies to cytokines. Samples are washed and analyzed by flow cytometry.

Quick reference protocols are included in this catalog. For detailed assay information, please refer to our immune function application notes: *Detection of Intracellular Cytokines in Activated Lymphocytes* and *Detecting Intracellular Cytokines in Activated Monocytes*. They are available on our website or from your local BD representative.



FastImmune intracellular cytokine procedure

Detection of Intracellular Cytokines in Activated Lymphocytes

Equipment

1. Disposable 12 x 75-mm capped polystyrene Falcon test tubes (BD Cat. No. **2058**), or equivalent.
2. 37°C incubator with 5% to 7% CO_2
3. Vortex mixer
4. Centrifuge
5. Pipetman, or equivalent pipettors
6. FACS brand flow cytometer

Cells

Whole Blood

Collect blood for whole blood activation assays into sodium heparin VACUTAINER tubes (BD VACUTAINER Cat. No. **367673**). FastImmune assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

For best results, assay blood within 8 hours of collection. If blood cannot be used within 8 hours, store VACUTAINER tubes horizontally at room temperature.

Peripheral Blood Mononuclear Cells (PBMCs) in Autologous Plasma

Prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) (Cat. No. **362753**) containing sodium heparin. See the VACUTAINER CPT product insert for detailed information. By gently inverting the tube, the leucocytes can be resuspended in the plasma and activated like whole blood in this assay.

Before storage, centrifuge CPTs and resuspend PBMCs in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature on its side. Assay the blood no later than 24 hours after collection.

PBMCs in Tissue Culture Medium

PBMCs can also be separated via Ficoll-Paque density-gradient centrifugation. Use standard techniques and resuspend at 2 x 10^6 cells/mL in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) for activation.

Cell Lines and T-Lymphocyte Clones

For activation, resuspend cells at 2 x 10^6 cells/mL in the fresh culture medium typically used to grow the cells.

NOTE: Heat inactivate FBS to denature complement.

Frozen Activated Whole Blood and PBMCs

Lyse and fix activated whole blood or PBMCs using 1X FACS Lysing Solution; wash with phosphate-buffered saline (PBS) and freeze in PBS with 1% bovine serum albumin (BSA) and 10% DMSO at –70°C. After thawing, aliquot cells into staining tubes. Wash cells by adding 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*; then permeabilize with 1X FACS Permeabilizing Solution and stain.

Detection of Intracellular Cytokines in Activated Lymphocytes

Reagents

The following procedures and reagents have been successfully used by the research laboratories at BD.

Reagents Used in Activation (Not Provided by BD)

1. Phorbol 12-myristate 13-acetate (PMA) (Sigma Cat. No. **P-8139**)
   a. Reconstitute in DMSO at 0.1 mg/mL.
   b. Store small aliquots (*eg*, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:100 in sterile PBS (without sodium azide) for each experiment.
   d. Use PMA at a final concentration of 10 ng/mL of cell suspension.
2. Ionomycin (Sigma Cat. No. **I-0634**)
   a. Reconstitute in EtOH at 0.5 mg/mL.
   b. Store at –20°C.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each experiment.
   d. Use ionomycin at a final concentration of 1 µg/mL of cell suspension.
3. Staphylococcal enterotoxin B (SEB) (Sigma Cat. No. **S-4881**)
   a. Reconstitute in sterile PBS (without sodium azide) at 0.5 mg/mL.
   b. Store at 4°C.
   c. Use SEB at a final concentration of 1 µg/mL of cell suspension.
4. Brefeldin A (BFA) (Sigma Cat. No. **B-7651**)
   a. Reconstitute in DMSO at 5 mg/mL.
   b. Store small aliquots (*eg*, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each assay.
   d. Use BFA at 10 µg/mL of cell suspension for the last 4 to 5 hours of activation.

NOTE: Extensive incubation with BFA will reduce cell viability.

5. RPMI-1640 (BioWhittaker Cat. No. **12-167F**)
6. PBS without sodium azide (NaN_3), sterile filtered
7. DMSO (Sigma Cat. No. **D-8779**)
8. EtOH (Gold Shield Ethyl Alcohol), 200 proof
9. Wash buffer, PBS with 0.5% BSA and 0.1% NaN_3. Store at 4°C.
10. 1% paraformaldehyde in PBS; store at 4°C.

Reagents for Immunophenotypic Staining (BD)

1. Monoclonal antibody conjugates for surface staining.
2. FACS Lysing Solution

   FACS Lysing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.
3. FACS Permeabilizing Solution

   FACS Permeabilizing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.
4. BD monoclonal antibody conjugates for intracellular staining.

Detection of Intracellular Cytokines in Activated Lymphocytes

Activation

Activation is performed in the presence of BFA which inhibits intracellular transport of proteins,[1,2,3] so antigens and cytokines produced during activation will be retained inside the cell. The unstimulated control sample should also contain BFA. See the Assay Control section in this procedure. All activation procedures outlined are performed in 12 x 75-mm capped polystyrene test tubes (Falcon Cat. No. **2058**). The reagent concentrations indicated are final concentrations in the activation mixture using reagent preparations described previously.

1. PMA + ionomycin (PMA + I)
   a. Dilute whole blood or PBMCs in plasma 1:1 with RPMI 1640 without serum. (This dilution procedure is required only for PMA + I activation. Cells that have already been resuspended at 2 x 10^6/mL in medium need not be further diluted with RPMI.)
   b. Stimulate with 10 ng/mL of PMA (10 µL of working solution described previously per mL of blood) and 1 µg/mL of ionomycin (20 µL of working solution per mL of blood) in the presence of 10 µg/mL of BFA (20 µL of working solution per mL of blood).
   c. Incubate for 4 hours at 37°C, 5% to 7% CO_2 with tube caps loosened to allow entry of CO_2-containing air. (While a CO_2 incubator is preferred to ensure proper control of pH, the incubation can also be carried out in a water bath with each tube tightly capped.)
2. SEB
   a. Activate undiluted blood with 1 µg/mL of SEB in the presence of 10 µg/mL of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
3. CD2/CD2R (BD Cat. No. **340366**)
   a. Activate undiluted blood with 20 µL of CD2/CD2R per mL of blood in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
4. CD3
   a. Activate undiluted blood with immobilized CD3[4] in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C. CD5 PerCP (BD Custom Conjugate) or CD45 PerCP (BD Cat. No. **347464**) are recommended for FL3 fluorescence triggering because the CD3 antigen is modulated by crosslinking of CD3.

NOTE: High-concentration, low-azide CD3 is available through the BD Custom Conjugate Program. Contact your local BD representative for more information.

5. CD28

   Use CD28 (BD Cat. No. **340975**) at 10 µg/mL to enhance activation responses to various stimuli, including SEB, CD3, and CD2/CD2R.

Assay Controls

Unstimulated Control

The unstimulated control is used to assess the level of residual cytokine synthesis from in vivo activation. Run this control for all samples. As the name implies, the unstimulated control is prepared by incubating the blood during the activation step with 10 µg/mL of BFA, but without a stimulus.

Isotype Controls

Fluorescent-conjugated isotype control antibodies are used at matching concentrations to detect non-specific binding to cells due to the class of the mouse monoclonal antibody. The FastImmune Cytokine System uses standard anti-KLH isotype controls specially formulated for intracellular detection systems.

Detection of Intracellular Cytokines in Activated Lymphocytes

Activation Control

The activation control uses surface expression of CD69 to assess whether activation has been achieved. If the expected level of CD69 is not seen, there is a problem with the activation step of the assay. Specifically, one of the reagents used in the activation step can be inactive, expired, or improperly prepared; or a solvent can be contaminated. Make fresh preparations of the stimuli and try again.

1. Activate one aliquot of blood with PMA + I (as described previously) but omit the BFA.

NOTE: Anti-secretory agents like BFA prevent surface expression of CD69 and must be omitted to permit surface expression and detection.

2. Surface stain only with CD69 PE/CD3 PerCP (BD Cat. No. **340368**). Omit the permeabilization and intracellular staining steps of the procedure.
3. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3-gated events. Surface staining for CD69 should be greater than 90% positive (Figure 1).

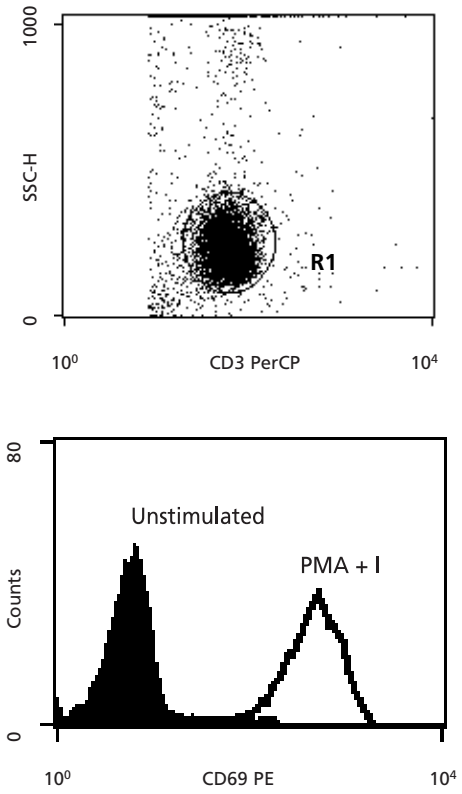


Figure 1 Activation control

Detection of Intracellular Cytokines in Activated Lymphocytes

Intracellular Staining Control

The intracellular staining control assesses intracellular staining of CD69 in conjunction with the results of the activation control to pinpoint whether permeabilization and intracellular staining are executed properly. If the activation control is greater than 90% positive, but a comparable level of CD69 is not detected by intracellular staining, there is a problem with the permeabilization or intracellular staining step of the assay. Make sure you have followed the procedure exactly as written, and try again.

1. Activate one aliquot of blood with PMA and ionomycin in the presence of BFA.
2. Omit the surface staining step. Stain intracellularly only with CD69 PE/CD3 PerCP (BD Cat. No. **340368**).
3. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Intracellular staining for CD69 should be greater than 90% positive (Figure 2).

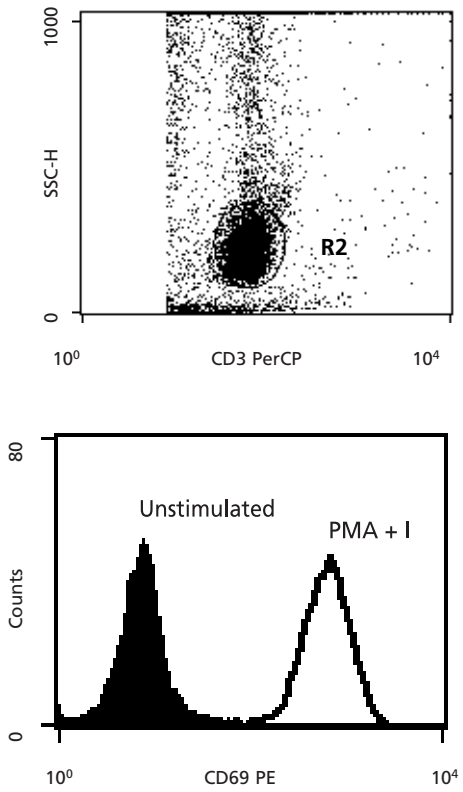


Figure 2 Intracellular staining control

Detection of Intracellular Cytokines in Activated Lymphocytes

Surface Staining

1. Add 20 µL of each BD surface staining reagent to 12 x 75-mm tubes.
2. Add 100 µL of diluted PMA + I activated blood or 50 µL of undiluted whole blood (activated by other stimuli) to the surface staining reagents. (Dilution of whole blood or PBMCs in plasma in RPMI is only required for PMA + I activation. Cells that have already been resuspended at 2 x 10^6 cells/mL in medium need not be further diluted with RPMI.)
3. Mix well and incubate for 15 minutes at room temperature in the dark.

Permeabilization and Intracellular Staining

1. Add 2 mL of 1X FACS Lysing Solution prepared according to the package insert. Incubate for 10 minutes at room temperature. When staining PBMCs or cultured cells, add FACS Lysing Solution to fix the surface epitopes and optimize the permeabilization process.

NOTE: PMA-activated whole blood does not always lyse completely.

2. Centrifuge for 5 minutes at 500 x *g* and remove the supernatant. Avoid disturbing the pellet. Add 500 µL of 1X FACS Permeabilizing Solution prepared according to the package insert and mix well. Incubate for 10 minutes at room temperature in the dark.
3. Add 2 to 3 mL of wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove the supernatant.
4. Add fluorescent-conjugated anti-cytokine mAbs. Mix well and incubate for 30 minutes at room temperature in the dark.
5. Add 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove supernatant and add 500 µL 1% paraformaldehyde.

NOTE: Samples can be stored for up to 24 hours at 4°C in the dark prior to analysis.

Analysis

1. Analyze on a FACS brand flow cytometer.
2. Use CaliBRITE beads and appropriate software (FACSComp, version 1.1 or later, or AutoCOMP, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Contact your BD representative if you have an older version of either FACSComp or AutoCOMP.

3. Acquire data with CellQuest or LYSYS II software, using a fluorescence or forward scatter (FSC) threshold. Typically, 10,000 gated events is sufficient.
4. Gate on $FL3^+$ cells. Display data as two-color dot plots to determine cytokine expression. Data can be analyzed using CellQuest, LYSYS II, PAINT-A-GATE, or Attractors software. With PMA activation, platelets can move into the $FL3^+$ gate. In this case, gate on FSC/SSC. In assays with a CD4 trigger, gate on FSC/SSC to exclude monocytes.

Calculate the Specific Response

As illustrated by the following formula, the specific response of cells to any stimulus is obtained by subtracting % positive events in the isotype control sample from % positive events in the anti-cytokine antibody-stained sample. Then subtract the isotype-corrected response of the unstimulated sample from that of the stimulated sample.

Formula: (AS – AIC) – (US – UIC)

where
AS = activated sample
AIC = activated isotype control
US = unstimulated sample
UIC = unstimulated isotype control

Detection of Intracellular Cytokines in Activated Lymphocytes

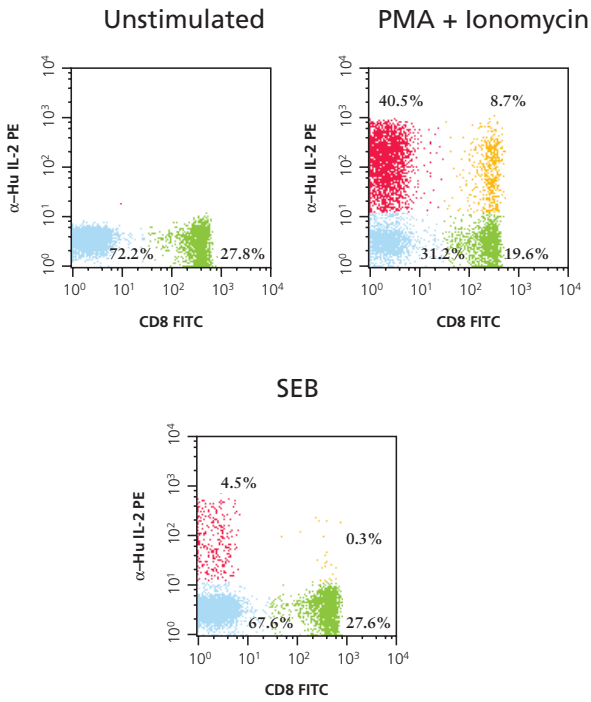


Figure 3 Functional subsets of CD8⁺ T cells

The FastImmune Cytokine System can be used to delineate functionally distinct subsets of CD8⁺ T cells. The two-color dot plots in Figure 3 compare the results of very general activation with PMA and ionomycin (PMA + I) versus more specific activation with superantigen staphylococcal enterotoxin B (SEB). Activation with PMA + I clearly induces IL-2 expression in the CD8⁺ and CD8⁻ (nominally CD4⁺) populations. In contrast, SEB stimulates IL-2 production in a less frequent population that is more strongly biased toward CD8⁻ cells. These subset-specific results cannot be observed in a bulk assay.

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1. Picker LJ, Singh MK, Zdraveski Z, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood*. 1995;86:1408-1419.
2. Openshaw P, Murphy EE, Hosken NA, et al. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J Exp Med*. 1995;182:1357-1367.
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Detection of Intracellular Cytokines in Activated Monocytes

Materials and Methods

Cells

Whole blood or peripheral blood mononuclear cells (PBMCs). Collect blood for whole blood activation assays into sodium heparin VACUTAINER tubes. FastImmune assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

Reagents

1. Brefeldin A (BFA, Sigma Cat. No. **B7651**), or equivalent. Reconstitute in DMSO at 5 mg/mL. Store in small aliquots at –20°C.
2. Lipopolysaccharide (LPS, Sigma Cat. No. **L2654**). Reconstitute in DMSO (or PBS) at 0.5 mg/mL. Store in small aliquots at –20°C.
3. BD fluorescent-conjugated monoclonal antibodies (mAbs) for lymphocyte and monocyte phenotyping. CD33 or CD14 can be used for monocyte identification.

NOTE: CD14 is an LPS receptor and can be downregulated by LPS treatment in some donors.

4. FACS Lysing Solution (10X), dilute 1:10 in deionized water. Refer to package insert for details.
5. FACS Permeabilizing Solution (10X), dilute 1:10 in deionized water. Refer to package insert for details.
6. Wash buffer: PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3
7. FastImmune anti-cytokine and control mAbs.
8. 1% paraformaldehyde

Equipment

1. Disposable 12 x 75-mm capped Falcon polypropylene test tubes (BD Labware Cat. No. **2063**), or equivalent.
2. Disposable 12 x 75-mm capped Falcon polystyrene test tubes (BD Labware Cat. No. **2058**), or equivalent.
3. 37°C incubator with 5% to 7% CO_2
4. Vortex mixer
5. FACS brand flow cytometer
6. Centrifuge
7. Micropipettor with tips (Pipetman, Rainin Instrument Co. Inc., or equivalent).

Procedure

Activation

Activation is done in the presence of BFA which inhibits intracellular transport so antigens and cytokines produced during the activation will be retained inside the cell. The unstimulated control sample should also contain BFA.

1. Label each of two 12 x 75-mm polypropylene tubes (with caps): Unstimulated and Activated.
2. Add 10 µg BFA to the Unstimulated tube.
3. Add 10 µg BFA and 1 µg LPS to the Activated tube.
4. Add 1 mL whole blood (sodium heparin), 1 mL PBMCs in autologous plasma (Prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) [BD VACUTAINER Cat. No. **362753**] containing sodium heparin), or 1 mL PBMCs in tissue culture medium (2 x 10^6 cells/mL) to each tube.

NOTE: This procedure provides enough cells for staining 20 samples, based on 50 µL/test.

5. Cap tubes loosely and vortex.
6. Incubate at 37°C, 5% to 7% CO_2 for 4 hours.

Detection of Intracellular Cytokines in Activated Monocytes

Staining

1. Label 12 x 75-mm polystyrene tubes.
2. Add surface antigen-specific fluorescent-conjugated mAbs to the appropriate tubes.
3. Add 50 µL activated or unstimulated blood to the tubes. Mix well. Incubate for 15 to 30 minutes at room temperature in the dark.
4. Add 2 mL of 1X FACS Lysing Solution to lyse the red cells (and fix white cells). Vortex gently. Incubate 10 minutes at room temperature in the dark.
5. Centrifuge at 500 x *g* for 5 minutes. Remove the supernatant; avoid disturbing the pellet.

NOTE: At this point, samples can be frozen in PBS with 1% BSA and 10% DMSO.

6. Add 500 µL of 1X FACS Permeabilizing Solution. Mix well to resuspend the pellet. Incubate 10 minutes at room temperature in the dark.
7. Add 2 to 4 mL wash buffer. Centrifuge at 500 x *g* for 5 minutes. Remove the supernatant; avoid disturbing the pellet.
8. Add intracellular antigen-specific fluorescent-conjugated mAbs. Mix well. Incubate for 30 minutes at room temperature in the dark.
9. Repeat step 7.
10. Resuspend cells in 500 µL of 1% paraformaldehyde.
11. Analyze on a FACS brand flow cytometer. Samples can be stored at 4°C in the dark for up to 24 hours prior to analysis.

Data Acquisition and Analysis

1. Use CaliBRITE beads and the appropriate software, such as FACSComp, version 1.1 or later, set on Lyse/NoWash, or AutoCOMP, version 3.0.2, for setting the photomultiplier tube (PMT) voltages and the fluorescence compensation, and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Please contact your BD representative if you have an older version.

2. Analyze prepared samples on a FACS brand flow cytometer. Acquire data with CellQuest or LYSYS II software, using a fluorescence or forward scatter (FSC) threshold. Acquire all events with at least 1,000 monocytes by setting an acquisition gate on $CD14^+$ or $CD33^+$ monocytes in SSC-monocyte marker dot plot.
3. Display data as two-color dot plots to determine cytokine expression. Analyze data using CellQuest, LYSYS II, PAINT-A-GATE^{PRO}, or Attractors software.

Detection of Intracellular Cytokines in Activated Monocytes

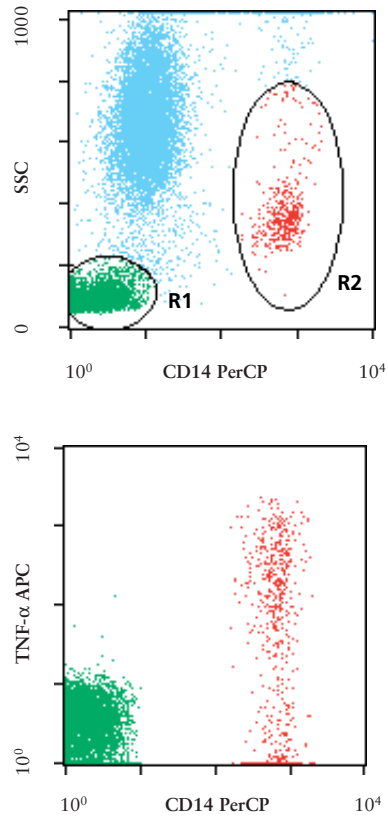

Figure 1—LPS stimulation of whole blood

LPS is a monocyte-specific stimulus. Figure 1 illustrates four-hour LPS-stimulated whole blood gated on $CD14^-$ lymphocytes (R1) and $CD14^+$ monocytes (R2). Only the monocyte population is activated and producing the cytokine TNF-α.

Platelet Activation, Staining, and Analysis

Scope

This procedure describes the preparation of fresh or fixed, unstimulated or in vitro activated platelets from unlysed whole blood; staining of platelets; and setting up the FACS brand flow cytometer prior to acquisition of platelet data.

When performing assays to detect in vivo platelet activation or analyzing in vitro platelet activation, it is critical that artifactual stimulation of the platelets be minimized during blood collection and sample handling. Even with careful preparation, low levels of inadvertent platelet activation can be difficult to avoid. Therefore, nominally unstimulated platelets should always be analyzed in parallel with in vitro stimulated samples to measure specific effects.

The investigator has several options when performing platelet assays by flow cytometry. Anticoagulant, blood collection, fixation, agonist, and gating/analysis strategies are independent variables; the only limiting factor is the antibody. Some guidelines are outlined here.

Reagents and Equipment Required

1. ACD VACUTAINER blood collection tube (BD Cat. No. **4816**).
2. Falcon disposable 12 x 75-mm capped polystyrene test tubes (BD Cat. No. **2058**) or equivalent.
3. Micropipettor with tips (BD Electronic Pipette, BD Cat. No. **343246**) or equivalent.
4. Adenosine diphosphate (ADP), 2 x 10^{-4} M (Bio/Data, Inc, Phila, PA, Cat. No. **101312**). Prepare according to manufacturer's instructions. Store at 2° to 8°C.
5. 1% paraformaldehyde solution prepared in PBS with 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week.

   WARNING: Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed. It is irritating to eyes and skin. Exposure can cause cancer. Possible risks of irreversible effects. Can cause sensitization by skin contact. Keep locked up and out of the reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show the container or label. Dispose of according to federal, state, and local regulations.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show this container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.
6. Centrifuge
7. Wash buffer: phosphate-buffered saline (PBS) with 0.1% sodium azide (Dulbecco's PBS without calcium, magnesium, or phenol red, pH 7.2 ± 0.2).[1] Filter the PBS through a 0.2-µm filter prior to use; store at 2° to 8°C.
8. Vortex mixer
9. Staining medium: PBS with 0.1% sodium azide and 2% fetal bovine serum (FBS). Store at 2° to 8°C.
10. BD fluorochrome-conjugated monoclonal antibodies to human platelet antigens. Refer to the appropriate reagent package insert for more information.
11. Arg-Gly-Asp-Ser (RGDS) (Sigma Cat. No. **A9041**). Prepare 10 mg/mL in PBS. Use RGDS with conjugated PAC-1 antibody.

NOTE: Some commercial preparations can include lipopolysaccharide (LPS), a potent platelet activator.

12. FACS brand flow cytometer. Refer to the appropriate instrument user's guide for detailed information.
13. CaliBRITE beads. Refer to the BD *CaliBRITE Beads* package insert for detailed information.
14. FACSComp software for instrument setup and CellQuest software for acquisition and analysis. Refer to the appropriate software user's guide for detailed information.

Platelet Activation, Staining, and Analysis

Procedure

Blood Collection

WARNING: All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing and gloves.

Our labs have successfully used ACD, sodium citrate, and EDTA anticoagulants. Other anticoagulants can also work, however. Heparin anticoagulant activates platelets and is not recommended for measuring in vivo platelet activation.

NOTE: PAC-1 binding to the fibrinogen receptor is pH and Ca^{++} sensitive.[2] PAC-1 will not bind to EDTA-treated blood and PAC-1 binding is typically higher in sodium citrate than in ACD.

The following protocol is designed to minimize artifactual activation of platelets during blood draw.[3]

1. Label collection tubes and organize them conveniently in a tube rack.
2. Collect ~2 mL of blood aseptically by venipuncture into any type VACUTAINER tube using a 20-gauge needle. Discard this blood because it contains activated platelets. Release tourniquet and collect blood into a labeled ACD VACUTAINER tube. This blood will be used for staining.
3. Perform activation and staining or fixation of platelets within 10 minutes of blood collection.[3,4]

Whole Blood Activation

This procedure is one example of a variety of methods that can be used to activate platelets. Our research labs have successfully used ADP, epinephrine, phorbol 12-myristate 13-acetate (PMA), thrombin, and thrombin-receptor agonist peptide (TRAP).

1. Within 10 minutes of blood collection, pipette 50 µL of ADP solution into a 12 x 75-mm test tube.
2. Add 0.45 mL of whole blood. Gently swirl to mix.
3. Incubate at room temperature (20° to 25°C) for 2 minutes.
4. Stain or fix immediately.

Fixation

Fixation of the blood with paraformaldehyde prior to staining inhibits spontaneous platelet activation. For clinical testing, fixing platelets can make the assay more manageable. Fixation has an effect on activation-dependent platelet antibodies. PAC-1 will not bind to paraformaldehyde-fixed platelets and CD62P binding is decreased. If fixation is not desired or possible, proceed to the next section, Direct Immunofluorescence Staining, and use fresh whole blood.

1. Within 10 minutes of blood collection, pipette 100 µL of unstimulated or activated whole blood into a 12 x 75-mm test tube containing 1 mL of cold (2° to 8°C) 1% paraformaldehyde solution.

NOTE: 100 µL of whole blood yields enough fixed blood for 20 tests. If additional fixed blood is required, prepare the appropriate number of tubes. Do not increase the volume of the blood or paraformaldehyde in the tube as this will increase the possibility of platelet aggregation.

2. Fix platelets at 2° to 8°C for a minimum of 2 hours. Fixed platelets are stable up to 5 days. Store at 2° to 8°C.
3. Before staining, centrifuge the fixed blood at 1200 x *g* for 5 minutes at room temperature (20° to 25°C).
4. Remove the supernatant, and add 1 mL of room-temperature wash buffer.
5. Resuspend the pellet by vortexing thoroughly. Centrifuge at 1200 x *g* for 5 minutes at room temperature.
6. Remove the supernatant and resuspend the pellet in 1 mL of room-temperature staining medium.

Platelet Activation, Staining, and Analysis

Direct Immunofluorescence Staining

Single- or multicolor staining can be used in the assay. With multicolor staining, one antibody conjugate can be used to threshold data acquisition to analyze only those blood cells that bind an activation-independent, platelet-specific antibody,[4,5] for example, CD61 or CD42a. Another antibody conjugated to a different fluorochrome can be used to simultaneously assess the binding of platelet-associated, activation-dependent antibodies, for example, CD62P or PAC-1. The combination of CD61, CD62P, and PAC-1 reagents represents a three-color assay that reports two aspects of platelet activation.[3] The following is a multicolor staining procedure.

1. Label 12 x 75-mm tubes.
2. Add appropriate volume of activation–independent, platelet-specific antibody to the appropriately labeled test tube.
3. Add appropriate volume of isotype control or platelet activation–dependent monoclonal antibody to the appropriately labeled test tubes. Test and control antibody concentrations should be matched. Consult the monoclonal antibody data sheet for each test antibody to determine the amount of control (in µg) to use.

NOTE: To demonstrate specific PAC-1 binding, include one tube with 10 µL RGDS solution in the staining mixture. RGDS peptide competitively inhibits PAC-1 binding. Refer to the PAC-1 data sheet.

4. Using a fresh micropipettor tip each time, carefully add to the bottom of each tube **either**:
   - 5 µL of unstimulated or activated fresh whole blood within 10 minutes of blood collection, or
   - 50 µL of unstimulated or activated fixed whole blood suspension within 5 days of fixation

   Exercise care to prevent blood from running down the side of the tube. If blood remains on the side of the tube, it might not be stained with the reagent. If this occurs, discard and try again.
5. Gently swirl the tubes to mix.
6. Incubate for 15 to 20 minutes at room temperature in the dark.
7. Add 1 mL of cold (2° to 8°C) 1% paraformaldehyde solution to each tube and vortex. Store stained and fixed cells at 2° to 8°C in the dark for at least 30 minutes, but not more than 24 hours.[3] Analyze samples on a flow cytometer.

Data Acquisition and Analysis

Acquisition and analysis can be performed on scatter gating (Figure 1 through Figure 4) or fluorescence gating (Figure 5 through Figure 8). Scatter gating (gating on forward scatter [FSC] and side scatter [SSC]) can be difficult when the platelet count is low or when there is aggregation in the sample. In both normal and disease states, and especially when activated, platelets and red blood cells can have overlapping light scatter signatures. For scatter gating, exclude debris and background noise by setting the appropriate FSC threshold.

Fluorescence gating (gating on FL3 and SSC) can be done on the activation-independent platelet marker, and then the light-scatter profile of the positive population can be independently analyzed. Venous blood typically demonstrates three subpopulations of particles (Figure 6). The majority of the particles consist of single intact platelets. A second population, typically representing 5% of all particles, exhibits greater light scatter than single platelets and represents platelets associated with large white blood cells (WBCs).[3,6] A third population, representing 5% to 15% of the particles whose light scatter is lower than single intact platelets, includes platelet-derived microparticles with an average diameter of 0.1 µm.[5] For fluorescence gating, exclude debris and background noise by setting the appropriate FL3 threshold.

Platelet Activation, Staining, and Analysis

The FACS brand flow cytometer should be calibrated using FACSComp software with CaliBRITE beads. The lyse/no-wash setup can be used for platelets. However, since platelets are much smaller than leucocytes, the events are not optimally displayed along each axis. The following procedure uses fluorescence gating and a setup that optimally displays platelet events.

1. Select logarithmic amplification for FSC and SSC gains.
2. Set the flow rate to low to minimize coincident events.
3. Trigger acquisition on positive events using an activation-independent platelet-specific antibody conjugated to an FL3 conjugate (Figure 5).
4. Use platelets stained with an isotype control or unstimulated platelets stained with activation-dependent monoclonal antibodies to adjust FL1 and FL2 PMT voltages. FL1/FL2 baseline signals should be depicted squarely in the first decade in an FL1 vs FL2 dot plot. For PAC-1, use resting platelets stained with PAC-1 and RGDS (Figure 7).
5. Use activated platelets stained with an activation-independent, platelet-specific antibody conjugate in FL3 and a mix of activation-dependent antibody and isotype control to adjust compensation. For example, use platelets stained with PAC-1 FITC/Mouse IgG_1 PE/CD61 PerCP to adjust compensation of FITC from FL2. To adjust compensation of PE from FL1 when using PAC-1 FITC, stain with PAC-1 FITC/CD62P PE/CD61 PerCP in the presence of RGDS to inhibit PAC-1 binding.
6. Acquire 5,000 to 10,000 activation-independent platelet events.
7. Display the total platelet population, including those associated with WBCs, or any light-scatter gated subpopulation as two-color dot plots and statistically analyze (Figure 8).

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Platelet Activation, Staining, and Analysis

Scatter Gating

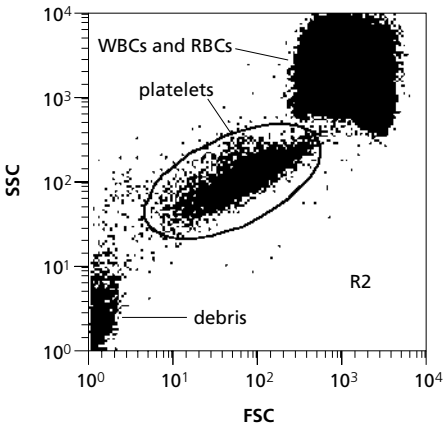

Figure 1 Unstimulated fresh whole blood

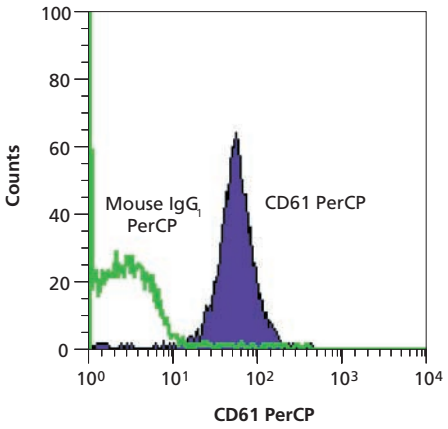

Figure 2 Unstimulated fresh whole blood (gated on R2)

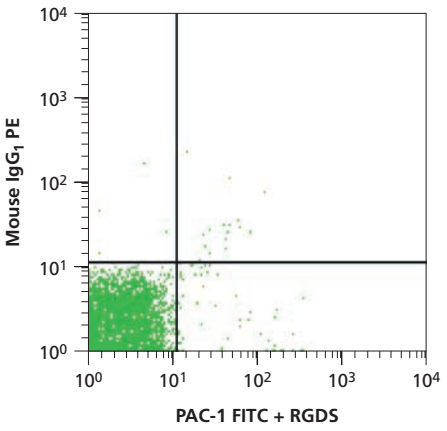

Figure 3 Unstimulated fresh whole blood (gated on R2)

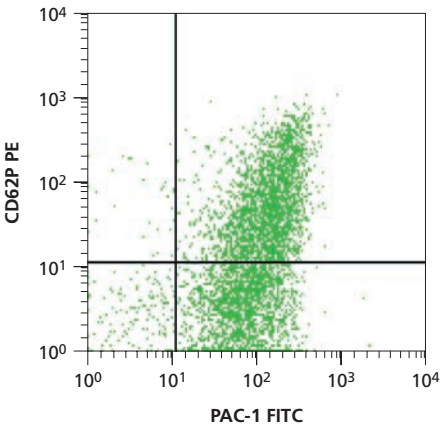

Figure 4 ADP-stimulated fresh whole blood (gated on R2)

Platelet Activation, Staining, and Analysis

Fluorescence Gating

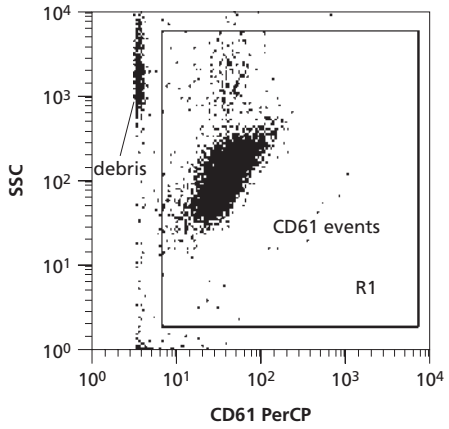

Figure 5 Unstimulated fresh whole blood

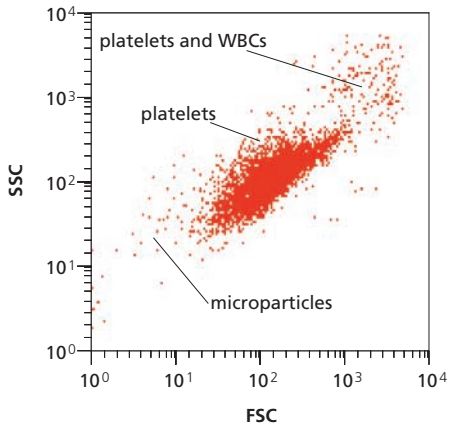

Figure 6 Unstimulated fresh whole blood (gated on R1)

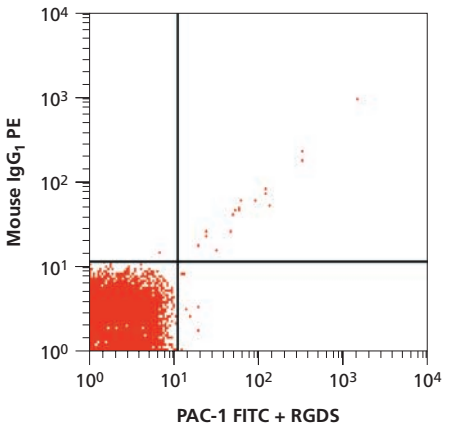

Figure 7 Unstimulated fresh whole blood (gated on R1)

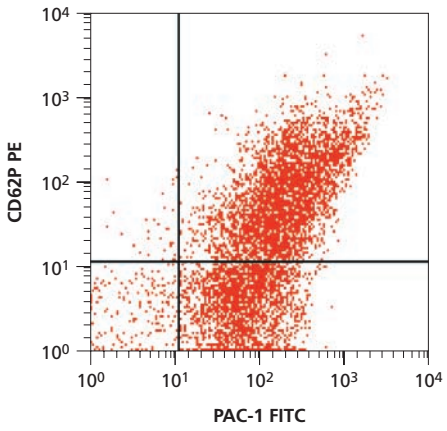

Figure 8 ADP-stimulated fresh whole blood (gated on R1)

The Escapee Phenomenon

Scope

This technical update provides information on the *escapee* phenomenon, an event that can occur when using monoclonal antibodies for flow cytometric analysis.

Definition

Escapees are aggregates of cells (including positively labeled cells) that are excluded from the flow cytometric analysis gate, thus altering the results of the analysis.[1]

Observations

The escapee phenomenon can account for the sum of %T + %B + %NK being less than 95% when the lymphocyte gate has been set correctly. This is due to the exclusion of the escapees from the standard lymphocyte gate.

Two dot plots illustrating the escapee phenomenon are shown below. Mononuclear cells from a normal donor were separated from peripheral blood using Ficoll-Paque. The monoclonal antibody used for staining was CD8 FITC from two different manufacturers. The forward light scatter (FSC) vs fluorescence-1 (FL1) dot plots obtained from each manufacturer's product reveal a population of $CD8^+$ aggregated cells that extend beyond the region of the lymphocyte gate. These events, which show an increased FSC signal, have been identified by sorting as the escapees or aggregated lymphocytes. Since these cells are not included in the standard lymphocyte analysis gate, the result is lower percentage positive values for the antigen of interest.

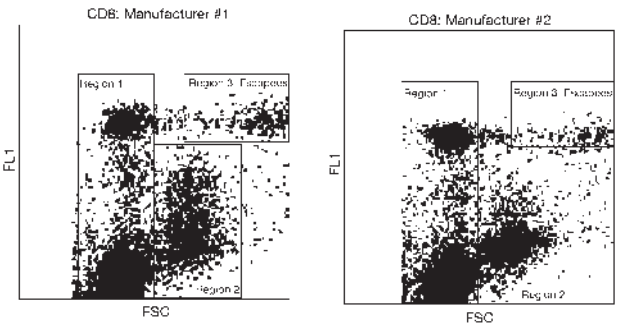

Figure 1 Region 1 includes the CD8-positive and CD8-negative single lymphocytes within the lymphocyte gate. Region 2 contains the monocyte population. Region 3 includes a population of bright CD8-positive events that are outside of the standard lymphocyte gate.

Use of various monoclonal antibodies such as CD8 and CD4[1] can cause cell aggregation in immunophenotyping assays, resulting in lower percentage positive values for the antigen of interest. The number of aggregates can vary depending on whether a FITC- or PE-conjugated monoclonal antibody is used. When analyzing lymphocyte-gated CD8-positive cells, CD8 FITC conjugates have been shown to yield lower percentage positive values than CD8 PE conjugates for the same donor.[1] Escapees can also be detected when using an unconjugated purified monoclonal antibody and a Goat Anti-Mouse (GAM) Ig FITC second-step reagent.

The number of escapees is partially dependent on the cell preparation method used. A peripheral blood mononuclear cell separation method will result in detection of more escapees than will a whole blood preparation stained and then lysed with FACS Lysing Solution.

The Escapee Phenomenon

Recommendations

1. To decrease the number of escapees present, BD recommends using whole blood preparations that have been lysed with FACS Lysing Solution prior to flow cytometric analysis. Refer to the *FACS Lysing Solution* package insert for a detailed procedure.
2. For cell suspensions prepared by Ficoll-Paque separation, it is recommended that a high-protein medium be used, such as phosphate-buffered saline (PBS) containing 2% fetal bovine serum and 0.1% sodium azide.
3. Cell suspensions should be vortexed at a low speed, and samples should not be centrifuged with forces greater than 300 x *g*. Rough handling of cells such as excessive washing or high-speed vortexing can cause increased cell aggregation.
4. Avoid the use of agents for monocyte depletion by phagocytosis (iron carbonyl particles) as these can increase the incidence of escapees.
5. Even when taking these precautions during sample preparation, cell aggregation can still occur.

For more information, contact the Customer Support Center at your local BD office.

References

1. Jackson A. Basic phenotyping of lymphocytes: selection and testing of reagents and interpretation of data. *Clin Immunol Newslett.* 1990;10:43-55.