

Advantages of the 561-nm (Yellow-Green) Laser on the BD FACSAria III

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Application Note

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Abstract

The increase in availability of monoclonal antibodies, new fluorochromes, and sophisticated data analysis software has increased the demand for flow cytometers with expanded multicolor and application capabilities. The development of more solid state lasers has resulted in greater wavelength choice and has facilitated the development of flow cytometers with increased laser choice. The incorporation of the 561-nm laser into the BD FACSAria™ III cell sorter provides the capability to physically separate the excitation and emission of two commonly used fluorochromes, FITC and PE, resulting in the elimination of spillover between the emission spectra of these fluorochromes. In addition, the 561-nm laser efficiently excites fruit fluorescent proteins such as mCherry.

Proof of principle data presented in this application note demonstrates the advantages of using the 561-nm laser to excite and detect PE-conjugated antibodies and mCherry-expressing cells. On average, the use of the 561-nm laser results in a 3.7-fold increase in the stain index of PE-conjugated antibodies and elimination of PE-FITC spillover. Preliminary data also demonstrates the increased resolution of dim staining or new populations using PE-conjugated markers. Finally, a single experiment shows the enhanced capability to detect mCherry with a 16.4-fold increase in stain index.

Taken together, these proof of principle experiments clearly demonstrate the advantages of the addition of the 561-nm laser as a standard laser on the BD FACSAria III.



Introduction

In the last few years there has been a huge increase in the availability of monoclonal antibodies, fluorochromes, and data analysis and management software that has driven the demand for flow cytometers with expanded multicolor and application capabilities.^{1,2} Recent advances in laser technology have resulted in the development of a greater range of wavelength choices for solid state lasers, thus increasing the choice of laser wavelengths available for flow cytometry applications.^{3,4} Some of these wavelengths minimize or eliminate spectral overlap issues (such as fluorescein isothiocyanate (FITC) overlap into R-phycoerythrin (PE)), thus increasing the resolution of dim staining populations in a multicolor experiment. In addition, scientists can take advantage of new research tools that have already gained wide acceptance for use in fluorescence microscopy, such as fruit fluorescent proteins, because flow cytometers can now be equipped with lasers capable of generating optimal excitation wavelengths.

Spillover and compensation

The most efficient way to excite and detect mixtures of fluorochromes with a minimal amount of interference is to use excitation wavelengths of light to excite a single given fluorochrome, and a single light path to detect only the light emitted from the single fluorochrome. Due to the limited numbers of lasers currently available, it has been necessary to design flow cytometers that excite multiple fluorochromes with a single laser. The emission spectra of the excited fluorochromes are discriminated using a mixture of bandpass filters and dichroic mirrors along a known (shared) emission light path to a known detector. As the number of fluorochromes available (and therefore, the number of antibody/fluorochrome conjugates) increases, the potential for spillover between fluorochromes with similar emission spectra also increases. Spillover occurs whenever the fluorescence emission of one or more fluorochromes is detected in a detector with a filter optimized to detect another specific fluorochrome. One common example is the FITC fluorochrome emission spectrum spillover into the PE channel.

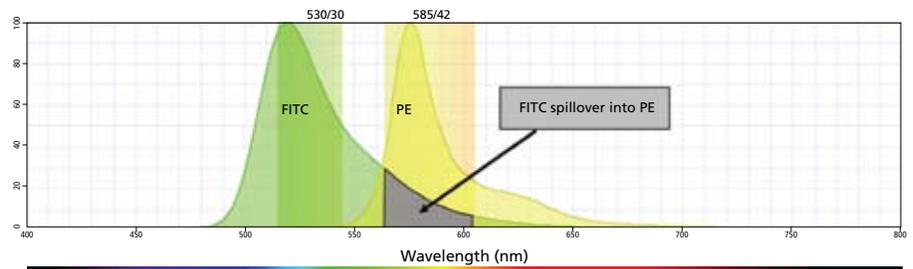


Figure 1. Example of FITC spillover into the PE channel.

PE detector (Figure 1).

More details of the spillover phenomenon, how compensation can be used to overcome this problem, and the effect on resolution sensitivity are covered extensively in the BD technical bulletin, *An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers*.⁵

Simultaneous excitation of FITC and PE with the 488-nm (blue) laser

The two commonly used fluorochromes FITC and PE have overlapping excitation absorption spectra as shown in Figure 2 and are normally simultaneously excited by the 488-nm (blue) laser. While FITC has a single absorption peak of 494 nm, PE has a primary absorption peak of 565 nm with secondary absorption peaks at 496 and 545 nm that have made it a versatile fluorochrome for multicolor flow cytometry for many years. The emission spectra of these fluorochromes is distinguished using different bandpass filters (for example, 530/30 for FITC and 585/42 for PE) (Figure 1) and the spillover of the FITC signal into the PE detector is overcome using compensation.⁵

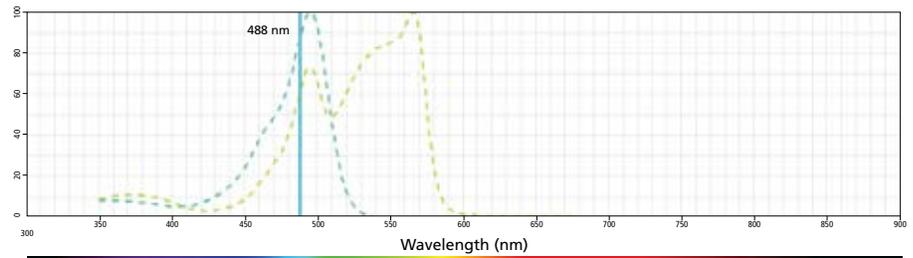


Figure 2. Simultaneous excitation of FITC and PE by the 488-nm laser.

Independent excitation of FITC and PE with the 488-nm (blue) and 561-nm (yellow-green) lasers

Since there is a large portion of the PE excitation spectrum that does not overlap with the FITC excitation spectrum, it is possible to “decouple” the excitation of these two commonly used fluorochromes using two different lasers which are spatially separated and have two different optical detection pathways. BD has incorporated this method for separating excitation and emission signals for many years. This permits the emission arising from each laser to be physically separated and directed along a different optical path which can then be detected free of interference from the other lasers. Thus, when a PE- and FITC-labeled event passes in front of a 561-nm (yellow-green) laser, only the PE fluorochrome is excited and emits light along a specific optical path which is transmitted to the 561-nm detection array through a suitable bandpass filter (for example, 585/42) to the detector (Figure 3).

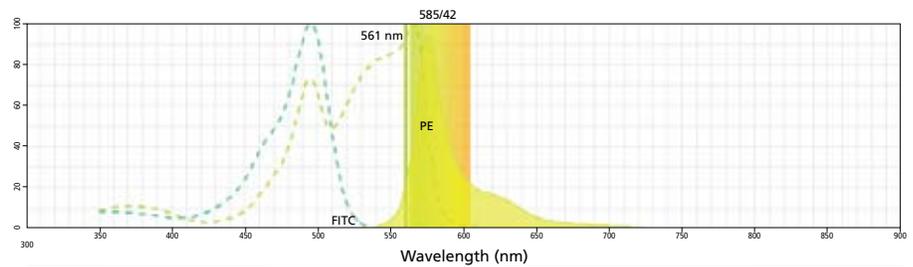


Figure 3. Excitation and emission of PE using the 561-nm (yellow-green) laser and detection array.

When the same PE- and FITC-labeled event passes in front of the 488-nm (blue) laser, both the FITC and PE fluorochromes are excited and emit light along a shared path which is transmitted to the 488-nm detection array. The light passes through a 530/30 bandpass filter to the FITC detector, but the light emitted by PE is not detected since the appropriate bandpass filter and detector are not present and there is minimal overlap of the PE emission spectrum into the 530/30 bandpass filter (Figure 4).

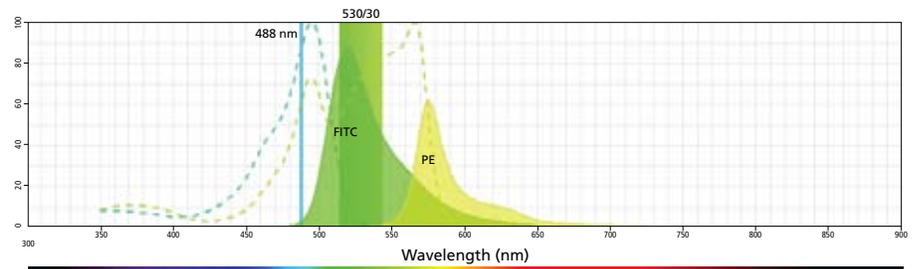


Figure 4. Excitation of FITC and PE by the 488-nm (blue) laser and detection of FITC alone.

Since there is no 582/42 bandpass filter present in the 488-nm emission light path, there is no inadvertent detection of FITC spillover into the PE channel and therefore no correction due to compensation is required. The excitation of PE and detection of PE emitted light is completely “uncoupled” from FITC excitation and emission. This phenomenon is not limited to the independent excitation of FITC and PE, but can also be applied to any fluorochrome combination that can be separated due to their different excitation characteristics. A consequence of the introduction of this new laser is a simplification of multicolor panel design. PE and FITC, the most commonly available fluorochrome conjugates, can now be used to stain dim populations without the risk of losing PE signal resolution from spillover of FITC into the PE detector.

Excitation and detection of mCherry

Fruit fluorescent proteins are becoming the fluorochromes of choice for the detection of gene expression in transfected cells and are commonly used in fluorescence microscopy studies. Resolution of these fluorochromes by flow cytometers is difficult on instruments that do not have lasers of a suitable wavelength to sufficiently excite these fluorochromes. It is possible to detect mCherry on the BD FACSAria™ II since the 488-nm (blue) laser provides minimal excitation (8%) at the very edge of the excitation spectrum as shown in Figure 5. It is, therefore, only possible to detect this fluorochrome in situations where the copy number is high. Since the level of excitation is very low, the light emission thus generated is very dim (Figure 5). The presence of spillover from other fluorochromes used to stain mCherry transfected cells requires compensation, which might further diminish the already dim signal.

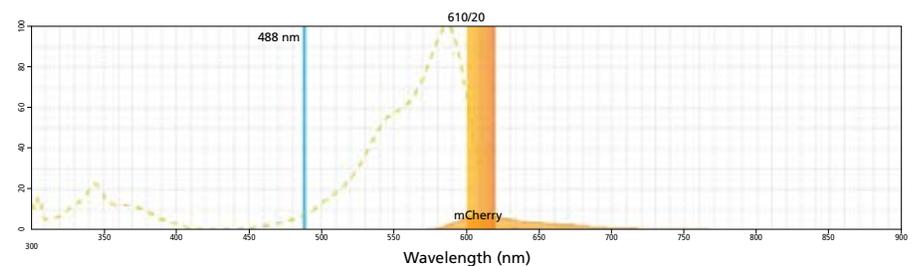


Figure 5. Excitation and detection of mCherry using the 488-nm (blue) laser and detector.

The 561-nm (yellow-green) laser results in more efficient excitation (64%) of mCherry because this wavelength is closer to the maximum excitation wavelength than the 488-nm (blue) laser. The 561-nm laser therefore results in a brighter emission signal as shown in Figure 6.

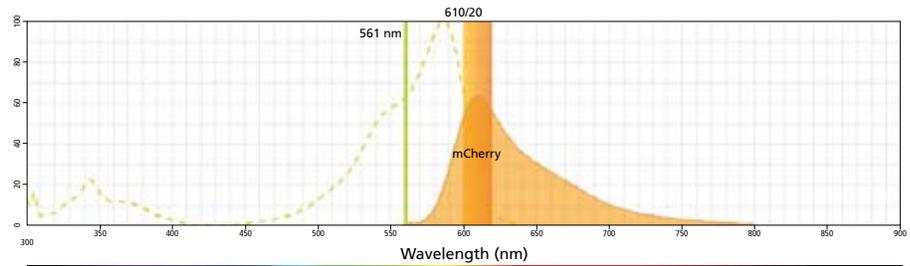


Figure 6. Excitation and detection of mCherry using the 561-nm (yellow-green) laser and detector.

BD FACSAria family of instruments

The BD FACSAria III cell sorter incorporates an innovative new X-mount optical plate to accommodate easy expansion to six lasers and four spatially separated beam spots. Four lasers can be used simultaneously for expanded multicolor application capability. Wavelength choices include the 561-nm, 633-nm, 488-nm, 445-nm, 405-nm, and 375-nm lasers. The instrument can be configured with up to 20 detector positions to measure up to 18 colors simultaneously. A new flow cell designed specifically for the BD FACSAria III improves cell cycle and side population resolution. Customers with a BD FACSAria™ or BD FACSAria II can obtain the new capabilities of the BD FACSAria III with a field upgrade. Upgraded instruments are identified by the addition of the letter “u” (for upgrade) after the new instrument name: for example, a BD FACSAria that has been upgraded to a BD FACSAria II is called a BD FACSAria IIu, and a FACSAria II upgraded to a FACSAria III is called a BD FACSAria IIIu.

In this application note we present proof of principle data generated on BD FACSAria IIu and BD FACSAria IIIu cytometers demonstrating the increased utility of the 561-nm laser in its ability to increase the sensitivity of PE detection by uncoupling the excitation and detection of this fluorochrome from FITC. We also present proof of principle data showing the utility of the 561-nm laser for the detection of mCherry-expressing mouse cells within a mixed population.

Objective

The objectives of this application note are to demonstrate:

- Increased PE detection sensitivity using the 561-nm laser compared to the 488-nm laser
- Elimination of PE-FITC spillover using the 561-nm laser compared to the 488-nm laser
- Improved resolution of fruit fluorescent proteins such as mCherry
- Increased ability of the cytometer to detect dim staining populations using the 561-nm laser compared to the 488-nm laser

Methods

Antibodies

Product Description	Fluorochrome	Clone	Species	Vendor	Product Number
CD3	FITC	SK7	Human	BD Biosciences	349201
CD3e	PE-Cy TM 7	145-2C11	Mouse	BD Biosciences	552774
CD4	PE	SK3	Human	BD Biosciences	347327
		RM4-5	Mouse	BD Biosciences	553049
CD8	BD Horizon TM V450	RPA-T8	Human	BD Biosciences	560347
CD8a	APC-Cy7	52-6.7	Mouse	BD Biosciences	557654
	APC	53-6.7	Mouse	BD Biosciences	553035
CD11b	FITC	M1/70	Mouse	BD Biosciences	553310
CD11b/Mac-1	PE	ICRF44	Human	BD Biosciences	555388
CD11c	PE	HL3	Mouse	BD Biosciences	553802
CD20	APC	2H7	Human	BD Biosciences	559776
CD45	APC-H7	2D1	Human	BD Biosciences	560178
CD45R/B220	Pacific Blue TM	RA3-6B2	Mouse	BD Biosciences	558108
CD45RA	FITC	HI100	Human	BD Biosciences	555488
CD56	Alexa Fluor [®] 488	B159	Human	BD Biosciences	557699
CD337 (NKp30)	PE	P30-15	Human	BD Biosciences	558407
F4/80	APC	BM8	Mouse	Invitrogen (Caltag Laboratories RM 2905)	MF48005
Integrin β_7	PE	FIB504	Human	BD Biosciences	555945
NK1.1	PerCP-Cy TM 5.5	PK136	Mouse	BD Biosciences	551114
TCR β chain	FITC	H57-597	Mouse	BD Biosciences	553171

Other Reagents

Product Description	Vendor	Product Number
BD Vacutainer [®] CPT TM tubes with sodium heparin	BD Medical	362753
BD TM Cytometer Setup and Tracking (CS&T) Bead Kit	BD Biosciences	641319

Instrument configurations

The fluorochromes used in this particular application are shown in bold.

Aria IIu (used for experiments on human cells)

Laser	Detector	Dichroic Mirror	Bandpass Filter (nm)	Fluorochrome
405 nm	A	502 LP	530/30	BD Horizon V500, AmCyan
	B	–	450/40	BD Horizon V450, DAPI, Pacific Blue™
488 nm	A	556 LP	576/26	PE (Blue)
	B	502 LP	530/30	FITC
	C	–	488/10	SSC
633 nm	A	735 LP	780/60	APC-H7, APC-Cy7
	B	–	660/20	APC

Aria IIIu (used for experiments on human cells)

Laser	Detector	Dichroic Mirror	Bandpass Filter (nm)	Fluorochrome
405 nm	A	502 LP	530/30	BD Horizon V500, AmCyan
	B	–	450/40	BD Horizon V450, DAPI, Pacific Blue™
488 nm	A	655 LP	695/40	PerCP-Cy5.5
	B	502 LP	530/30	FITC
	C	–	488/10	SSC
561 nm	A	735 LP	780/60	PE-Cy7
	B	630 LP	670/14	PE-Cy™5
	C	600 LP	610/20	mCherry, PE-Texas Red®, PI
	D	–	582/15	PE (yellow-green)
633 nm	A	735 LP	780/60	APC-H7, APC-Cy7
	B	–	660/20	APC

Aria IIu (used for experiments on mouse cells)

Laser	Detector	Dichroic Mirror	Bandpass Filter (nm)	Fluorochrome
405 nm	A	535 LP	585/32	Qdot® 585
	B	502 LP	530/30	Alexa Fluor® 430
	C	–	450/50	Alexa Fluor® 405, Cascade Blue™, DAPI, Pacific Blue™
488 nm	A	735 LP	780/60	PE-Cy7
	B	655 LP	675/20	PE-Cy5, PerCP, PerCP-Cy5.5 , 7AAD, PE-Cy5.5
	C	610 LP	616/23	PE-Texas Red®, PI, mCherry
	D	556 LP	585/42	DsRed, PE
	E	502 LP	498E-521E	FITC , GFP, YFP
	F	–	488/10	SSC
633 nm	A	735 LP	780/60	APC-Cy7 , APC-H7
	B	685 LP	730/30	Alexa Fluor® 680, Alexa Fluor® 700
	C	–	660/20	APC , Alexa Fluor® 647

Aria IIIu (used for experiments on mouse cells)

Laser	Detector	Dichroic Mirror	Bandpass Filter (nm)	Fluorochrome
405 nm	A	502 LP	530/30	AmCyan
	B	–	450/40	DAPI, Pacific Blue™ , BD Horizon V450
488 nm	A	655 LP	695/40	PerCP-Cy5.5
	B	505 LP	530/30	FITC , GFP, Alexa Fluor® 488
	C	–	488/10	SSC
561 nm	A	735 LP	780/60	PE-Cy7
	B	630 LP	670/14	PE-Cy5, PerCP, 7AAD
	C	600 LP	610/20	Texas Red®, mCherry , PI
	D	–	582/15	PE
633 nm	A	735 LP	760/60	APC-Cy7 , APC-H7
	B	–	660/20	APC , Alexa Fluor® 647

Table 1. Eight-color panel used to stain mouse spleen cells.

Marker	Fluorochrome
CD11c	PE
CD11b	FITC
NK1.1	PerCP-Cy5.5
B220	Pacific Blue™
F4/80	APC
CD8a	APC-Cy7
CD3e	PE-Cy7
NA	mCherry

Table 2. Panel used to stain human PBMCs.

Tube	Antigen	Fluorochrome
1	CD3	FITC
	CD4	PE
2	CD56	FITC
	CD337	PE
3	CD45RA	FITC
	CD11b	PE

Isolation of mouse spleen cells

Six-week old C57/B6 mice with mCherry under the control of the SpiC promoter were used for the mCherry study. Cells were isolated from intact spleens according to Bezbradica, Stanic, and Joyce (2006).⁶

Surface staining of mouse spleen cells

Spleen cells were stained with surface markers according to the panel in Table 1.

Five million (5×10^6) mouse spleen cells were stained with antibody at a final antibody concentration of 5 µg/mL in 100 µL of phosphate buffered saline (PBS) (without Mg/Ca) + 0.1% bovine serum albumin (BSA) (PBS/BSA), except for CD3e PE-Cy7 which was used at a concentration of 2.5 µg/mL. Cells were incubated in the presence of the antibody for 30 minutes at 4°C, washed in PBS/BSA (200g, 5 minutes, at room temperature (RT)). The supernatant was discarded and cells resuspended in 0.5 mL of 2% paraformaldehyde in PBS.

Isolation of Human PBMCs

Blood specimens were collected from normal healthy volunteers who consented to participate in an Institutional Review Board approved protocol. Blood was collected into BD CPT Vacutainer tubes and peripheral blood mononuclear cells (PBMCs) were isolated according to the technical data sheet (TDS).⁷ PBMCs were washed in PBS (w/o Mg/Ca) + 1% fetal bovine serum (FBS) + 0.09% sodium azide (PBS/FBS/azide) (200g, 5 min, RT) and resuspended at a concentration of 2×10^7 cells/mL. Fifty (50) µL cells were stained with the appropriate amount of antibody as shown in Table 2 according to the antibody TDS. Cells were incubated in the presence of antibody for 20 minutes in the dark at RT, then washed in PBS/FBS/azide (200g, 5 min, RT). The supernatant was discarded and cells resuspended in 0.5 mL PBS/FBS/azide.

Data acquisition and analysis

Data from the same sample of stained cells was acquired using the BD FACSAria IIu and BD FACSAria IIIu using the configurations shown above. The flow cytometers were set up using BD™ Cytometer Setup & Tracking (CS&T), application settings were created and applied, then compensation was performed using BD™ CompBeads prior to each experiment. BD FACSDiva™ software v6.1.3 was used for acquisition and BD FACSDiva v6.1.3 or FCS Express 3 was used for analysis.

Stain index was calculated using the following formula, where MFI is the median fluorescent intensity and rSD is the robust standard deviation.

$$\text{Stain Index} = \frac{(\text{MFI}_{\text{positive}} - \text{MFI}_{\text{negative}})}{2 \times \text{rSD}_{\text{negative}}}$$

Results

Note: The figures presented in the results section use blue dots, lines, or shading to represent data generated from the 488-nm (blue) laser and detectors and yellow-green dots, lines, or shading to represent data generated from the 561-nm (yellow-green) laser and detectors.

Increased PE sensitivity

The data presented in Figures 7, 8, and 9 compares the detection of PE-conjugated antibodies on the surface of human PBMC lymphocytes (CD4) and mouse splenic monocytes (CD11c) when excited with the 488-nm versus 561-nm lasers. In both cases there is an increase in stain index when PE is excited by the 561-nm laser (summarized in Figure 9).

- In Figure 7, the stain index of human CD4 PE (lymphocytes) increased 3.3-fold from 149.2 to 489.8.
- In Figure 8, the stain index of mouse CD11c PE (splenic monocytes) increased 4.9-fold from 28.6 to 139.9.

As expected, the different wavelengths of the excitation lasers had no effect on the FITC stain index (data not shown).

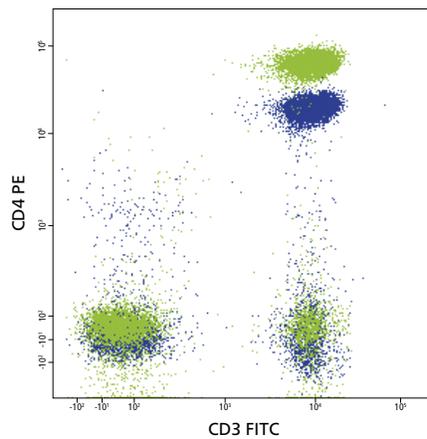


Figure 7. CD3 and CD4 staining of human lymphocytes.

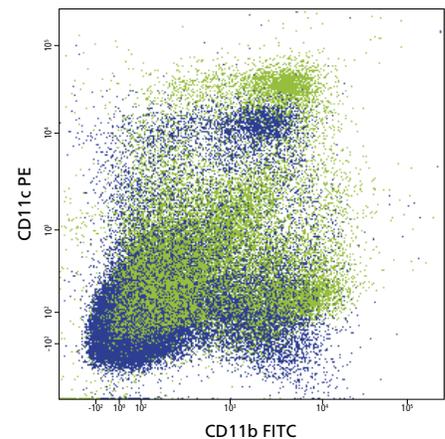


Figure 8. CD11b and CD11c staining of mouse splenic monocytes.

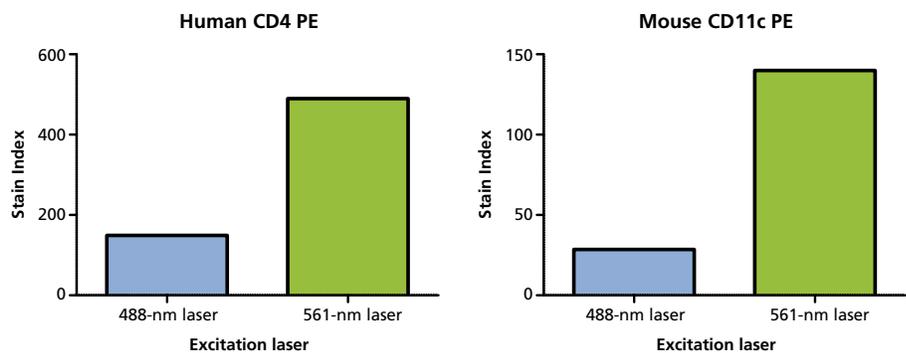


Figure 9. Human CD4 PE and mouse CD11c PE stain index.

Elimination of PE-FITC spillover

The data presented in Figure 10 shows the spillover of FITC into the PE detector. In the BD FACS Aria IIu configuration, the FITC and PE emission share the same optical detection pathway, resulting in 12.9% and 27.9% spillover, respectively. In the BD FACS Aria IIIu configuration, the FITC and PE emission have different optical detection pathways resulting in 0.0% spillover on both instruments.

As expected, PE-FITC spillover is not affected by the different optical configurations (data not shown).

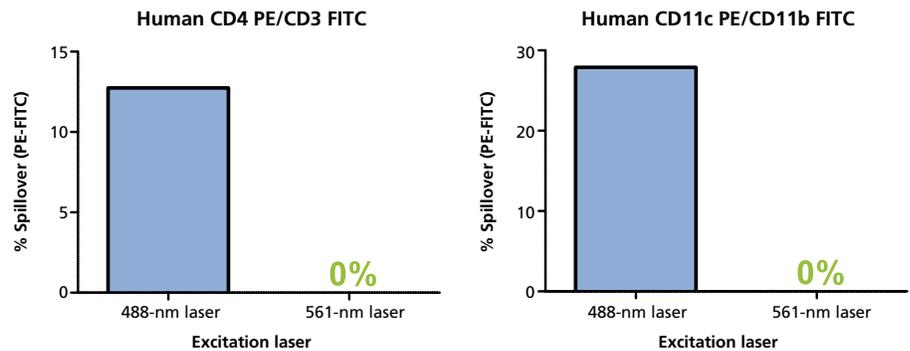


Figure 10. PE-FITC spillover.

Increased mCherry sensitivity

The data presented in Figure 11 compares the detection of mCherry in SpiC1 mCherry-expressing mouse cells when excited with the 488-nm and 561-nm lasers. Use of the 561-nm laser resulted in a 16.4-fold increase in mCherry stain index (from 5.7 to 93.2) resulting in a clearer resolution of a mCherry^{hi} population (Figure 12).

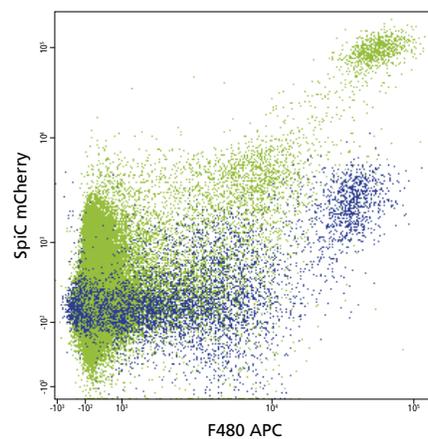


Figure 11. SpiC1 mCherry expression of mouse splenic monocytes.

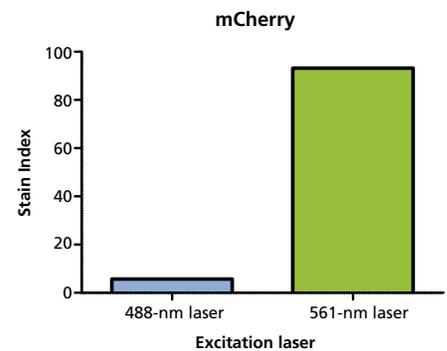


Figure 12. mCherry stain index.

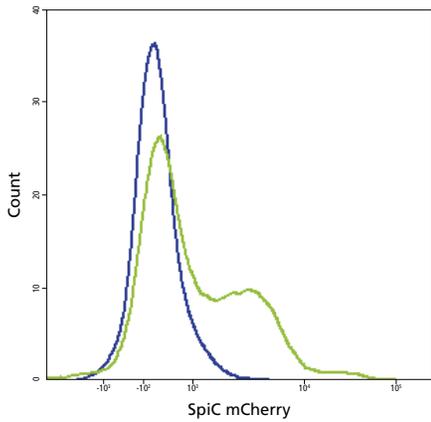


Figure 13. mCherry expression of F4/80⁺CD11b⁺ mouse spleen cells.

Increase in ability to detect dim signals

Mouse spleen cells

The data presented in Figures 13, 14, and 15 compares the detection of SpiC mCherry, CD11c PE in F4/80⁺CD11b⁺ mouse spleen cells, and the detection of CD3e PE-Cy7 in CD8a⁻NK1.1⁺ mouse spleen cells when excited with the 488-nm and 561-nm lasers. This very preliminary data shows the presence of SpiC mCherry⁺, CD11c^{dim}, and CD3e⁺ populations when the 561-nm laser was used that were not present when the 488-nm laser was used for fluorochrome excitation.

These are preliminary observations, and these populations may not have any biological significance and have the potential to be artifactual observations. Additional work is required to verify that the detection of these populations is reproducible and do indeed represent new, distinct populations.

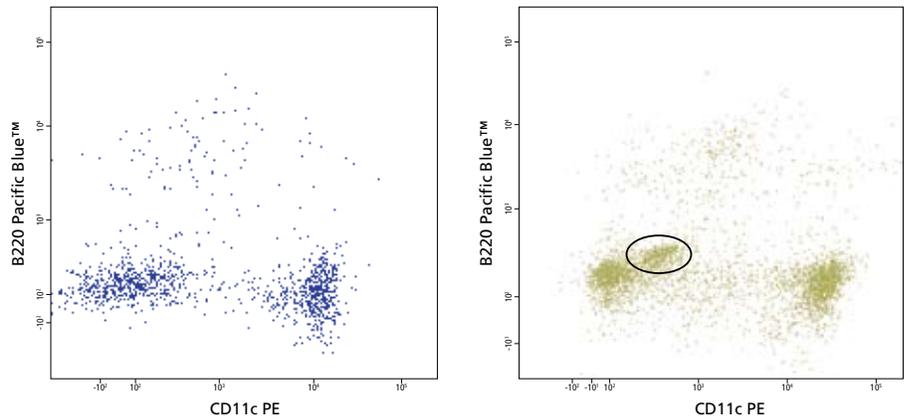


Figure 14. CD11c expression of F4/80⁺CD11b⁺ mouse spleen cells.

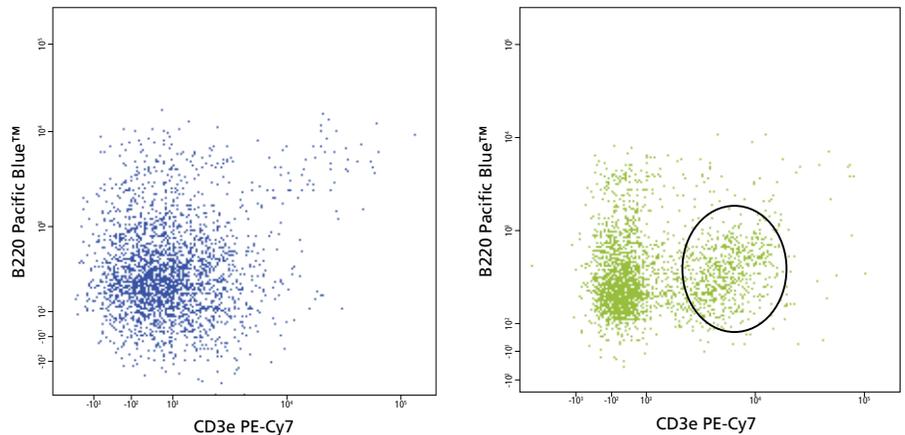


Figure 15. CD3e expression of CD8a⁻NK1.1⁺ mouse spleen cells.

Human lymphocytes

The data presented in Figures 16, 17, and 18 compares the detection of dim CD56⁺CD377⁺, CD45RA⁺CD11b⁺, and Integrin B₇⁺ human lymphocyte populations when excited with the 488-nm and 561-nm lasers. This preliminary data shows the increased resolution of these populations with the 561-nm laser compared to when the 488-nm laser was used for excitation. A comparison of the stain indices is shown in Figure 19.

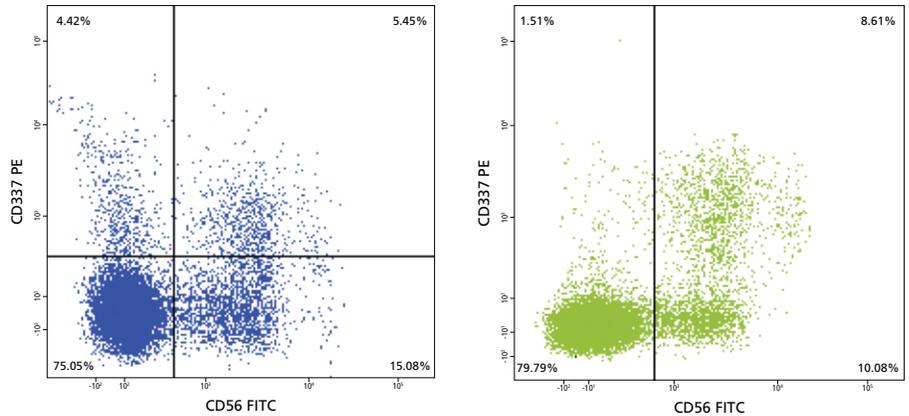
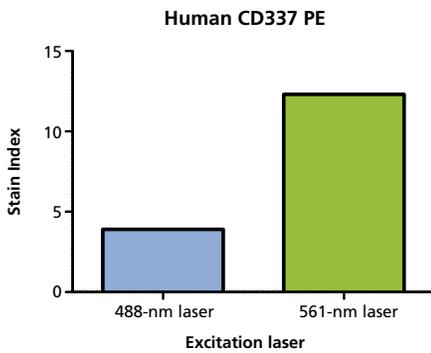


Figure 16. Detection CD56⁺CD377⁺ human lymphocyte populations.

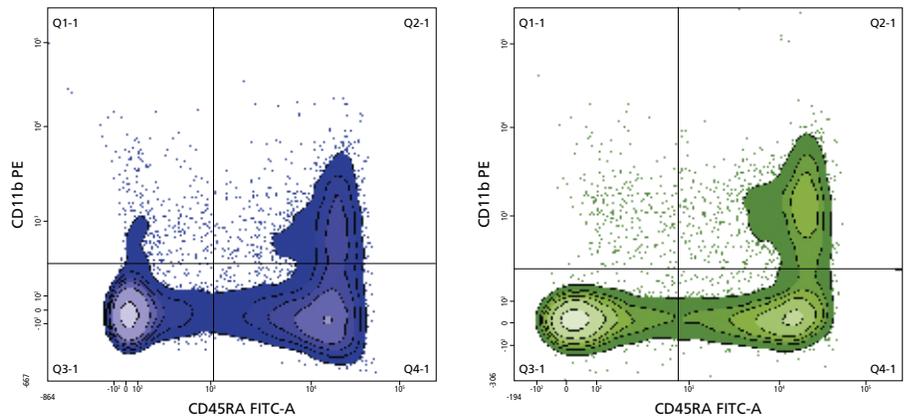
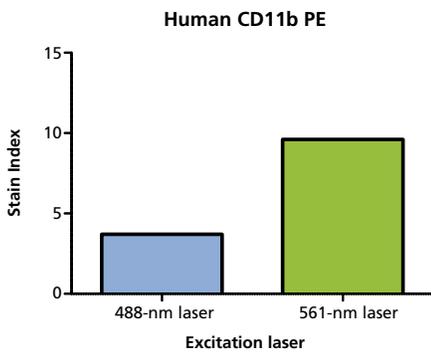


Figure 17. Detection of human CD45RA⁺CD11b⁺ lymphocyte populations.

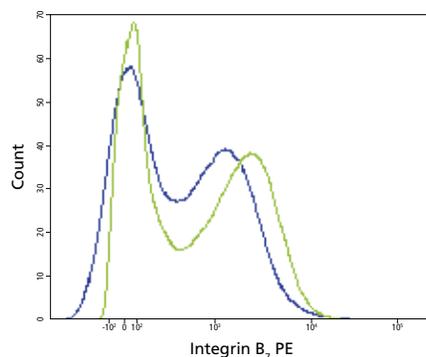
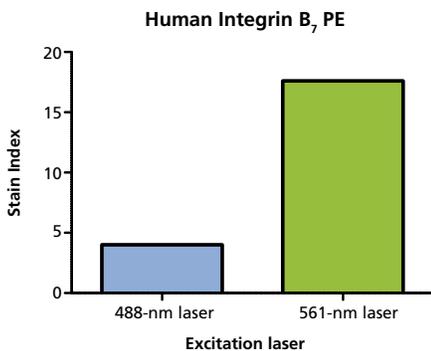


Figure 19. Stain indices.

Figure 18. Detection of Integrin B₇ in human lymphocytes.

Discussion

As outlined in the introduction, FITC and PE have overlapping excitation spectra and are normally simultaneously excited by the 488-nm (blue) laser. In the 488-nm detector (octagon), the emission spectra of these fluorochromes are separated by dichroic mirrors and different bandpass filters. However, the emission spectrum from FITC still spills over into the PE detector but is overcome using compensation. The physical separation of FITC and PE excitation and emission using the 561-nm laser and detectors, and the more efficient excitation of PE by the 561-nm laser, combined with the elimination of PE-FITC spillover results in an increase in PE signal. This is manifested by an increase in PE stain index (Figures 9 and 19), and the increased ability to resolve dim staining PE populations in a multicolor panel (Figures 14, 16, 17, and 18). The preliminary data presented in this application note demonstrates that, on average, the stain index of PE-conjugated surface antibodies is 3.7-fold higher when the 561-nm laser is used compared to the 488-nm laser (n = 5).

The fruit fluorescent proteins, such as mCherry, are becoming the fluorochromes of choice for the detection of gene expression in transfected cells and are commonly used in microscopy studies. Resolution of these fluorochromes using a flow cytometer is difficult on instruments that do not sufficiently excite these fluorochromes. It is possible to detect mCherry on a BD FACSAria II since the 488-nm (blue) laser provides minimal excitation (8%) at the very edge of the excitation spectrum. When this suboptimal excitation is combined with significant spectral overlap from other fluorochromes that require compensation, the detection of dim mCherry expression by transfected cells becomes extremely challenging. The 561-nm laser provides much more efficient excitation of this fluorochrome (64%), and when combined with a physical separation from fluorochromes with overlapping emission spectra, can result in a stronger mCherry signal as shown by an increase in stain index (Figures 11 and 12). The preliminary data presented in this application note shows a 16.4-fold increase in mCherry stain index resulting in clearer resolution of an mCherry^{hi} population.

Further analysis of the 8-color mouse panel shows that, in addition to an increase in stain index of CD11c⁺ PE and SpiC mCherry^{hi} populations, the 561-nm laser and detectors also reveal the presence of SpiC mCherry^{lo}, CD11c^{dim} PE, and CD3e⁺ PE-Cy7 populations that could not be visualized when the 488-nm laser and detectors were used (Figures 13, 14, and 15). Finally, a comparison of some dim staining human populations shows that the resolution of CD56⁺CD337⁺, CD45RA⁺CD11b⁺, and Integrin B₇⁺ human lymphocyte populations can be enhanced using the 561-nm laser (Figures 16, 17, and 18). This is very preliminary data and will need additional investigation.

Conclusions

In conclusion, the preliminary data presented in this application note demonstrates that compared to the 488-nm laser, the use of a 561-nm laser and detector results in:

- Increased PE detection sensitivity as demonstrated by an average 3.7-fold increase in stain index
- Elimination of PE-FITC spillover to 0%
- Improved resolution of mCherry-expressing populations as demonstrated by a 16.4-fold increase in stain index and the resolution of a SpiC mCherry^{int} population
- Increased ability of the cytometer to detect dim or previously unseen populations

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Tips for developing a multicolor research panel

- Successful development of multicolor flow cytometry panels can be time consuming and can require careful application of complex design principles. Be sure to allow sufficient time for testing and design rework.
- Information with suggested guidelines on choosing reagents and reducing compensation problems is available in the application note, “Selecting Reagents for Multicolor Flow Cytometry”.⁸ As a general rule:
 - Use fluorochromes with a high stain index for low density markers and fluorochromes with a lower stain index for higher density markers.
 - As you add new fluorochromes to an existing panel, check that your original markers continue to perform as expected and are not compromised by spectral overlap. Watch for the appearance of false positives.^{9,10}
- Consider the utilization and objective of each marker when making a decision on fluorochrome choice.
 - Markers used to gate or define high-percentage populations may not need to be as bright as those markers used to probe the biology of the cells or to detect rare events.
- Take care in your experimental design and ensure that your controls are appropriate. Refer to Maecker and Trotter (2009) for additional information.⁸
- When acquiring data that will be subsequently utilized in the analysis of rare event phenotypes, ensure that sufficient data is acquired. As a general rule, a minimum of 2,500 events for the rarest phenotype of interest might be sufficient for analysis.
- For consistent results, maintain your flow cytometer and perform QC on a regular basis. BD CS&T beads are designed to automate setup and track the performance of your cytometer.
- Technical support for the design of multicolor panels is provided by BD Biosciences. Visit bdbiosciences.com/research/multicolor/index.jsp for additional information.

BD Biosciences is constantly expanding its portfolio of conjugates for flow cytometry. If you can't find a catalog conjugate, please try our Custom Conjugation Service which offers more choices for multicolor experiment design. Contact your BD Sales representative for more information about the Custom Conjugation Program or visit bdbiosciences.com/services/customtechnologyteam/index.jsp.

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