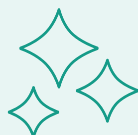
Fluorochrome Faceoff

See how your favorite fluorochromes stand up to the competition

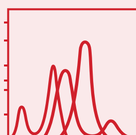
Explore the performance of various flow cytometry fluorochromes excited primarily by the blue and yellow-green lasers as they face off against the competition. In this series of faceoffs, we highlight key fluorochrome attributes to keep in mind when designing flow cytometry panels and review performance data to shine a light on the best-performing fluorochromes within each category.

FACEOFF 1



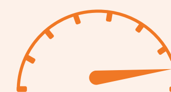
Resolution

FACEOFF 2



Spillover

FACEOFF 3

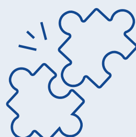


Photostability

FACEOFF 4

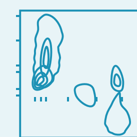
Intracellular Staining

FACEOFF 5



Buffer Compatibility

FACEOFF 6



Monocyte Background

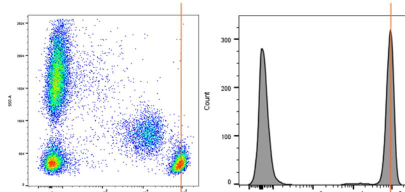
BD

FACEOFF 1 | Resolution

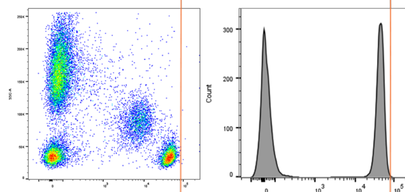
Competitors: RB780 | PerCP/Fire™ 780 | PE-Cy7 | cFBYG781

Fluorochrome resolution is the degree of separation between the negative and positive populations as calculated by Stain Index, (MFIpos – MFIneg)/2(rSDneg). CD4 is commonly used as a reference for Stain Index because of its robust expression and regular availability of the same clone in different formats. Here we measure CD4 (SK3) Stain Index values for several fluorochromes excited by the blue-laser with a peak emission around 780 nm.

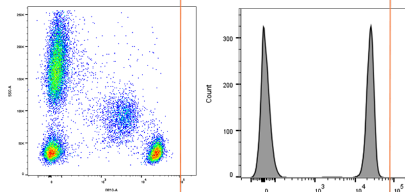
RB780
hCD4 (SK3)



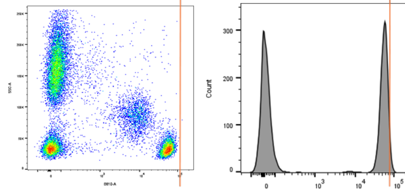
PE-Cy7
hCD4 (SK3)

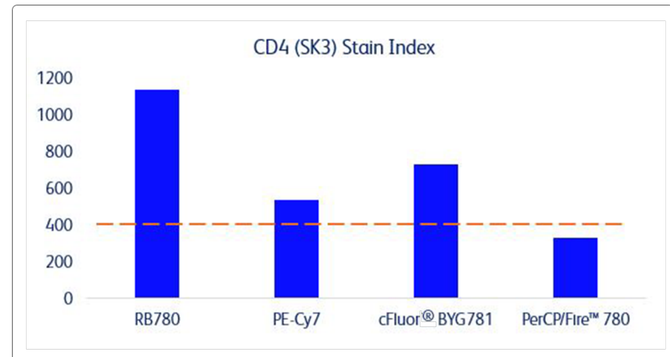


PerCP/Fire™ 780
hCD4 (SK3)



cFBYG781
hCD4 (SK3)





Human whole blood was stained with BD Horizon™ RB780 Mouse Anti-Human CD4 SK3 (Cat. No. 568675), BD Pharmingen™ PE-Cy7 Mouse Anti-Human CD4 SK3 (Cat. No. 557852), BioLegend PerCP/Fire™ 780 Mouse Anti-Human CD4 SK3 or Cytek cFluor® BYG781 Mouse Anti-Human CD4 SK3 Reagent. Erythrocytes were lysed with BD Pharm Lyse™ Lysing Buffer (Cat. No. 555899). The bivariate pseudocolor plots showing the expression of CD4 versus side light-scatter (SSC-A) signals were derived from gated events with the forward and side light-scatter characteristics of viable leukocytes. Histograms were derived from gated events based on light scattering characteristics for lymphocytes. Flow cytometry was performed conventionally using a BD FACSymphony™ A5 SE Cell Analyzer System. Data were generated from the Blue 810 detector using FlowJo™ Software.

Winner's Circle

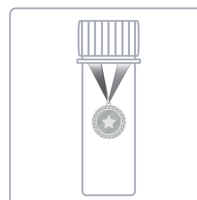
While all the fluorochromes used in this experiment provide good population resolution of the CD4 positive cells, there are some differences in terms of the stain index values. We, therefore, ranked RB780, cFBYG781 and PE-Cy7 as the winners, with PerCP/Fire™ 780 as the runner up.

Pro Tip

Be sure to pair bright dyes (high CD4 stain index values) with lower antigen-expression markers to get the best possible biological resolution of your data!

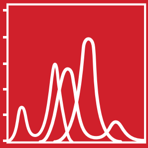
Winners:

RB780, cFBYG781 and PE-Cy7



Runner-Up:

PerCP/Fire™ 780

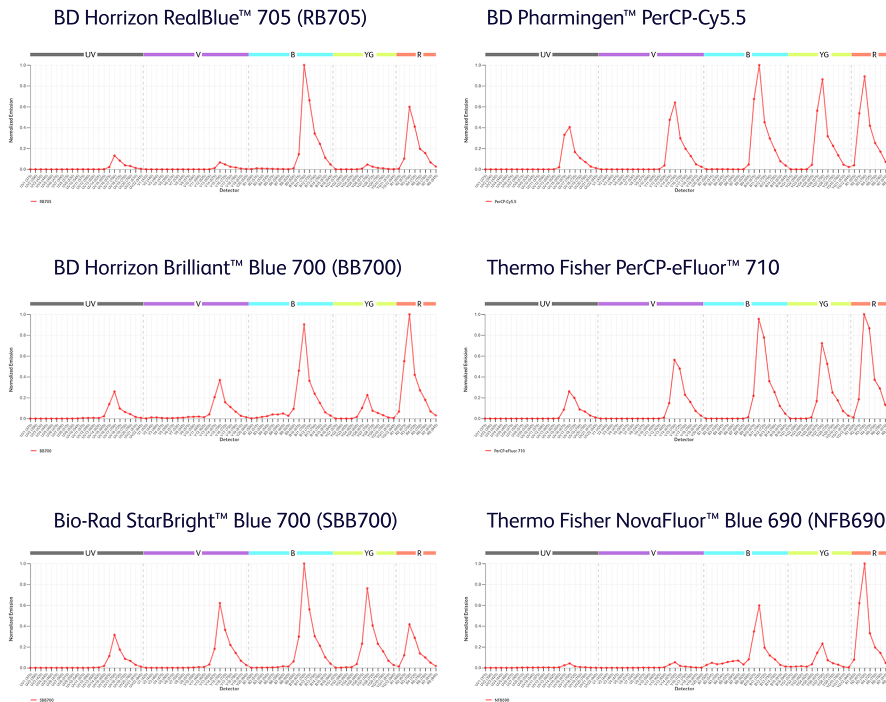


FACEOFF 2 | Spillover

Competitors: RB705 | PerCP-Cy5.5 | SBB700 | PerCP-eFluor™ 710 | BB700 | NFB690

Fluorescence spillover is the fluorescent signal measured in adjacent, residual and cross-laser detectors. Spectral overlap between fluorochromes can be managed through compensation or spectral unmixing to prevent data artifacts. However, even with compensation and unmixing, spectral overlap of two fluorochromes in a panel can lead to compromised resolution through spillover spreading and unmixing error, making spillover an important consideration in panel design.

Spillover here is evaluated and ranked based on the analysis of a given fluorochrome's full emission profile across five lasers. Fluorochromes with less cross-laser excitation are cleaner and will have lower impact on other fluorochromes. We evaluated several fluorochromes primarily excited by the blue laser that have peak emission around 700 nm:



Freshly isolated human PBMCs were stained with either BD Horizon™ RB705 Mouse Anti-Human CD4 SK3 (Cat. No. 570221), BD Pharmingen™ PerCP-Cy5.5 Mouse Anti-Human CD4 SK3 (Cat. No. 566923), BD Horizon™ BB700 Mouse Anti-Human CD4 SK3 (Cat. No. 566392), Thermo Fisher PerCP-eFluor™ 710 Mouse Anti-Human CD4 SK3, Bio-Rad StarBright™ Blue 700 Mouse Anti-Human CD4 RPA-T4, or Thermo Fisher NovaFluor™ Blue 690 Anti-Human CD4 SK3 Reagent. Spectral profiles are generated from the positive population with the negative subtracted and are normalized to peak detector. Spillover percentages are dependent on the configuration of an instrument. The rankings were based on analysis on three instruments (BD FACSDiscover™ S8 Cell Sorter, BD FACSymphony™ A5 SE Cell Analyzer and Cytek Aurora). For the data shown, flow cytometry and data analysis were performed using a BD FACSDiscover™ S8 Cell Sorter and FlowJo™ Software.

Winner's Circle

Using normalized emission profiles, winners were determined based on the number of lasers with an additional peak of signal greater than 15% of the main peak signal. RB705 and NFB690 had the least amount of emission into other channels and are therefore declared the winners!

Pro Tip

When assigning a fluorochrome to a marker, it is important to consider how spillover spreading may affect resolution of other markers in your panel.

Winners:

RB705 and NFB690

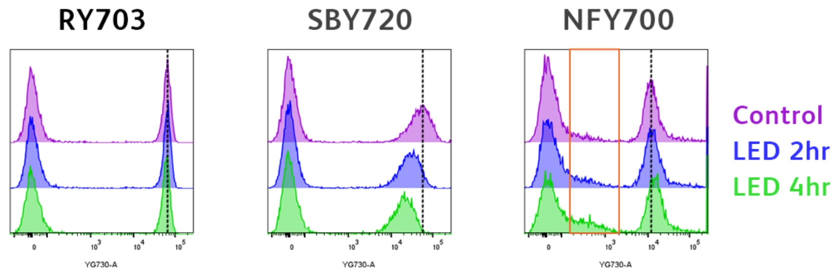
Runner-Up:

BB700

FACEOFF 3 | Photostability

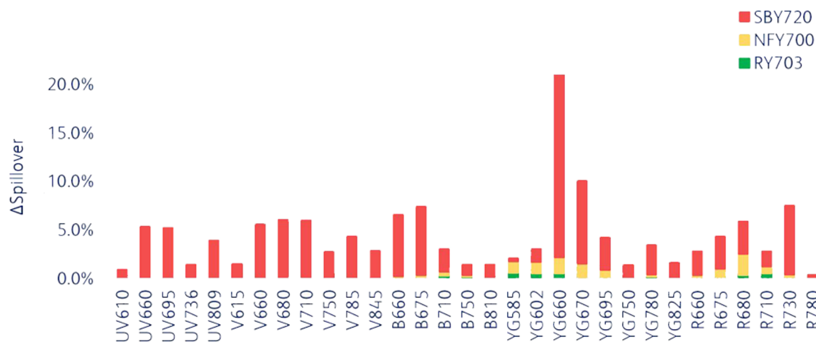
Competitors: RY703 | SBY720 | NFY700

Fluorescent dyes can undergo photobleaching (dimming) when exposed to light. This can cause a decrease in resolution. Additionally, some dyes may undergo spectral changes when exposed to light. In this Fluorochrome Faceoff, we evaluate the impact of light on fluorochromes that are primarily excited by the yellow-green laser with peak emission around 700 nm.



Human whole blood was stained with BD Horizon™ RY703 Mouse Anti-Human CD4 SK3 (Cat. No. 571427), Bio-Rad StarBright™ Yellow 720 Mouse Anti-Human CD4 RPA-T4 or Thermo Fisher NovaFluor™ Y700 Mouse Anti-Human CD4 SK3 Reagent. Erythrocytes were lysed with BD Pharm Lyse™ Lysing Buffer (Cat. No. 555899). After staining was completed, cells were either kept in the dark at room temperature (purple histogram) or exposed to 200 lux of LED light at room temperature for 2 (blue histogram) or 4 hours (green histogram). Histograms were derived from gated events based on light scattering characteristics for lymphocytes. Flow cytometry and data analysis were performed using a BD FACSymphony™ A5 SE Cell Analyzer System and FlowJo™ Software.

Note that in the histograms shown, both MFI loss and an increase in background with light exposure can be observed. Both changes result in lower stain index and resolution.



Change in spillover was evaluated following light exposure. This graph shows the absolute change in percent spillover into each channel after 2 hours of LED light exposure when compared to control samples kept in the dark.

Winner's Circle

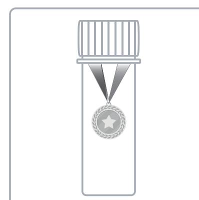
Exposure to light can cause changes in a fluorochrome's emission profile, measured here as changes in spillover into other detectors. In an experimental setting, this can impact researchers as differences in spectral profile between reference controls and samples can lead to compensation or unmixing errors and data artifacts. This example shows changes in spillover in several detectors after 2 hours of light exposure, with SBY720 being the fluorochrome impacted the most. SBY720 also exhibited the greatest loss in resolution after light exposure. Our winner for this Fluorochrome Faceoff is RY703 because this fluorochrome exhibited the least amount of spillover change and MFI loss across the dyes tested.

Pro Tip

Keep your tubes protected from light during sample acquisition to prevent changes in spectral profiles over time.

Winners:

RY703



Runner-Up:

NFY700

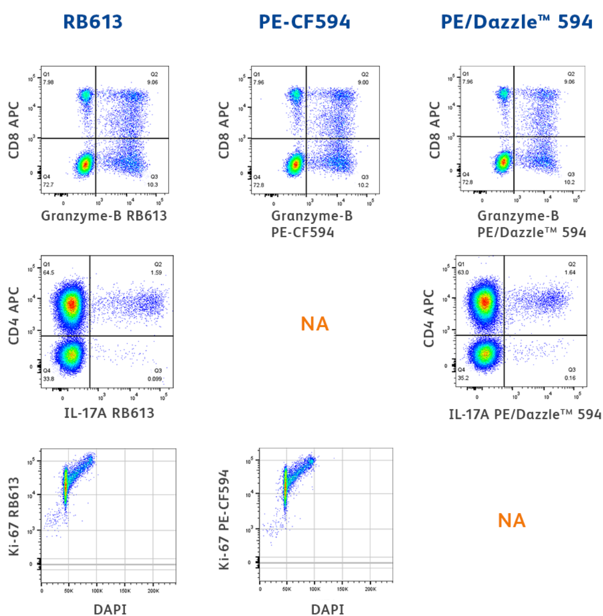
FACEOFF 4 | Intracellular Staining

Competitors: RB613 | PE-CF594 | PE/Dazzle™ 594

When you assess intracellular markers in your assay, fluorochrome choice can have a significant impact on your results. Not all fluorochromes work well for intracellular staining, as some fluorochromes are impacted by buffers used to fix and permeabilize the cells.

In this Fluorochrome Faceoff, we pair fluorochromes that are excited off the blue laser and emit around 610 nm. We selected several different intracellular markers to evaluate and stained them with three different fluorochromes. As you can see, each dye provides good population resolution and the expected staining pattern. Please note that there are clone differences between the dyes.

Top: Two-color flow cytometric analysis of Granzyme B expression in human peripheral blood lymphocytes. Human whole blood was fixed with BD Phosflow™ Lyse/Fix Buffer (Cat. No. 558049). The cells were then washed and stained in BD Perm/Wash™ Buffer (Cat. No. 554723) with APC Mouse Anti-Human CD8, clone RPA-T8 (Cat. No. 561953) and BD Horizon™ RB613 Mouse Anti-Human Granzyme B, clone GB11 (Cat. No. 571117), BD Horizon™ PE-CF594 Mouse Anti-Human Granzyme B, clone GB11 (Cat. No. 562462) or BioLegend PE/Dazzle™ 594 Mouse Anti-Human Granzyme B, clone QA18A28 Reagent. The bivariate pseudocolor density plots showing the correlated expression of Granzyme B versus CD8 were derived from gated events with the forward and side light-scatter characteristics of intact lymphocytes.



Middle: Two-color flow cytometric analysis of IL-17A expression in stimulated human peripheral blood lymphocytes. Human peripheral blood mononuclear cells were stimulated for 5 hours with Phorbol 12-Myristate 13-Acetate (Sigma P-8139; 50 ng/ml final concentration) and Ionomycin (Sigma I-0634; 1 µg/ml final concentration) in the presence of BD GolgiStop™ Protein Transport Inhibitor (containing Monensin) (Cat. No. 554724). The cells were harvested, washed with BD Pharmingen™ Stain Buffer (FBS) (Cat. No. 554656) and fixed with BD Cytofix™ Fixation Buffer (Cat. No. 554655). The cells were washed then permeabilized and stained in BD Perm/Wash™ Buffer (Cat. No. 554723) with APC Mouse Anti-Human CD4 SK3 antibody (Cat. No. 555349) and either BD Horizon™ RB613 Mouse Anti-Human IL-17A, clone N49-653 (Cat. No. 571130) or BioLegend PE/Dazzle™ 594 Mouse Anti-Human IL-17A, clone BL168 Reagent. The bivariate pseudocolor density plots showing the correlated expression of IL-17A versus CD4 were derived from gated events with the forward and side light-scatter characteristics of intact lymphocytes. NA indicates that the specificity/format combination was not available to us at the time of testing.

Bottom: Two-color flow cytometric analysis of Ki-67 expression by proliferating human MOLT-4 cells. Proliferating cells from the human MOLT-4 (T lymphoblastic leukemia, ATCC® CRL-1582™) cell line were permeabilized and fixed with 70% ice-cold ethanol. The cells were washed twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656), stained with BD Horizon™ RB613 Mouse Anti-Ki-67, clone B56 (Cat No. 571126), BD Horizon™ PE-CF594 Mouse Anti-Ki-67, clone B56 (Cat. No. 567120) or BioLegend PE/Dazzle™ 594 Mouse Anti-Ki-67, clone 11F6 Reagent and counterstained with BD Pharmingen™ DAPI Solution (Cat. No. 564907) to stain DNA. Bivariate pseudocolor density plots showing the correlated expression of DAPI staining versus Ki-67 expression were derived from gated events with the forward and side light-scatter characteristics of MOLT-4 cells. NA indicates that the specificity/format combination was not available to us at the time of testing.

Flow cytometry and data analysis were performed using a BD FACSymphony™ A5 SE Cell Analyzer System and FlowJo™ Software.

Winner's Circle

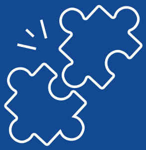
Because all three of the dyes tested perform well for intracellular staining, we have classified them all as winners! Please note that as of July 26, 2024, StarBright™ and NovaFluor™ Dye-conjugated antibodies are currently only available against cell surface markers and are not currently conjugated to intracellular markers. They were, therefore, not included in this Fluorochrome Faceoff.

Pro Tip

In addition to fluorochrome sensitivity, different clones are also differentially impacted by treatments with different permeabilization protocols and therefore it is important to make sure you are using an antibody clone that can withstand these protocols.

Winners:

RB613, PE-CF594 and PE/Dazzle™ 594



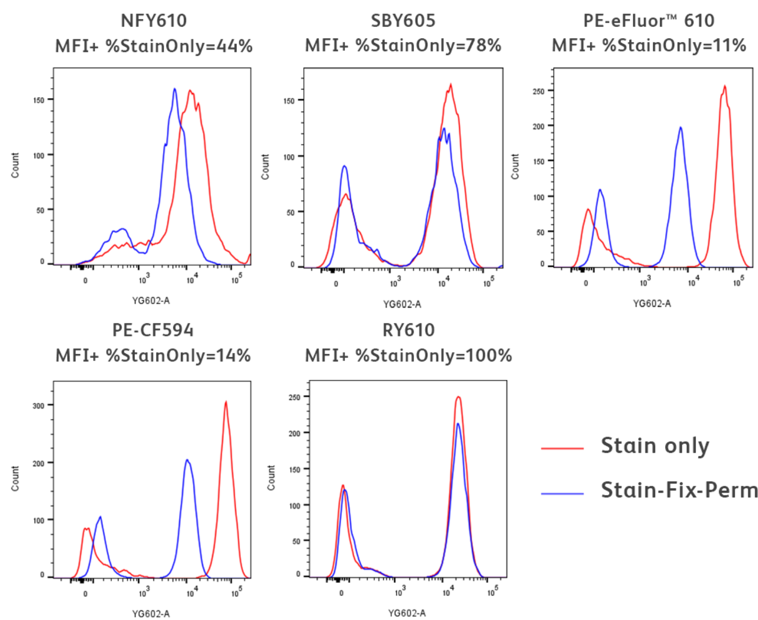
FACEOFF 5 | Buffer Compatibility

Competitors: RY610 | PE-CF594 | SBY605 | NF610 | PE-eFluor™ 610

Once you determine the markers you want to investigate in your flow cytometry panel and the protocol you want to use, you will need to determine what fluorochromes are compatible with the staining, fixation and permeabilization buffers you will use.

If you are interested in investigating markers within the cell, your cells may need to undergo treatments to make those antigens accessible to your staining reagents. It is important to consider how using different buffers affects the staining pattern of your markers and biological resolution of your data.

Here we demonstrate an example using a common fixation and permeabilization protocol. The staining of several fluorochromes excited by the yellow-green laser with peak emission around 610 nm is evaluated in cells treated with fixative and methanol-based permeabilization buffer after surface staining (blue line). Untreated cells are used as a reference (red line).



Freshly isolated human PBMCs were stained for 30 minutes with either BD Horizon™ RY610 Mouse Anti-Human CD3 UCHT1 (Cat. No. 571134), BD Horizon™ PE-CF594 Mouse Anti-Human CD3 UCHT1 (Cat. No. 562280), Thermo Fisher NovaFluor™ Yellow 610 Mouse Anti-Human CD3 UCHT1, Bio-Rad StarBright™ Yellow 605 Mouse Anti-Human CD3 UCHT1 or Thermo Fisher PE-eFluor™ 610 Mouse Anti-Human CD3 UCHT1 Reagent. The stained PBMCs were then washed twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656) (red histograms). For the treated condition (blue histograms), after washing twice with BD Pharmingen™ Stain Buffer, cells were fixed using BD Cytofix™ Fixation Buffer (Cat. No. 554655) and permeabilized with BD Phosflow™ Perm Buffer III (Cat. No. 558050) for 30 minutes on ice. Cells were then washed twice with BD Pharmingen™ Stain Buffer. Histograms were derived from gated events based on light scattering characteristics for lymphocytes. Flow cytometry and data analysis were performed using a BD FACSymphony™ A5 SE Cell Analyzer System and FlowJo™ Software.

Winner’s Circle

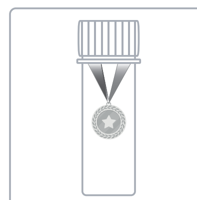
You can tell if the biological resolution of your data is impacted by the buffer you’re using by comparing the staining profiles of the untreated (red) and fix-perm treated samples (blue). As you can see in the data above, there is varying impact to the fluorochromes used in this experiment, from little-to-no impact for RY610 and StarBright™ Yellow 605 to a significant reduction in signal for PE-CF594 and PE-eFluor™ 610.

Pro Tip

It is important to note that PE-tandem dyes are notorious for being impacted by these harsher staining conditions.

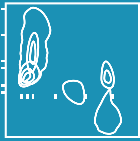
Winners:

RY610 and SBY605



Runner-Up:

NFY610



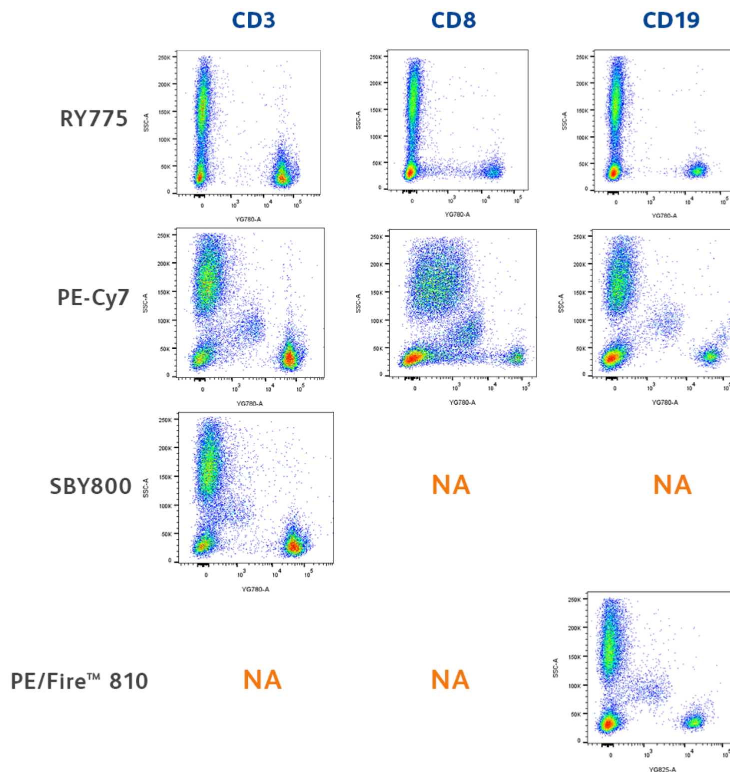
FACEOFF 6 | Monocyte Background

Competitors: RY775 | PE-Cy7 | SBY800 | PE/Fire™ 810

It has been shown that some cyanine (Cy™)—and cyanine-like—dyes can nonspecifically bind to monocytes. Additional nonspecific binding to the human Fc receptor has been observed. Nonspecific monocyte binding can decrease data resolution by increasing background and/or causing a false positive signal.

In this Fluorochrome Faceoff, we evaluate dyes excited by the yellow-green laser with peak emission around 780 nm on several clones.

As expected, PE-Cy7 demonstrated significant non-specific monocyte binding, irrespective of the antibody clone it was conjugated to. Bio-Rad StarBright™ Yellow 800 and BioLegend PE/Fire™ 810 also demonstrated varying degrees of nonspecific binding to the monocyte population.



Human whole blood was stained with BD Horizon™ RY775 Mouse Anti-Human CD3 UCHT1 (Cat. No. 571376), BD Horizon™ RY775 Mouse Anti-Human CD8 RPA-T8 (Cat. No. 571380), BD Horizon™ RY775 Mouse Anti-Human CD19 SJ25C1 (Cat. No. 571374), BD Pharmingen™ PE-Cy7 Mouse Anti-Human CD3 UCHT1 (Cat. No. 563423), BD Pharmingen™ PE-Cy7 Mouse Anti-Human CD8 RPA-T8 (Cat. No. 557746), BD Pharmingen™ PE-Cy7 Mouse Anti-Human CD19 SJ25C1 (Cat. No. 557835), Bio-Rad StarBright™ Yellow 800 Mouse Anti-Human CD3 UCHT1 or BioLegend PE/Fire™ 810 Mouse Anti-Human CD19 SJ25C1 Reagent. Erythrocytes were lysed with BD Pharm Lyse™ Lysing Buffer (Cat. No. 555899). The bivariate pseudocolor plots showing the expression of CD3, CD8 and CD19 versus side light-scatter (SSC-A) signals were derived from gated events with the forward and side light-scatter characteristics of viable leukocytes. Flow cytometry and data analysis were performed using a BD FACSymphony™ A5 SE Cell Analyzer System and FlowJo™ Software. N/A means the corresponding clone/specificity was unavailable conjugated to that fluorochrome at the time of our testing.

Winner's Circle

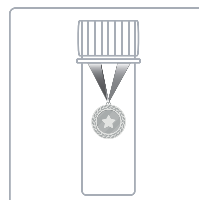
BD Horizon RealYellow™ 775 was the only fluorochrome that did not demonstrate nonspecific background binding to monocytes and is declared the winner of this Fluorochrome Faceoff!

Pro Tip

There are several different blocking buffers available to help improve these nonspecific interactions, such as the BD Pharmingen™ MonoBlock™ Leukocyte Staining Buffer (Cat. No. 570002).

Winners:

RY775



Runner-Up:

SBY800

Fluorochrome Family Summary

As you can see, not all fluorochromes are created equal. Some are bright, some are dim; some are excited by many lasers, some are excited by only one or two; some work well for intracellular staining, some do not; and so on. For example, the StarBright™ Dyes are generally bright with unique spectral profiles but are not available on intracellular markers. The NovaFluor™ Dyes generally have clean spillover profiles but tend to be dimmer and have nonspecific background staining. PE and PerCP tandem dyes have cross laser excitation that limits their utility in multicolor flow cytometry panels. In comparison, the BD Horizon RealYellow™ and BD Horizon RealBlue™ Dyes generally perform well across all categories measured in this competition. In the chart below, we have summarized the overall performance of the various families of dyes that competed in this Fluorochrome Faceoff.

Dye Family	Spillover	Resolution	Buffer Compatibility	Intracellular Staining	Photostability	Monocyte Background
What Good Looks Like	1-2 Peak/s	3- Relative Brightness	Perm III; >50% MFI Compared to Control after Treatment	Catalog Availability	<25% MFI Loss vs Time 0	No Nonspecific Binding
BD Horizon RealYellow™ and RealBlue™ Reagents	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓
StarBright™ Blue and Yellow Reagents	✓	✓✓	✓✓	⊘	✓	✓
NovaFluor™ Blue and Yellow Reagents	✓✓	✓	✓	⊘	✓✓	✗
BD Horizon Brilliant™ Blue Reagents	✓	✓✓	✓✓	✓	✓✓	✓
PE and PE Tandems	✓	✓✓	✗	✓✓	✓	✗
PerCP and PerCP Tandems	✗	✗	✗	✓	✓	✓

✓✓ Most dyes pass ✓ Some dyes pass ✗ Most dyes fail ⊘ No clones available for testing

Note: Additional data supporting the generation of this chart are not shown as part of the Fluorochrome Faceoff series.

Disclaimer: Results and conclusions shown throughout the Fluorochrome Faceoff are based on experiments performed under the conditions described. Users should evaluate reagents with their specific protocols as results may vary with different experimental conditions.

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