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New fluorochromes offer full utilization of the blue and yellow-green laser lines – the BD Horizon RealBlue™ and RealYellow™ development story

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Abstract

For many years, flow cytometry has leveraged PE and PE tandems that are excited by both the blue (488nm) and yellow-green(YG)/green (561nm/532nm) lasers. On modern 5 laser systems, this led to the underutilization of the blue laser line, with many systems only offering 2 fluorochromess out of the blue laser. To fully utilize both the blue and YG lasers, we have created the BD Horizon RealBlue™ and RealYellow™ family of fluorochromes. Here, we present the multiyear development journey, from concept to final product family.

To develop dyes that would demonstrate the best performance when combined with other dyes in multicolor flow cytometry, we used AI-guided optimization to determine the optimal number and emission maxima of fluors on these two laser lines. This resulted in a roadmap of 14 BD Horizon RealBlue™ and RealYellow™ dyes, all of which are now available. In addition to striving for dyes with excellent resolution and clean spillover profiles, our robust development process includes stress testing the dyes for photostability, thermostability, compatibility with a variety of lysis buffer systems and fixation and permeabilization systems, and intracellular compatibility. This resulted in fluorochromes that are designed and tested to work in the most common use cases. Our comprehensive analysis shows advantages in resolution, spillover, photostability, buffer compatibility, intracellular compatibility, and nonspecific background binding when compared to legacy and other commonly used dye families.

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Methods

Normalized emission profiles and CD4 overlays in Results 1A

Human whole blood was stained with either *BD Horizon™ RB780 Mouse Anti-Human CD4 SK3 (Cat. No. 568675), BD Horizon™ RY775 Mouse Anti-Human CD4 SK3 (Cat. No. 571373),* or *BD Pharmingen™ PE-Cy™7 Mouse Anti-Human CD4 SK3 (Cat. No. 560909)* and lysed using *BD FACS™ Lysing Solution (Cat. No. 349202).* Samples were acquired on a FACSDiscover S8. Spectral profiles are generated from the positive population with the negative subtracted and are normalized to peak detector. Plots shown are CD4 overlays, shown with an unmixing matrix that is appropriate for the combination of dyes displayed.

Normalized emission profiles shown in Results 2B

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

Spillover changes shown in Results 2B

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. Change in spillover was evaluated following light exposure. The 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

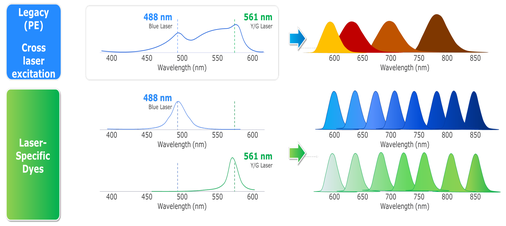
Normalized emission profiles and complexity scores in Results 2C

BD Spectrum Viewer was used to display normalized profiles of different dye combinations as well as the complexity scores for the FACSDiscover™ A8. Spectral profiles are generated from the positive population with the negative subtracted and are normalized to peak detector.

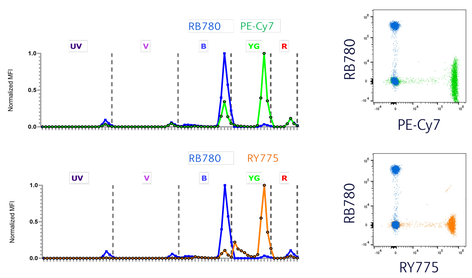
BD Spectrum Viewer can be found at: *https://www.bdbiosciences.com/en-us/resources/bd-spectrum-viewer*

Results (1)

1A Our Goal: Reduce Cross Laser Excitation and Fully Utilize Both the Blue and Yellow-Green Laser Lines

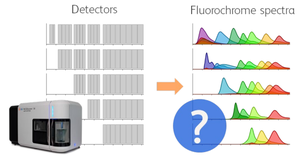


PE and PE tandems are excited by the Blue and YG lasers. Our goal was to create a series of Blue excited and a series of YG excited dyes, maximizing the use of those lasers.

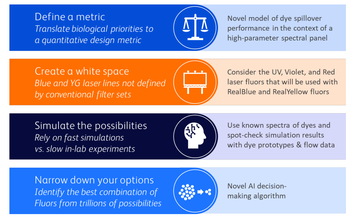


Top: Normalized emission profile for RB780 and PE-Cy 7 from a FACSDiscover™ S8, and the CD4 overlay, demonstrating the spread observed from the blue excitation of PE-Cy7.
Bottom: Normalized emission profile for RB780 and RY775, and the CD4 overlay, showing the reduction in spread observed with RY775 because it has less Blue cross-laser excitation.

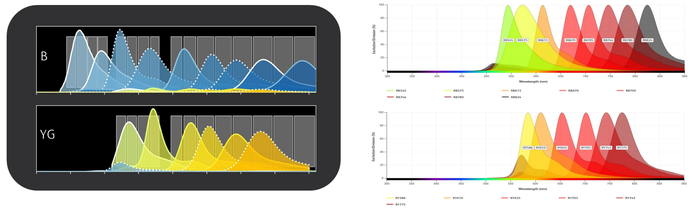
1B A New Approach to Dye Development – Creating Our Dye Roadmap



Given how spectral instruments work, how should you design a set of fluorochromes?

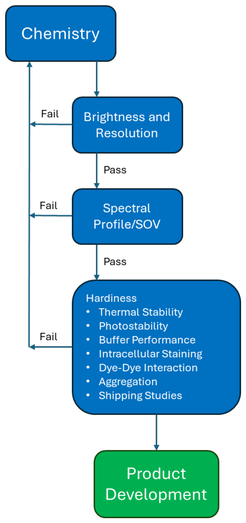


We used computational tools to help create our blue and yellow-green roadmap. Most importantly, we wanted to define the optimum number of dyes off each laser line as well as their spectral positioning. This work was done in the context of a 5-laser system with existing dyes excited by the UV, Violet, and Red lasers.



Left Image: The dye roadmap that resulted from our computational work. We used this throughout the development of the RealBlue™ and RealYellow™ dyes to target dyes at particular wavelengths/positions.
Right Image: Spectrum Viewer esport of the 14 RealBlue™ and RealYellow™ dyes that were developed following our roadmap.

1C Robust Tech Dev Process Ensures High Performing Dyes



Our robust tech-dev process allows us to iterate on the chemistry of the prototype dyes, making sure we select the best candidates for product development.

Results (2)

2A Full Suite of RealBlue™ and RealYellow™ Dyes Available

Format	Spectral	Conventional	Relative Brightness	Spillover (1-low, 4-high)
Blue Laser				
RB545	✓	–	●●○○	1
RB575	✓	✓	●●○○	1
RB613	✓	✓	●●●●	2
RB670	✓	✓	●●●●	2
RB705	✓	✓	●●●●	2
RB744	✓	✓	●●●●	1
RB780	✓	✓	●●●●	1
RB824	✓	✓	●●●○	1
Yellow-Green Laser				
RY586	✓	✓	●●●●	1
RY610	✓	✓	●●●○	1
RY655	✓	✓	●●●●	3
RY703	✓	✓	●●●●	2
RY743	✓	✓	●●●●	2
RY775	✓	✓	●●●●	2

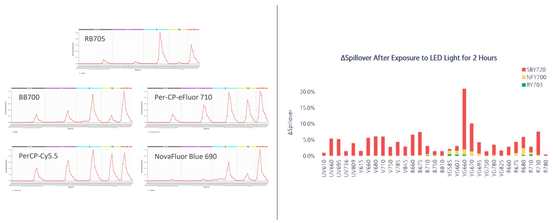
Summary table showing all 14 RealBlue ™ and RealYellow ™ dyes available for sale. Table shows the application of the dye (spectral of conventional flow), the relative brightness (4-point scale), as well as the spillover score for each dye (cross-laser spillover determined by assessing the number of significant peaks on a normalized emission graph).

2B RealBlue™ and RealYellow™ Dye Performance Compared to Other Dye Families

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

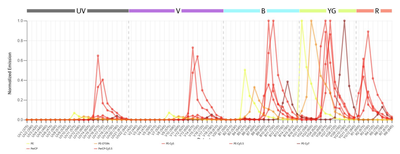
Note: Additional data supporting the generation of this chart are not shown as part of the Fluorochrome Faceoff series.



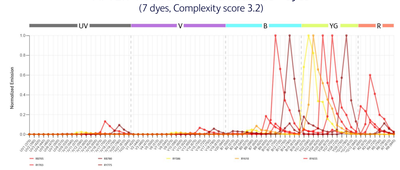
Top: Summary table showing RB and RY dye performance compared to other in-market dye families. Categories assessed were Spillover, Resolution, Buffer Compatibility, Intracellular Staining, Photostability and Monocyte Background.
Bottom: Data examples for the Spillover Category (left) and the Photostability Category (right)

2C Cleaner Dyes Reduce Complexity Scores

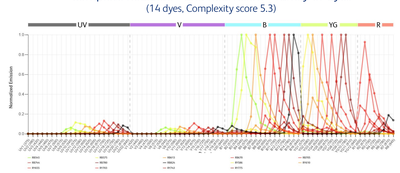
BEFORE: legacy PE and PerCP tandem dyes
(7 dyes, Complexity score 6.0)



AFTER: RealYellow™ and RealBlue™ dyes
(7 dyes, Complexity score 3.2)



Complete RealBlue™ and RealYellow™ family of dyes
(14 dyes, Complexity score 5.3)



Normalized emission profiles of different dye combinations and the complexity scores from a FACSDiscover ™ A8. Top combination: Legacy dyes including PE and PerCP tandem dyes. Middle combination: RB and RY direct replacements for the dyes in the top image. Bottom combination: All 14 RB and RY dyes.

Conclusions

- RealBlue™ and RealYellow™ dyes have successfully overcome the cross-laser challenges associated with PE and PE-tandems
- Computational and AI tools helped us define the roadmap – determining the optimum number of dyes and their spectral positioning
- Our rigorous tech-dev process, where we iterate on the chemistry until we achieve the performance characteristics desired, has resulted in a family of high-performance dyes
- 14 dyes are available, with more to come, for use in both spectral and conventional flow cytometry
- A thorough comparison to other dye families/platform technologies shows that RealBlue™ and RealYellow™ dyes perform well in all categories that were assessed (SOV, Resolution, Buffer Compatibility, Intracellular Staining, Photostability, and Monocyte Background)
- With less cross-laser excitation, RealBlue™ and RealYellow™ dyes produce lower complexity scores than legacy dyes