Expanding the capacity of complex spectral panels by using NovaFluor-conjugated antibodies

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Abstract

When creating a high-complexity multiparameter flow cytometry panel, one must consider fluorophore brightness, antigen density, expression patterns, emission and excitation wavelengths, spectral patterns, and other influential factors. As spectral cytometry gains popularity and the need for larger, more comprehensive panels grows, it may be necessary to use more challenging fluorophore combinations in a flow cytometry panel.

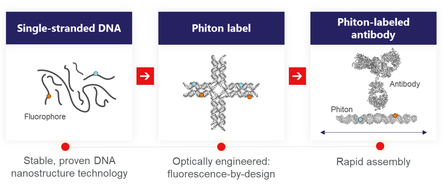
Challenging fluorophore combinations can be considered as fluorophores that are spectrally similar, with emission wavelengths detected on the same or adjacent detectors. When used together, these combinations can lead to high levels of negative spreading and poor resolution. In addition, it can be difficult to find the right fluorophore-antigen combinations, with limited options sometimes available. As a potential solution to this problem, we investigated the incorporation of Invitrogen™ NovaFluor™ dyes in spectral panels, by creating small, 2-3 color panels and observed the interactions between spectrally similar fluorophores for both high- and low-density antigen markers.

NovaFluor dyes are comprised of small molecule fluorophores embedded in a nucleic acid macrostructure and have been specifically designed to be used for both traditional and spectral cytometry. The data presented highlights the capacity of NovaFluor dyes to facilitate multiparameter panel design given their narrow emission profiles and unique structure that minimizes cross-laser excitation. There were instances in which challenging fluorophore combinations were able to be resolved cleanly and successfully, despite their similarities, and would be recommended for panel development.

In conclusion, NovaFluor dyes expand the repertoire of available tools that are fundamental for a successful spectral panel and provide additional fluorophore-antigen combinations.

Introduction

NovaFluor dyes are created using Invitrogen™ Phiton™ technology and are compatible with both conventional and spectral flow cytometry workflows. The Phiton label is comprised of small molecule fluorophores embedded in a nucleic acid macrostructure. The NovaFluor dyes possess uniquely engineered fluorescent signatures that minimize laser cross-excitation and spectral spillover, enabling panels of increasing complexity and size without sacrificing resolution. Lower spectral spillover and overlap lessens the need for compensation, decreases spreading error, and increases opportunities to add new markers.



Materials and methods

Sample preparation and test method

All flow cytometric staining was performed on lysed whole blood or peripheral blood mononuclear cell (PBMC) samples from multiple human donors. The optimal titer of each antibody was aliquoted into appropriate flow tubes with eBioscience™ Flow Cytometry Staining Buffer (00-4222-26, Thermo Fisher Scientific) in addition to 5 microliters of Invitrogen™ CellBlox™ Blocking Buffer (B001T02F01, Thermo Fisher Scientific).

Data analysis

All data was acquired on a Cytek™ Aurora and unmixed using SpectroFlo™ software. After collection, data was analyzed using FlowJo™ (BD Biosciences). All data was gated on lymphocytes. Lymphocytes were gated using Forward Scatter Area (FSC-A) vs. Side Scatter Area (SSC-A) parameters and then gated to exclude doublets. All plots show the effects of the unmixing algorithm and have no additional compensation in post-processing.

Results

NovaFluor conjugates can provide flexibility in panel design.
Several NovaFluor dyes peak in laser channels that currently have limited or few available fluorophore options. A notable example is Invitrogen™ NovaFluor Blue 610-70S™, a dye that experiences maximal emission on the blue laser line at 615 nm, or the 'B6' channel (**Figure 1A**). NovaFluor Blue 610-70S dyes are designed to be specific to the blue laser, unlike PE and PE-tandem dyes that have considerable emission peaks in both blue and yellow-green laser channels.

This unique characteristic allows for NovaFluor Blue 610-70S dyes to act as replacements for PE-tandem fluorophores or as an easy addition to panels containing them, ultimately creating flexibility in multiparameter panel design by maximizing channel usage (**Figure 1B-C**).

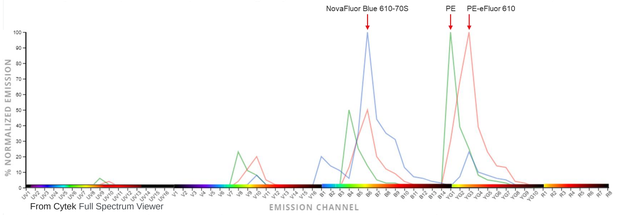


Figure 1A. The overlaid spectral signature profiles of NovaFluor Blue 610-70S, PE, and PE-eFluor 610 fluorophores visualized on the Cytek Full Spectrum Viewer.

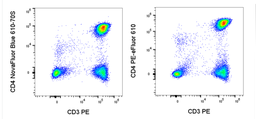


Figure 1B. Comparable data resolution of CD4 (SK3) NovaFluor Blue 610-70S and Invitrogen™ PE-eFluor™ 610 dye separately co-stained in a 2-color panel with CD3 (OKT3) PE.

Figure 1C. The successful substitution of CD4 (SK3) NovaFluor Blue 610-70S in a 3-color panel with CD19 (HIB19) PE-eFluor 610 and CD3 (OKT3) PE.

NovaFluor conjugates can compare to traditional formats in terms of brightness and resolution, while simultaneously reducing spreading and unmixing errors.
Using challenging fluorophore combinations increases the potential for spillover, leading to decreased resolution and potential unmixing or compensation errors. To remedy this, NovaFluors are uniquely tailored to minimize spillover, facilitating their use with other dyes in adjacent channels that may be spectrally similar**.** FITC and Invitrogen™ NovaFluor™ Blue 510 fluorophores peak on neighboring channels and share considerable spectral overlap (**Figure 2A**). When co-stained with Invitrogen™ Alexa Fluor™ 532 dye or Invitrogen™ NovaFluor™ Blue 530 dye, FITC-positive populations incurred spreading and unmixing errors (**Figure 2B, 2D**). When replaced with a NovaFluor Blue 510 format, spreading error was visually reduced and unmixing error did not occur (**Figure 2C, 2E**).

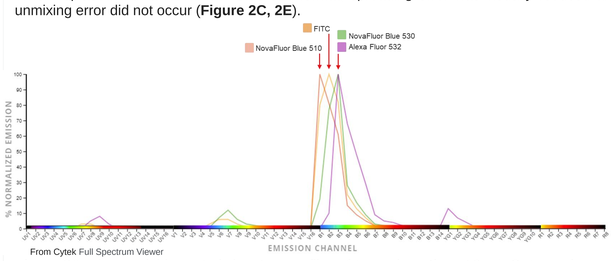
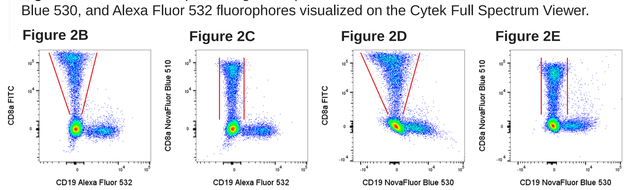


Figure 2A. The overlaid spectral signature profiles of NovaFluor Blue 510, FITC, NovaFluor Blue 530, and Alexa Fluor 532 fluorophores visualized on the Cytek Full Spectrum Viewer.



Figures 2B-E. 2-color combinations of CD8a (OKT8) FITC or NovaFluor Blue 510 vs. CD19 (HIB19) Alexa Fluor 532 or NovaFluor Blue 530. The combination of NovaFluor Blue 510 dye and NovaFluor Blue 530 dye provides clear resolution and a drastic reduction in spread of positive populations, in place of the traditional combination of FITC dye and Alexa Fluor 532 dye.

NovaFluor conjugates can be added to panels containing very spectrally similar dyes that emit in the same or neighboring channels.
In several cases, NovaFluor dyes were able to be cleanly resolved with other formats that shared similar primary peak emission channels and spectral signatures. Invitrogen™ NovaFluor™ Red 685 dye shares an emission peak with Invitrogen™ Alexa Fluor™ 660 dye and is very spectrally similar to Invitrogen™ Alexa Fluor™ 700 dye (**Figure 3A**), two dyes often used to build complex panels. Despite high similarity index and complexity values calculated between the three fluorophores (**Figure 3B**), the addition of NovaFluor Red 685 dye to this combination showed cleanly resolved populations and would be recommended to be included in panel development (**Figures 3C**).

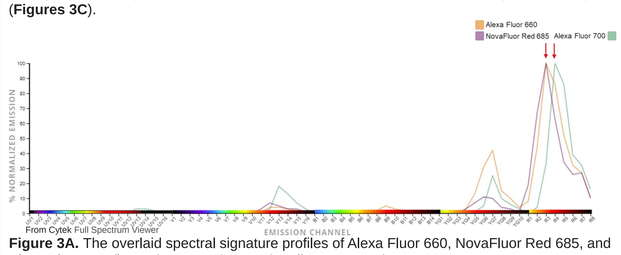


Figure 3A. The overlaid spectral signature profiles of Alexa Fluor 660, NovaFluor Red 685, and Alexa Fluor 700 fluorophores on the Cytek Full Spectrum Viewer.

	Similarity™ Indices Theoretical		
Alexa Fluor 660	1		
Alexa Fluor 700	0.82	1	
NovaFluor Red 685	0.94	0.69	1
	Alexa Fluor 660	Alexa Fluor 700	NovaFluor Red 685
Complexity™ Index	8.37		

Figure 3B. The calculated similarity indices and complexity index of Alexa Fluor 660, NovaFluor Red 685, and Alexa Fluor 700.

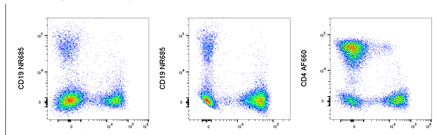


Figure 3C. 3-color panel with CD4 (SK3) Alexa Fluor 660, CD19 (HIB19) NovaFluor Red 685, and CD8a (RPA-T8) Alexa Fluor 700.

NovaFluor conjugates maintain resolution in challenging fluorophore combinations, even with medium-low density markers and co-expressed populations.
In addition to facilitating clear resolution when staining spectrally similar combinations of high-density antigen markers, the NovaFluor formats can maintain high clarity and separation in similarly challenging combinations even when conjugated to medium-density antigen markers that are co-expressed, demonstrated by a 3-color panel with CD19 (HIB19) Invitrogen™ PE-Alexa Fluor™ 610, CD27 (O323) Invitrogen™ NovaFluor™ Yellow 660 and CD28 (CD28.2) Invitrogen™ NovaFluor™ Blue 660-120S (**Figures 4A-C**).

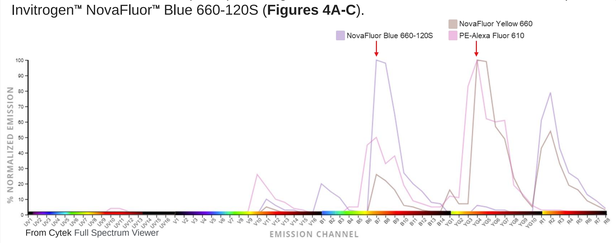


Figure 4A. The overlaid spectral signature profiles of NovaFluor Blue 660-120S, NovaFluor Yellow 660, and PE-Alexa Fluor 610 fluorophores on the Cytek Full Spectrum Viewer.

	Similarity™ Indices Theoretical		
NovaFluor Blue 660-120S	1		
NovaFluor Yellow 660	0.46	1	
PE-Alexa Fluor 610	0.36	0.75	1
	NovaFluor Blue 660-120S	NovaFluor Yellow 660	PE-Alexa Fluor 610
Complexity™ Index	3.04		

Figure 4B. The calculated similarity indices and complexity index of NovaFluor Blue 660-120S, NovaFluor Yellow 660, and PE-Alexa Fluor 610.

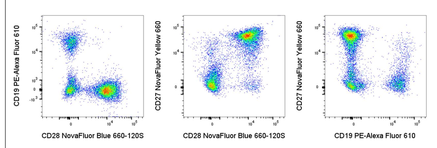


Figure 4C. 3-color panel with (HIB19) PE-Alexa Fluor 610, CD27 (O323) NovaFluor Yellow 660 and CD28 (CD28.2) NovaFluor Blue 660-120S.

NovaFluor conjugates can be integrated into complex panels.
NovaFluor conjugates can be integrated into complex multiparameter panels in combination with traditional fluorophores, replace common conventional formats, and provide great resolution and clear separation of populations with minimal spillover. To further illustrate this point, a 10-color panel was designed to showcase the NovaFluor fluorophores' representation of familiar patterns seen in simple NK- and T-cell immunophenotyping. Note the clear resolution of populations of interest, including those representing low percentages of total cells, such as the CD4+CD25+ population, and distinct definition of multiple NK subsets defined by CD16 and CD56.

Approx. nm	UV 349	Approx. nm	Violet 405	Approx. nm	Blue 488	Approx. nm	Yellow-Green 561	Approx. nm	Red 640
UV1 (372/15)		-----	-----	-----	-----	-----	-----	-----	-----
UV2 (388/15)		-----	-----	-----	-----	-----	-----	-----	-----
UV3 (427/15)		V1 (428/15)		-----	-----	-----	-----	-----	-----
UV4 (443/15)		V2 (443/15)		-----	-----	-----	-----	-----	-----
UV5 (458/15)		V3 (458/15)	**CD45RO eFluor 450**	-----	-----	-----	-----	-----	-----
UV6 (473/15)		V4 (473/15)		-----	-----	-----	-----	-----	-----
UV7 (514/28)		V5 (508/20)	**CD16 eFluor 506**	B1 (508/20)	**CD8a NovaFluor Blue 510**	-----	-----	-----	-----
-----	-----	V6 (525/17)		B2 (525/17)		-----	-----	-----	-----
UV8 (542/28)		V7 (542/17)		B3 (542/17)	**CD45 NovaFluor Blue 530**	-----	-----	-----	-----
UV9 (581/31)		V8 (581/19)		B4 (581/19)		YG1 (577/20)	**CD25 PE**	-----	-----
-----	-----	V9 (598/20)		B5 (598/20)		YG2 (598/20)		-----	-----
UV10 (612/31)		V10 (615/20)		B6 (615/20)	**CD4 NovaFluor Blue 610-70S**	YG3 (615/20)		-----	-----
UV11 (664/27)		V11 (664/27)		B7 (660/17)		YG4 (660/17)	**CD3 NovaFluor Yellow 660**	R1 (660/17)	**CD45RA-APC**
-----	-----	-----	-----	B8 (678/18)		YG5 (678/18)		R2 (678/18)	
UV12 (691/28)		V12 (692/28)		B9 (697/19)		YG6 (697/19)		R3 (697/19)	**CD19-NovaFluor Red 685**
UV13 (720/29)		V13 (720/29)		B10 (717/20)	**CD56 PerCP-eFluor 710**	YG7 (720/29)		R4 (717/20)	
-----	-----	-----	-----	B11 (738/21)		-----	-----	R5 (738/21)	
UV14 (750/30)		V14 (750/30)		B12 (760/23)		YG8 (750/30)		R6 (760/23)	
UV15 (780/30)		V15 (750/30)		B13 (783/23)		YG9 (750/30)		R7 (783/23)	
UV16 (812/34)		V16 (812/34)		B14 (812/34)		YG10 (812/34)		R8 (812/34)	

Figure 5A. Spectral panel design grid for 10-color panel on a Cytek Aurora with 5-laser configuration: 16UV-16V-14B-10YG-8R.

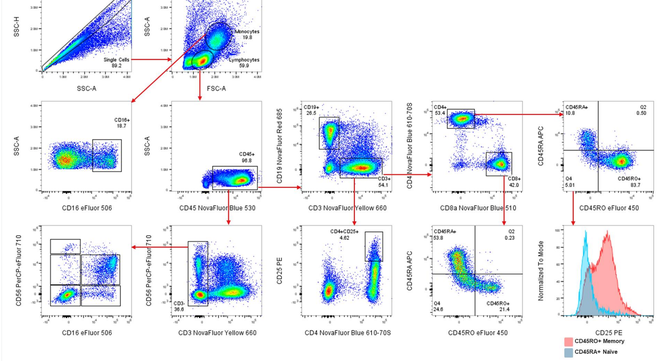


Figure 5B. 10-color panel stained on normal human PBMCs to demonstrate basic NK- and T-cell immunophenotyping using a combination of NovaFluor and traditional formats. Following single cell gating, lymphocytes and monocytes were identified by FSC-A and SSC-A parameters. CD16+ cells were gated off monocytes. CD45+ were gated off lymphocytes and then differentiated with CD3-/CD3+ cell populations and CD19+ to exclude B-cells. CD3- was further gated to display an NK subset defined by CD16 and CD56. CD3+ was gated for CD4+CD25+, CD4+ and CD8+ populations. CD4+ and CD8+ cell populations were further gated to display Memory and Naïve T-cell subsets. CD4+CD45RA+ and CD4+CD45RO+ were displayed in a histogram for CD25+ to further define the subset.

Discussion

In several instances of challenging fluorophore combinations, the NovaFluor dye-antibody conjugates were able to maintain brightness, clarity, and resolution with minimal unmixing issues or spreading errors. NovaFluor dyes allow for maximum channel usage and are recommended for multiparameter panel design due to their narrow excitation and emission wavelengths that allow for minimal cross-laser excitation in spectral and traditional flow cytometry workflows.

Benefits of NovaFluor dyes include:

- Compatibility in all sized panels for conventional and spectral flow cytometry
- A purposeful design aimed towards minimal cross-laser excitation, decreased spillover spread, and high resolution
- Uniquely engineered spectral signatures with narrow emission peaks to allow for more fluorophores per panel
- Stable dyes that can be stored long-term without a loss of fluorescence as compared to tandem dyes

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