Leveraging DNA in fluorescent reporter technology to enable higher plex flow cytometry

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Abstract

Flow cytometry has long been used to identify cellular populations of interest and investigate changes in immune subsets at the cellular level. With the advent of spectral flow cytometry, the ability to expand flow staining panels to 30 or 40 color panels has allowed for investigation into previously unstudied or neglected cell types that play important roles in the immune response. **Purpose:** To increase the number of fluorescent probes used in a multicolor memory T cell panel, with proper compensation and minimal cross-laser excitation and spectral spillover. **Methods:** Specific placement of fluorophores within a DNA-based macromolecule, was used to fine-tune both excitation and emission spectra of DNA-based fluorescent reporters. These novel reporters were then used in conventional flow cytometric analysis of a 14-color panel built using a combination of classic dyes and DNA-based fluorescent reporters and compared to conventional flow cytometric analysis of a 12-color panel built with classic dyes. **Results:** Replacing cross-laser excited fluors allowed for the resolution of 2 additional chemokine receptors, while maintaining clear distinction of cellular subpopulations. The use of DNA/fluorophore-based dyes can aide to optimize flow cytometry panels and add depth to immunological studies previously not possible**.**

Introduction

Flow cytometers are critical tools frequently used for immunophenotyping and analysis of single cells. The number of parameters that can be measured simultaneously in flow cytometry experiments are limited due to cross-laser excitation that occurs with commonly used conventional fluors. The problem of cross-laser excitation is particularly apparent when fluors, such as PE and PerCP tandems, are used in flow cytometers equipped with blue (488nm) and yellow-green (561nm) excitation lasers. Previously, fluors with similar excitation and emission spectra as PE and PerCP tandems, with less cross-laser excitation, have not existed. Therefore, panel design that enables the full detection capabilities of conventional flow cytometers has been difficult and often impossible. Novel fluorescent reporters have been developed using a DNA nanostructure, which allows for the manipulation of excitation and emission spectra. The fine tuning of spectra allows for DNA-based fluorescent reporters with similar emission as PE and PerCP tandem dyes with much less cross-laser excitation.

Materials and methods

Sample Preparation

Frozen human peripheral blood mononuclear cells (PBMCs) from an apparently healthy donor were thawed and plated at a concentration of $5x10^6$ cells/mL in a 96 well tissue culture plate. Briefly, PBMCs were washed twice with phosphate buffered saline and stained for viability using a violet (405 nm) excited amine reactive viability dye for 20 minutes. PBMCs were washed twice using flow cytometry staining buffer and stained with primary conjugated antibodies. The 12-color panel was stained against human CD8a, CD19, CD25, CD4, CD3, CCR7, CD127, CD27, CD56, CD16, CD45RA. The 14-color panel was stained against human CD127, CD56, CD25, CD4, CD27, CD19, CCR7, CD3, CD8a, CD184, CD16, CD28, CD45RA. All samples were stained for 45 minutes, on ice, and protected from light. All samples were fixed for 30 minutes and analyzed immediately afterward.

Test Method

Detector voltage settings were optimized prior to data collection. Stained PBMCs were interrogated using an Invitrogen™ Attune™ NxT V4 equipped with Violet (405 nm), Blue (488 nm), Yellow-Green (561 nm), and Red (637 nm) excitation lasers. The standard 590/40 nm BL2 bandpass filter was replaced with a 620/15 nm filter to better capture peak dye emission. All other filters were standard manufacturer recommendations. PBMCs were interrogated at a flow rate of 100 µL/min and 80,000 events were collected in the lymphocyte scatter gate.

Data Analysis

Panels were analyzed using FlowJo™ Software 10.8.1. Spectral compensation was performed using single color stained PBMCs. Fluorescence minus one controls were used to set positive gates for cell surface markers on cell populations that did not show a clear separation between positive and negative. Population separation and the percentages of positive populations were compared between the 12 and 14-color panels.

Results

Panel Design

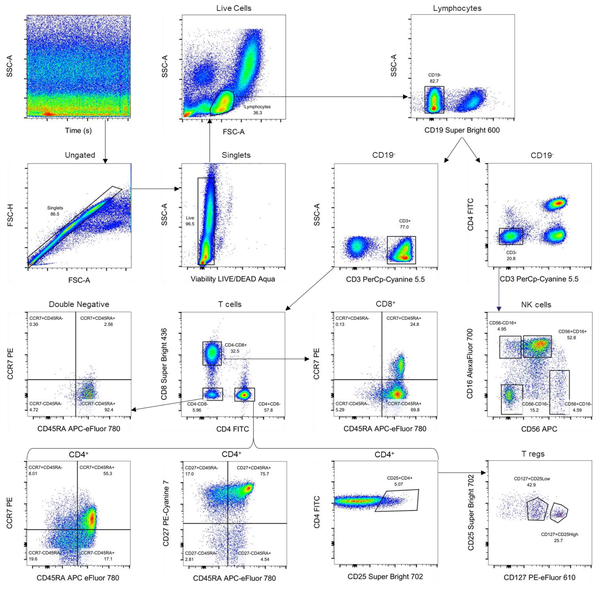
Common memory T cell and NK cell surface markers were used in both panels. All antigens analyzed in the 12-color panel were also included within the 14-color panel. Precise dye placement within a DNA nanostructure permits engineering of FRET properties and allows for specific control of fluorescent excitation and emission. Replacing cross-laser excited PerCp-Cyanine5.5 and PE-eFluor™ 610 with low cross-laser excited DNA fluorescent reporters, NovaFluor™ Blue 660-120S and NovaFluor Yellow 610, allowed for the addition of two colors and subsequent analysis of chemokine receptors (CD27 & CXCR4 [CD184]) within the 14-color panel. Antigen density was matched to dye brightness, with poorly expressed antigens matched to bright emitting dyes and highly expressed antigens matched to dyes with dimmer fluorescent emission. Fluorescence minus one controls were used to set gates for populations lacking clear separation between positive and negative populations.

Table 1. 12-color panel

VL1	VL2	VL3	VL4	BL1	BL2	BL3	YL1	YL2	YL3	YL4	RL1	RL2	RL3
440/50	512/25	603/48	710/50	530/30	590/40	695/40	585/16	620/15	695/40	780/60	670/14	720/30	780/60
Super Bright™ 436	LIVE/DEAD™ Aqua	Super Bright™ 600	Super Bright™ 702	FITC	PE-eFluor™ 610	PerCP-Cyanine 5.5	PE	PE-eFluor™ 610	PE-Cyanine 5.5	PE-Cyanine 7	APC	Alexa Fluor™ 700	APC-eFluor™ 780
CD8	Viability	CD19	CD25	CD4		CD3	CCR7	CD127		CD27	CD56	CD16	CD45RA

Table 1. Antigen-Dye Combinations. Antigen-dye combinations and bandpass filters for 12-color memory T cell panel. The greyed-out detectors (BL2 & YL3) show detectors that cannot be used due to cross-excited dyes being used in BL2 & YL2.

Figure 1. 12-color panel.



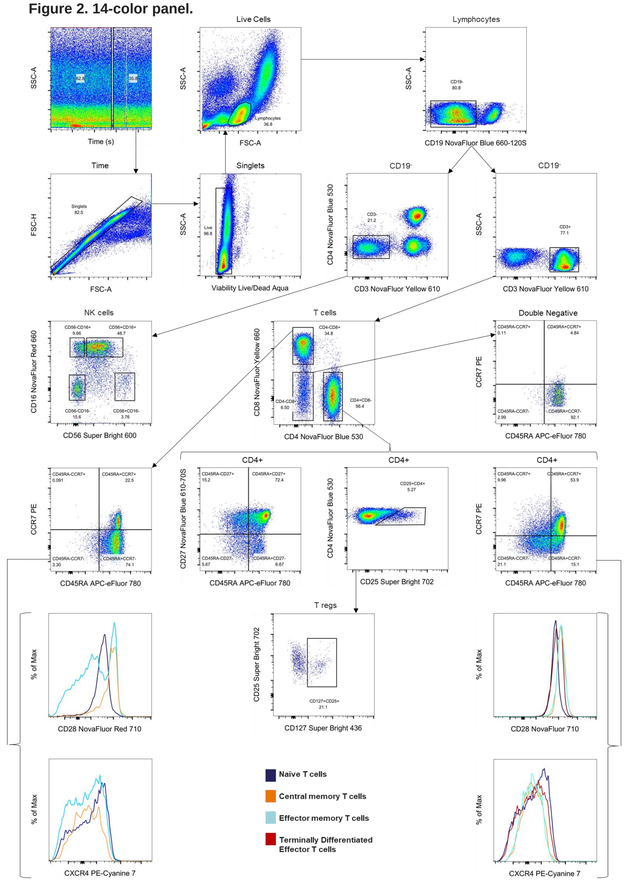
Results (continued)

Table 2. 14-color panel

VL1	VL2	VL3	VL4	BL1	BL2	BL3	YL1	YL2	YL3	YL4	RL1	RL2	RL3
440/50	512/25	603/48	710/50	530/30	590/40	695/40	585/16	620/15	695/40	780/60	670/14	720/30	780/60
Super Bright 436	LIVE/DEAD Aqua	Super Bright 600	Super Bright 702	NovaFluor™ Blue 530	NovaFluor™ Blue 610/70S	NovaFluor™ Blue 660/120S	PE	NovaFluor™ Yellow 610	NovaFluor™ Yellow 660	PE-Cyanine7	NovaFluor™ Red 660	NovaFluor™ Red 710	APC-eFluor 780
CD127	Viability	CD56	CD25	CD4	CD27	CD19	CCR7	CD3	CD8	CXCR4	CD16	CD28	CD45RA

Table 2. Antigen-Dye combinations. Antigen-dye combinations and bandpass filters for a 14-color memory T cell panel.

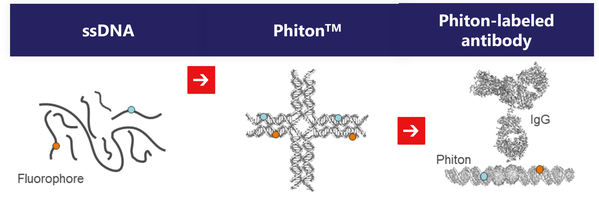
Figure 2. 14-color panel.



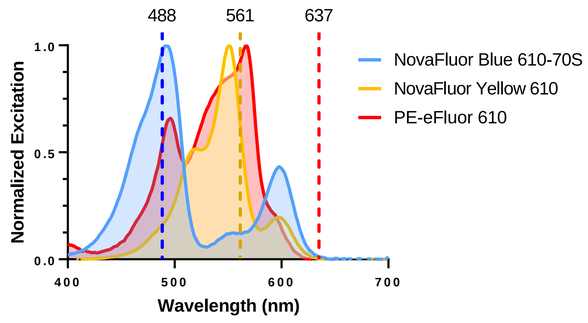
Conclusions

Replacing cross-laser excited conventional dyes with DNA-based fluorescent reporters allows for the expansion of a 12-color flow cytometry panel to 14 colors and full utilization of available detectors.

- DNA-based fluorescent reporter technology allow for precise control of excitation and emission spectra.



- PE and PerCP tandem dyes display significant excitation by blue (488 nm) and yellow (561 nm) lasers, which prevents full utilization of available detectors.



- The addition of 2 markers allows for deeper phenotyping through the enablement of higher plex flow cytometry.

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