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TECHNICAL NOTE

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Novel PE and APC tandems: Additional near-infrared fluorochromes for use in spectral flow cytometry

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Abstract

Recent advances in flow cytometry instrumentation and fluorochrome chemistries have greatly increased fluorescent conjugated antibody combinations that can be used reliably and easily in routine experiments. The Cytek Aurora flow cytometer was first released with three excitation lasers (405, 488, and 640 nm) and incorporated the latest Avalanche Photodiode (APD) technology, demonstrating significant improvement in sensitivity for fluorescent emission signals longer than 800 nm. However, there are limited commercially available fluorochromes capable of excitation with peak emission signals beyond 800 nm. To address this gap, we engineered six new fluorochromes: PE-750, PE-800, PE-830 for the 488 nm laser and APC-750, APC-800, APC-830 for the 640 nm laser. Utilizing the principal of fluorescence resonance energy transfer (FRET), these novel structures were created by covalently linking a protein donor dye with an organic small molecule acceptor dye. Additionally, each of these fluorochrome conjugates were shown to be compatible with fixation/permeabilization buffer reagents, and demonstrated acceptable brightness and stability when conjugated to antigen-specific monoclonal antibodies. These six novel fluorochrome reagents can increase the numbers of fluorochromes that can be used on a spectral flow cytometer.

KEYWORDS
flow cytometry, fluorochrome, high content, high dimensional, near infrared, spectral

1 | INTRODUCTION

An advantage of near-infrared emission (wavelengths from 780 to 900 nm) is the limited interference from cellular autofluorescence sometimes associated with shorter wavelengths (400 to 550 nm).[1] As a result, the use of fluorochromes with near-infrared emissions may result in higher sensitivity and improved fluorescent staining indexes. Near-infrared emission fluorochromes also tend to introduce minimal spillover into detectors designated for fluorochromes with shorter wavelength emission spectra.

Despite these advantages, there has been limited application of fluorochromes with near-infrared emission spectra in flow cytometry. Most commercial cytometers are commonly manufactured with a 488 nm blue laser and 640 nm red laser, but not a near-infrared laser. To make fluorochromes with peak excitation in the 488 to 640 nm range and peak emission within the near-infrared region, manufacturers rely on the principle of fluorescence resonance energy transfer (FRET), coupling a donor base fluorochrome (e.g. PE or APC) with an acceptor fluorochrome that emits near-infrared fluorescence (e.g. Cy7).[2] These are commonly referred to as "tandem dyes".

Historically, PE-Cy7 and APC-Cy7 are among the most widely used tandem dyes with peak emission wavelengths near 780 nm. Up until recently, there were no other commercial phycoerythrin (PE) and allophycocyanin (APC) tandem fluorochromes with emission wavelengths longer than 780 nm that could be easily distinguished from PE-Cy7 and APC-Cy7[1]. Very low quantum efficiency of photomultiplier tube (PMT) detector for emissions longer than 800 nm could explain the limited commercial availability of fluorochromes with near-infrared emission in flow cytometry.[3] Recent advances in optic technology led to the commercial adoption of Avalanche Photodiodes (APD) (e.g., Beckman Coulter CytoFLEX LX and Cytek Aurora), which have improved quantum efficiencies over photomultiplier tubes for fluorescent emission in the infrared region.[4]

In this technical note, we describe and characterize the performance of the following six novel fluorochromes: PE-750, PE-800, PE-830, APC-750, APC-800, and APC-830 with peak emission wavelengths at 750, 800, and 830 nm, respectively.

2 | MATERIALS AND METHODS

2.1 | Subjects and samples

The study was approved by the Institutional Review Board at Abbvie Biotherapeutics. The human blood samples were collected from healthy donors who registered for AbbVie Biotherapeutics Employee Blood Collection Program in Redwood City, CA. Whole blood was obtained in heparin-anticoagulated tubes (BD Biosciences) and then processed for staining on the same day of collection.

2.2 | Preparation of PE- and APC-linked fluorochromes

The conjugation method was adapted from the previously described protocol.[5] Prior to conjugation, R-Phycoerythrin (R-PE, 240 kDa) (Prozyme) was extensively dialyzed into phosphate-buffered saline (PBS, pH 7.2) (GE Life Sciences) using the Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher) according to the manufacturer's instructions. Briefly, the R-PE is dialyzed for 2 h at room temperature and this process is repeated with fresh PBS for another 2 h. The dialysis buffer was then replaced with fresh PBS, and the dialysis continued overnight at 4°C. The final concentration of dialyzed R-PE was 5.54–12.2 mg/ml. Lyophilized cross-linked Allophycocyanin (APC, 105 kDa) (AAT Bioquest, Sunnyvale) was resuspended with PBS to a final concentration of 2.5 mg/ml.

Organic small molecule N-hydroxysuccinimide (NHS) ester fluorophores such as Dy704-P4, Dy704 (Dyomics), Dy800-P4 (Thermo Fisher), and iFluor810 (AAT Bioquest) were dissolved with anhydrous DMSO (Thermo Fisher) to a final concentration of 1000 nmol/ml. For example, 1 mg of Dy800-P4 NHS ester was dissolved in 952 μl of anhydrous DMSO. Meanwhile, 1 M sodium bicarbonate (pH 8.3–8.5) was prepared by dissolving sodium bicarbonate (Sigma-Aldrich) in deionized water.

The reaction condition is summarized in Table 1. The reaction was rotated at room temperature for at least 60 min. The absorbance spectra of the fluorochromes were preliminarily measured with SpectraMax M5 microplate reader (Molecular Devices) using 1:100 dilution in PBS. Desalting procedure is followed using the Zeba Spin 7 K MWCO desalting columns (Thermo Fisher) according to the manufacturer's instruction.

2.3 | Characterize the brightness of the tandem fluorochrome molecules

Anti-mouse Ig, κ compensation particles (BD Biosciences) were stained with 1 μg of purified anti-Phycoerythrin antibody (clone PE001, BioLegend, La Jolla, CA), and then washed twice with staining buffer (1X PBS with 0.5% BSA). The compensation particles were then stained with PE tandem fluorochrome molecules.

Similarly, anti-mouse Ig, κ compensation particles were stained with 1 μg of purified anti-Allophycocyanin antibody (clone APC003, BioLegend), and then washed twice with staining buffer. The compensation particles were then stained with APC tandem fluorochrome molecules. Stained samples were acquired on the spectral flow cytometer Aurora (Cytek).

2.4 | Click-chemistry to generate novel fluorochromes-conjugated antibodies

Conjugation of a monoclonal antibody and the tandem fluorochrome was achieved by click chemistry reaction between methyl-tetrazine and trans-cyclooctene-tetrazine (TCO) (Click Chemistry). Click Chemistry works well in conjugation with tandem dyes because the conjugation can occur at near neutral pH conditions (pH = 7.2). TCO was tagged to 100 μg of each antibody. Each of the anti-human CD3 (Clone UCHT1), CD8α (Clone OKT-8), CD19 (Clone 4G7), CD20 (Clone 2H7) (Bio X Cell, West Lebanon, NH), CD16 (Clone 3G8, Leinco Technologies), and CD45 (Clone HI30, BioLegend) antibodies were mixed with 5 μl (5% of the total reaction volume) of 1 M $NaHCO_3$ with 100 μl of the PBS-based solution. Then, 20 nmol of TCO-PEG4-NHS ester was added to the mixture.

In the same manner, methyl-tetrazine was tagged to the fluorochromes. 100 μg of tandem fluorochromes were mixed with 5 μl of 1 M $NaHCO_3$ with 100 μl of the PBS-based solution. Then, 20 nmol of methyl-tetrazine-PEG4-NHS ester was added to the mixture. Those reaction mixtures were kept at room temperature for 60 min. Desalting procedure is followed for both mixtures using spin desalting columns (Thermo Fisher). The recovery protein amount after desalting was calculated as ~75 μg.

Cross-linking reaction was initiated by mixing the two reaction mixtures. Antibody-TCO was mixed with fluorochrome-methyl-

[1]Biolegend has recently released PE-Fire810 and APC-Fire810 as near-infrared fluorochrome options for channel B14 and channel R8 respectively on the Cytek Aurora.

TABLE 1 Reaction condition for pairs of a donor protein and an acceptor small molecule dye

Channel	Fluorochrome	1 M $NaHCO_3$ (μl)	Donor protein conc. (mg/ml)	Mixture volume (μl)				Molar ratio in reaction (dye: Protein)	Final conjugate (dye: protein F:P ratio)
				Donor		Acceptor (1000 nmol/ml)			
B11	PE-750	10	6.818	PE	35.2	Dy704-P4	15	15:1	3.2
B14	PE-800	10	5.54	PE	21.7	Dy800-P4	30	60:1	6.6
B16	PE-830	10	12.2	PE	9.8	iFluor810	20	80:1	5.9
R5	APC-750	10	2.5	APC	41.6	Dy704	25	25:1	5.0
R8	APC-800	10	2.5	APC	41.6	Dy800-P4	15	15:1	5.3
R10	APC-830	10	2.5	APC	41.6	iFluor810	30	30:1	7.0

tetrazine ester in 1:2 molar ratio. The reaction mixture was stored at 4°C overnight. The next day, protein-stabilizing cocktails (Thermo Fisher) and bovine serum albumin were added. The final products were stored at −20°C.

Large-scale conjugation was done by AAT Bioquest for PE-750, APC-750 and by BioLegend for PE-800, PE-830, APC-800, and APC-830.

2.5 | Conjugation of antibodies to commercially available fluorochromes

Prior to the conjugation procedure, concentration of the antibodies should be higher than 1 mg/ml for an optimal reaction condition. If needed, monoclonal antibodies were concentrated by the Amicon Ultra centrifugal filter (Millipore).

Anti-human CCR6 (Clone G034E3, BioLegend) was conjugated with CF680 NHS ester (Biotium) in 1:10 molar ratio. 1 M sodium bicarbonate (pH 8.3–8.5) was added to 10% of total volume. After 2-h incubation at room temperature, the mixture went through the desalting spin column.

Anti-human CCR3 (Clone 5E8, BioLegend) was conjugated to biotin using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher). 2 mg of antibody was mixed with 150 μg of biotin reagent. The mixture was incubated at room temperature for 30 min and subsequently desalted.

Anti-human CD11b (Clone M1/70, Leinco Technologies) was conjugated with Alexa Fluor 532 NHS ester (Thermo Fisher) according to the manufacturer's instructions. 0.75 nmol of CD11b antibody was added to the pre-mix and 1 M sodium bicarbonate (pH 8.3–8.5) was added to 10% of total volume. After 2-h incubation at room temperature, the mixture went through the desalting spin column.

2.6 | Comparison of sensitivity between PMT-based and APD-based flow cytometers

PMT-based flow cytometer was compared against APD-based flow cytometer to evaluate the sensitivity of novel antibody-conjugated fluorochromes in near-infrared region. Compensation beads were stained with each novel antibody-conjugated fluorochromes and acquired either on the APD-based spectral flow cytometer (Beckman Coulter 6-laser CytoFLEX LX and Cytek Aurora) or the PMT-based flow cytometer BD FACSymphony A5. As for BD FACSymphony, 710/50, 780/60, 820/60 bandpass filters on both blue laser and red laser were used for acquisition. For the CytoFLEX LX system, 710/50 bandpass filters on the blue and the red lasers were used to measure PE-750 and APC-750 respectively; 819/44 bandpass filters on the blue and red lasers were used to measure the remaining tandem dyes. Of notes, 780/60 bandpass was not available on the CytoFLEX LX as an optics option due to possible leakage from the 808 nm IR laser. Stain index from each measurement of novel antibody-conjugated fluorochromes was calculated as previously described.[6]

$$\text{Stain index} = \frac{\text{MFI}_{\text{positive}} - \text{MFI}_{\text{negative}}}{2 \times (\text{SD}_{\text{negative}})}.$$

For Figure 3A,B, stain index for the Cytek Aurora was calculated based on raw FCS data files. In Figure 3C, as noted, stain index for the Cytek Aurora was calculated based on raw and unmixed FCS data files.

2.7 | Stability test of the novel fluorochromes after fixation

Peripheral blood mononuclear cells (Veri-Cells PBMC, BioLegend) were stained with each novel fluorochrome for 30 min and fixed with various fixatives according to the manufacturer's instructions—cell staining buffer (PBS + 10% Fetal Bovine Serum [FBS]), FACS lysing solution (BD Biosciences), Fixation buffer and Intracellular Staining Perm Wash buffer (BioLegend), and Foxp3/Transcription factor staining buffer with Permeabilization buffer (Thermo Fisher). Samples were analyzed directly after fixation and washing (marked as "fresh") and analyzed after overnight incubation at 4°C (marked as "Overnight"). All samples were acquired on Aurora spectral flow cytometry and stain index of each fluorochrome was calculated.

2.8 | Whole blood staining of immune cell subsets for flow cytometry

Monocyte blocking solution (BioLegend) and Brilliant Stain Buffer (BD) were added prior to multicolor staining. A cocktail of novel in-house antibody-conjugated fluorochromes and other antibody-conjugated fluorochromes was added to 300 μl of whole blood in a 12 × 75 mm tube. Single color compensation bead controls were stained with each antibody-conjugated fluorochrome. 0.5–1 μg of in-house antibody-conjugated fluorochromes per test was used. 5 μl of commercially available antibody-conjugated fluorochromes per test was used as instructed. 1 μl of HLA-DR-APC-eFluor780 and 0.25 μl of CD4-BB660 per test was used due to its intense brightness.

Antibody-conjugated fluorochromes for the violet 405 nm laser includes: CD33-BV421 (Clone WM53), CD28-BV510 (Clone 28.2), CD27-BV570 (Clone O323), CD123-BV650 (Clone 7G3), CXCR5-BV711 (Clone J252D4), CD56-BV750 (Clone 5.1H11) (BioLegend); CD22-SuperBright436 (Clone 4 KB128), CD57-eFluor450 (Clone TBO1), Qdot 585 Streptavidin conjugate (Invitrogen); CD138-BV480 (Clone MI15), CD127-BV605 (Clone HIL-7R-M21), CD45RA-BV786 (Clone HI100) (BD); CD14-KromeOrange (Clone RMO52) (Beckman Coulter).

Antibody-conjugated fluorochromes for the blue 488 nm laser includes: DNAM-BB515 (Clone DX11), CXCR3-PE (Clone 1C6/CXCR3), CCR7-PE-CF594 (Clone 150503), CD4-BB660 (Clone SK3) (BD); IgD-FITC (Clone IA6-2), CD11b-Alexa Fluor 532 (Clone M1/70), CD11c-PE-Cy5 (Clone 3.9), CD38-PerCP-eFluor710 (Clone 90) (Invitrogen); PD1-PC5.5 (Clone PD1.3) (Beckman Coulter); CCR4-PE-Vio770 (Clone REA279) (Miltenyi Biotec).

Antibody-conjugated fluorochromes for the red 640 nm laser includes TCRγδ-APC (Clone B1), CD303-AlexaFluor647 (Clone 201A) (BioLegend); CD25-APC-R700 (Clone 2A3) (BD); HLA-DR-APC-eFluor780 (Clone LN3) (Invitrogen).

Fresh whole blood was first incubated with CXCR3, CCR7, and CCR4 for 10 min at 37°C and incubated with rest of the antibodies for 30 min in the dark at room temperature. 2 ml of 1X FACS lysing solution (BD) were added to the mixture and incubated for another 10 min in the dark at room temperature. The tubes were centrifuged at 500×*g* for 5 min. Qdot 585 VIVID streptavidin conjugate (Thermo Fisher) was added to the samples and then incubated for 15 min. The samples were washed with 2 ml of FACS buffer (1× PBS with 1% FBS) and centrifuged at 500×*g* for 5 min. Washing step was repeated twice. In the end, the samples were resuspended in 350 μl of FACS buffer.

2.9 | Flow cytometry and high-dimensional data analysis

The samples and single-color controls were acquired on a the Cytek Aurora spectral flow cytometer (Cytek Biosciences) with custom configuration (additional channels B15, B16, R9, and R10 as described in Tables S1, S2) at AbbVie Biotherapeutics. The custom cytometer was first built with the violet, blue, and red lasers and formed the foundation for this body of work, while the UV laser was added at a much later time without changing the existing optical layout. Using single-color controls and voltage titration method, gain of each channel in the violet, blue and red lasers was adjusted for optimal sensitivity.[7] Sample QC and unmixing was run on SpectroFlo.

Acquired data was analyzed using FlowJo analysis software (BD Biosciences). viSNE plots and FlowSOM plots were created in Cytobank (www.cytobank.org). All parameters were displayed with an arcsinh transformation.

3 | RESULTS

3.1 | Conjugation of protein dye with a small molecule dye

Novel tandem fluorochromes were generated by a principle of FRET. Distance-dependent energy transfer from a donor molecule to an acceptor molecule creates a unique emission wavelength. Here we used PE and APC as a donor molecule due to their high solubility, brightness, and stability. In addition, a variety of acceptor dyes were evaluated, and highly water-soluble fluorescent dyes were selected as acceptor dyes for covalent labeling.[2]

Tandem dyes were synthesized by a reaction between the NHS ester on the small molecule dye and the amine residue on the protein dye. As previously described,[5] for each tandem dye, various molar ratios of a protein dye and a small molecule dye (ranging from 1:10 to 1:80) were evaluated. By comparing relative brightness, residual donor emission, and emission spectrum at distinctive wavelength, optimal ratio for each tandem dye was determined (Table S3). Depicted in Table 1 is the optimal reaction condition for each tandem dye (Table 1). Overall, PE tandems tend to require higher molar ratio supposedly due to its higher molecular weight.

The excitation and emission wavelength were measured (Figure 1). When APC and PE were conjugated with Dy704 and Dy704-P4 respectively, their peak emission wavelength was 750 nm. When APC or PE was conjugated with Dy800-P4, the peak emission wavelength was 800 nm. Conjugates prepared with iFluor810 showed a peak emission wavelength of 830 nm in near-infrared range. We named these novel fluorochromes as a combination of donor molecule and its peak emission wavelength PE-750, PE-800, PE-830 and APC-750, APC-800, APC-830.

3.2 | Conjugation of novel-fluorochromes to a monoclonal antibody

The novel fluorochromes are conjugated with antibodies using click chemistry reaction.[8] Click chemistry is a simple and robust reaction

[2]Recently Biotium Inc. has just released a new series of near-infrared fluorescent dyes (CF800, CF820, CF850, CF870) with polyethyleneglycol (PEG) modifications which may drastically enhance the fluorochrome water solubility and brightness. Unfortunately, we did not have a chance to evaluate these fluorochromes as tandem dye partners to PE and APC at the time of the study.

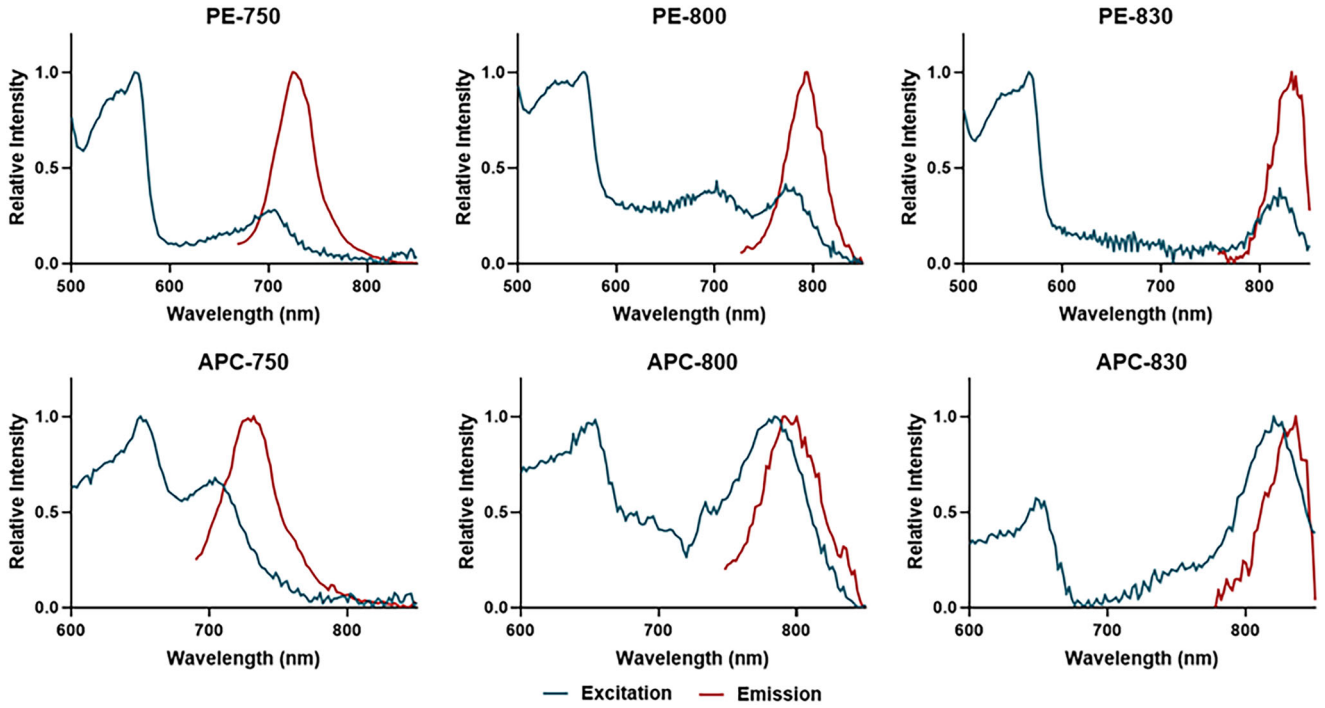

FIGURE 1 Excitation and emission curves of the novel fluorochromes [Color figure can be viewed at wileyonlinelibrary.com]

that is commonly used in bioconjugation. It generates conjugated product with quick, high-yield, and high-reaction specificity.

First, an antibody was linked to the TCO tag, and the novel fluorochromes were linked to methyl-tetrazine, respectively. Then, the antibody-TCO structure and fluorochrome-methyl-tetrazine were crosslinked by mixing two reagents in 1:2 molar ratio, respectively. The reaction is completed in 1–2 h at room temperature or overnight at 4°C.

We selected lineage markers for antibody conjugation with the novel fluorochromes. Their expression pattern is very predictable, so the quality of the novel antibody-conjugated fluorochromes can be easily evaluated.

Initial assessment of the Cytek Aurora reveals the following available channels: B11, B14, B16 for the blue laser and R5, R8, R10 for the red laser (Table S2). The fluorochromes that we created demonstrated peak emission spectra at the desired channels on the Cytek Aurora (Figure 2). We further performed stability testing on the final conjugates and found their stability and brightness at 6-month post-conjugation (data not shown).

3.3 | Comparison of sensitivity between APD-based and PMT-based flow cytometers, and assessment of the novel antibody-conjugated fluorochromes stability in fixatives

PMT-based system has been widely used in flow cytometry. Here, APD-based flow cytometers were compared against the PMT-based system, specifically for performance in the near-infrared region.

On the FACSymphony system, 710/50 bandpass filters off the blue laser (488 nm) and red laser (640 nm) were used to measure PE-750 and APC-750 fluorescent signals. PE-800 and APC-800 were measured with the 780/60 bandpass filters, while 820/60 bandpass filters were used to measure PE-830 and APC-830 fluorescent signals.

On the CytoFLEX LX system, 710/50 bandpass filters off the blue laser (488 nm) and red laser (640 nm) were used to measure PE-750 and APC-750 fluorescent signals. PE-800, PE-830, APC-800, APC-830 were measured with 819/44 bandpass filters, as 780/60 bandpass filter is not an available optic option on the CytoFLEX LX due to possible leakage from the 808 nm IR laser.

The Cytek Aurora and the BD FACSymphony demonstrated comparable performance for PE-750, APC-750, and PE-800 channels. There were most striking differences in the near-infrared channels (PE-830, APC-800, and APC-830) where the Cytek Aurora demonstrated higher staining index than the FACSymphony (Figure 3A). These differences were also noticed when the CytoFlex LX's performance in these near-infrared channels was compared against the FACSymphony, suggesting that APD may have superior sensitivity over PMT for emission wavelength beyond 800 nm. Of notes, on the CytoFlex, because PE-750 and APC-750 are read off the 710/50 bandpass filters off the blue and red lasers, which are also responsible for PerCP/Cy5.5 and Alexa Fluor 700 respectively, it is probably difficult to incorporate PE-750 and APC-750 into existing panels that already contains PerCP/Cy5.5 and Alexa Fluor 700. However, PE-800, PE-830, APC-800, APC-830 are read off the 819/44 bandpass filters off the blue and red lasers, which are not currently occupied by any fluorochromes; therefore, PE-800, PE-830, APC-800, APC-830 may represent new fluorochrome options on the CytoFlex platforms.

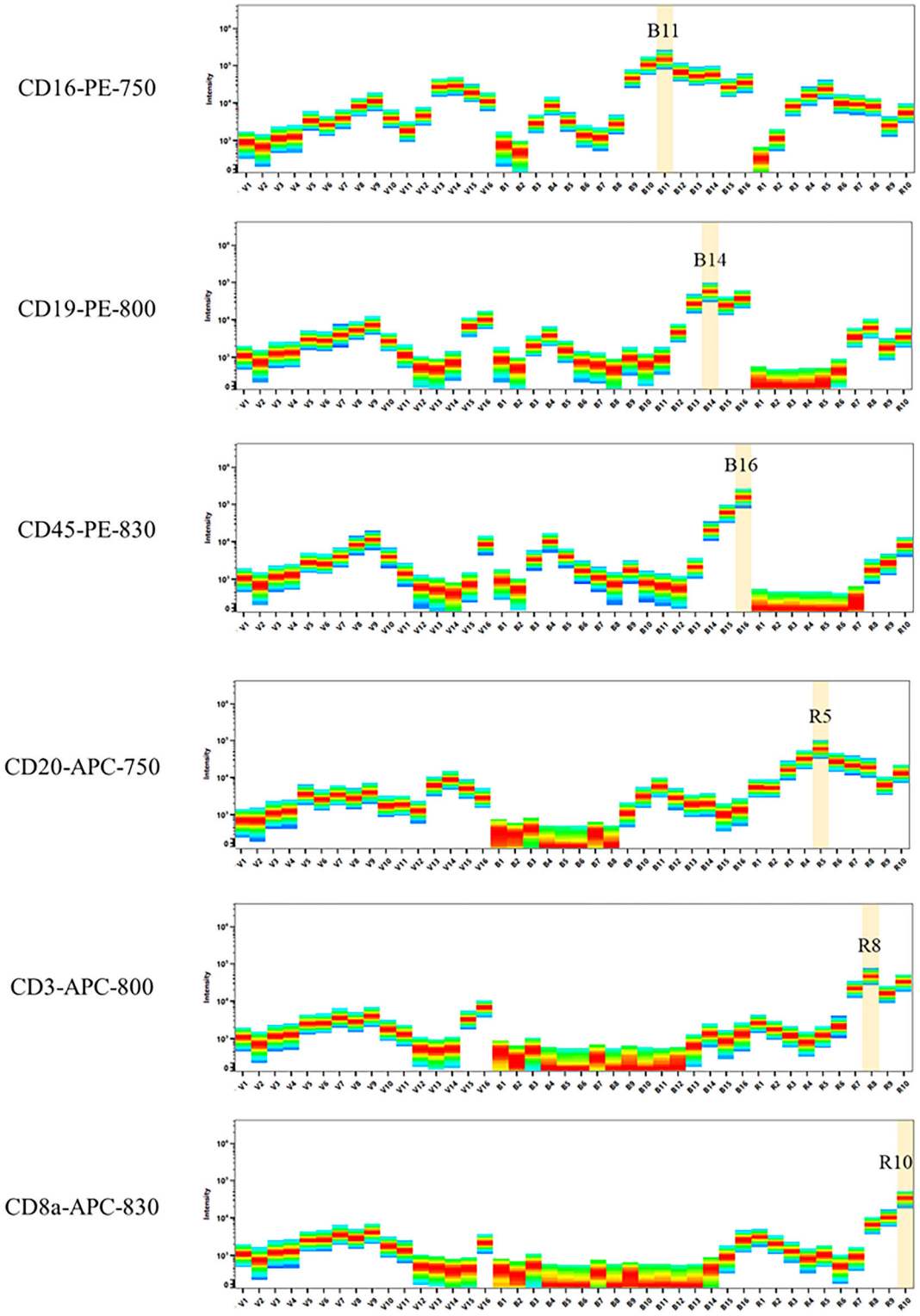

FIGURE 2 Spectral plots of the novel fluorochromes. Each spectral plot shows its peak emission and spectrum signatures in the violet, blue, and red lasers. To generate the figure, UltraComp bead was stained with the novel fluorochrome-conjugated antibodies. The autofluorescence profile of UltraComp bead is demonstrated in Figure S4 [Color figure can be viewed at wileyonlinelibrary.com]

Fluorochrome stability after fixative treatment was evaluated on the Cytek Aurora in Figure 3B. We selected three representative fixative buffer systems that are commonly used in flow cytometry: BD FACS Lysing Solution (1% paraformaldehyde and 3% diethylene glycol, commonly used for whole blood cell surface staining), BioLegend Fixation Buffer (4% paraformaldehyde, commonly used for intracellular cytokine staining), and Thermo Fisher Foxp3 Transcription Factor Buffer kits (proprietary fixative formula, commonly used

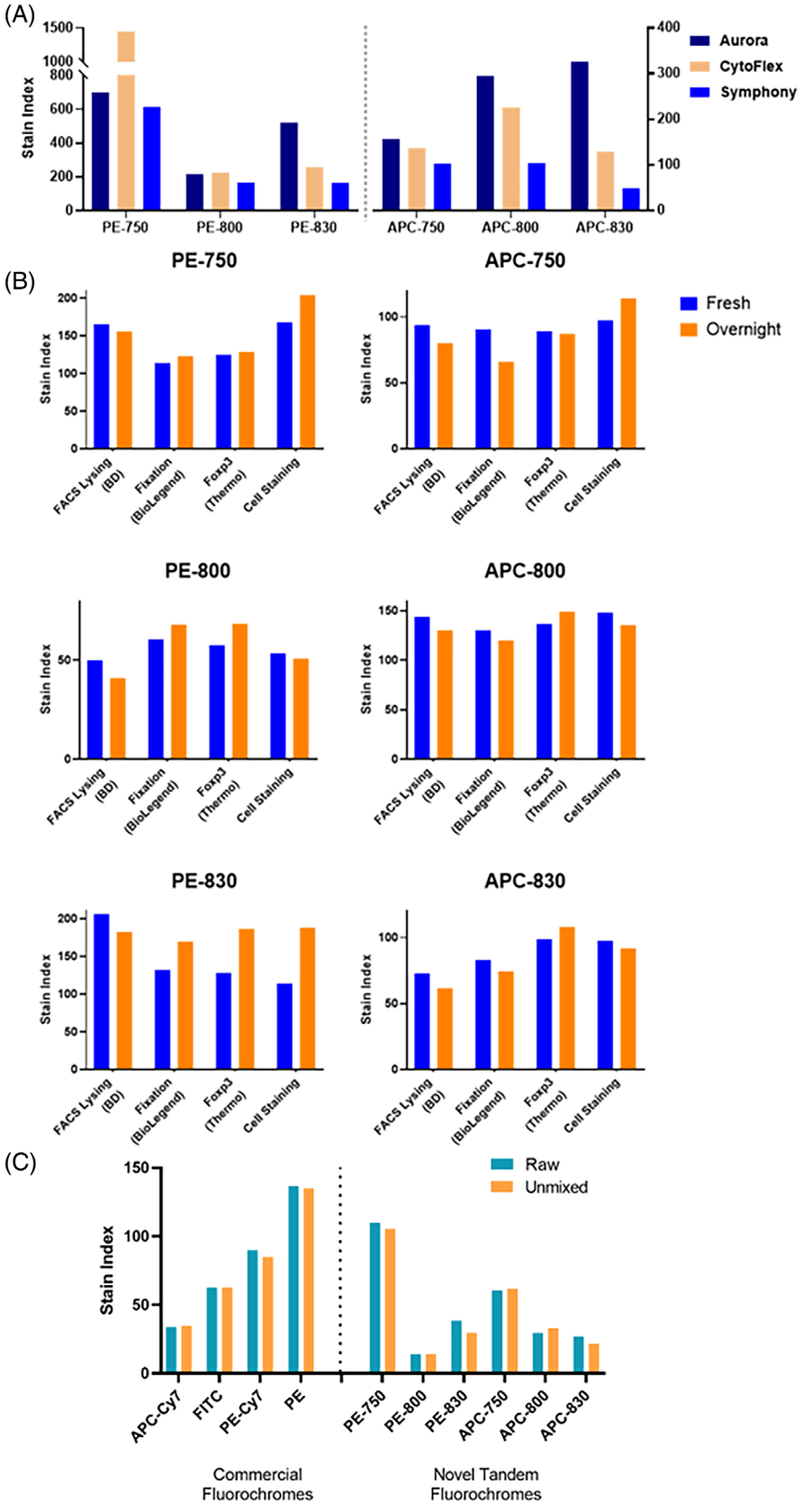

FIGURE 3 Novel antibody-conjugated fluorochromes showed higher sensitivity in near-IR region in spectral flow cytometry and maintained stability after fixation followed by storage overnight. (A) Stain indexes were obtained for each of the novel antibody-conjugated fluorochromes after binding to compensation beads and acquired using Aurora (dark blue columns), CytoFLEX LX (orange column), and BD FACSymphony (blue columns); for the Cytek Aurora, stain indexes were calculated based on raw data files. (B) Stability for each antibody-conjugated fluorochrome was evaluated in the presence of BD FACS lysing solution, BioLegend fixation buffer, Foxp3/transcription factor staining buffer set from Thermo fisher, and cell staining buffer (PBS + 10% FBS), respectively, by comparing the stain indexes for PBMCs when stained and acquired fresh (blue columns) versus following storage 4°C overnight (orange columns). (C) Stain index measurement of the novel fluorochromes from raw FCS files and spectrally-unmixed FCS files was shown along with the conventional fluorochromes to demonstrate relative brightness [Color figure can be viewed at wileyonlinelibrary.com]

for transcription factor staining). Single-color cell staining of each novel fluorochrome conjugated with anti-CD4 antibody was either fixed and immediately acquired on the cytometer, or fixed overnight at 4°C and acquired on the next day. As a control, we also stained the cells and resuspend it in cell staining buffer (PBS + 1% FBS). In general, the stain index of the novel antibody-conjugated fluorochromes was not

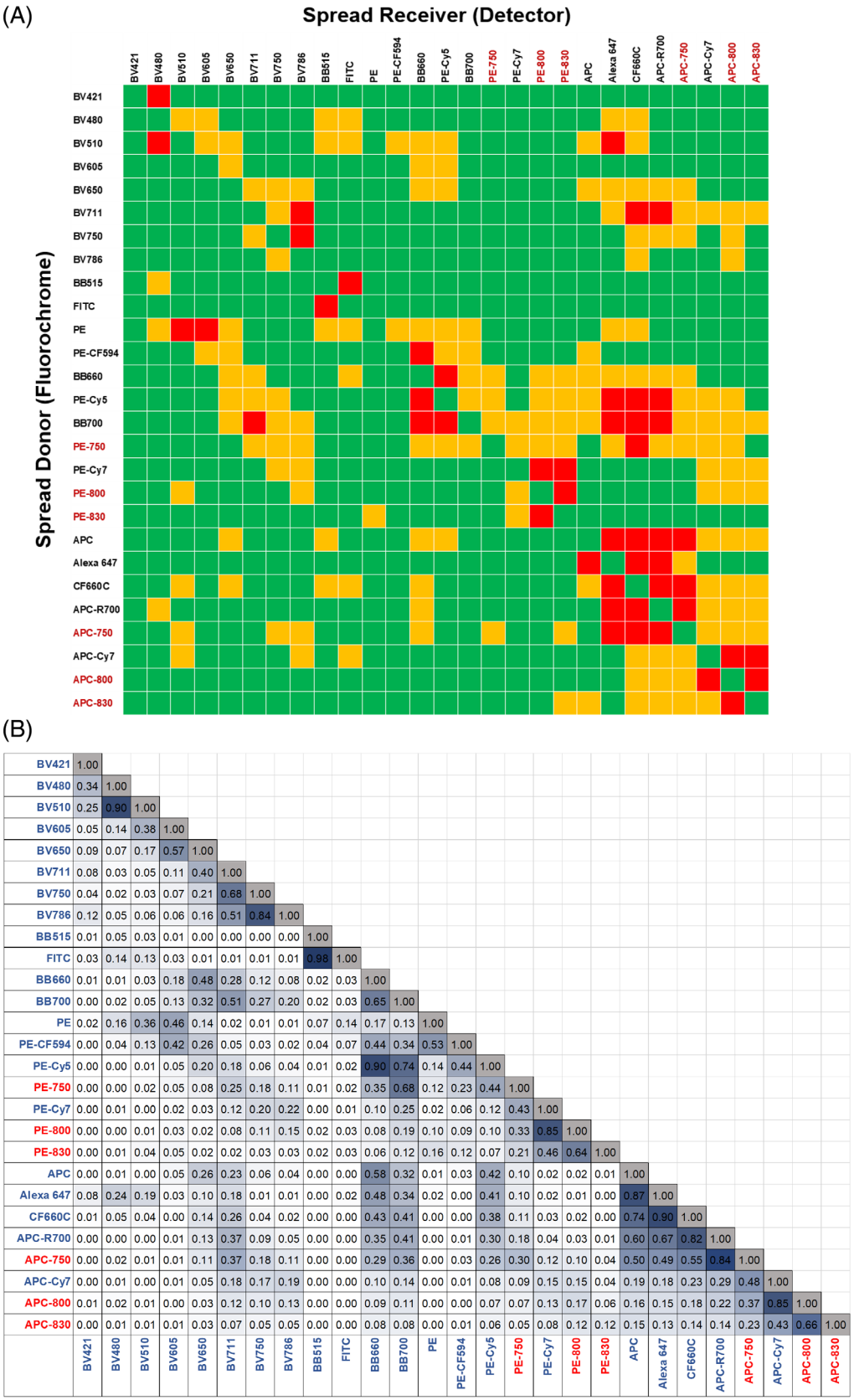

FIGURE 4 Spillover spread matrix (SSM) and similarity index. (A) SSM calculated with the reagents listed in the first column on spectral flow cytometry. SSM values are color-coded by green-yellow-red color transition; SSM values <2 (green), between 2 and 6 (yellow), and 6 > (red). The novel antibody-conjugated fluorochromes are written in red. (B) Similarity index measures a dye pair's uniqueness ranging from 0 to 1. Dye pair with very distinct spectral signature (BV421 and PE) will have a similarity index of 0, whereas highly overlapping dyes (FITC and BB515) will have a similarity index closer to 1 [Color figure can be viewed at wileyonlinelibrary.com]

significantly changed after overnight incubation with various fixatives. In addition, stain index was similar among treatment with different fixatives. These results suggest that these novel-fluorochromes can be adopted in protocols including fixation and permeabilization steps.

In Figure 3C, we demonstrated the relative brightness of the novel fluorochromes compared with conventional fluorochromes. This is a handy tool to further help cytometrists pair these fluorochromes with antigens of interest.

3.4 | Spillover spread matrix and similarity index including the novel antibody-conjugated fluorochromes

Spillover spread matrix (SSM) is a useful tool to characterize dye and instrument performance, which helps cytometrist choosing optimal fluorochrome combinations in a panel design. Commonly used 21 fluorochromes-conjugated anti-CD4 antibody, along the six novel fluorochromes (also conjugated with anti-CD4 antibody) were used to generate the spread matrix in FlowJo, as previously described.[7] SSM value lower than 2 is considered as minimal spread (green), between 2 and 6 as moderate spread (yellow), and above 6 as high spread (red) (Figure 4A). As demonstrated by the SSM matrix, PE-800, PE-830, APC-800, APC-830 fluorochromes introduce minimal spread into other detectors, indicating that they could be easily adopted into an existing multicolor panel. In contrast, PE-750 and APC-750 introduce a significant level of spread into other detectors, indicating that thoughtful panel design is needed to incorporate these fluorochromes into an existing multicolor panel.

On the other hand, the similarity index measures a dye pair uniqueness ranging from 0 to 1. The matrix was calculated in SpectroFlo, as previously described. Dye pair with very distinct spectral signature (BV421 and PE) will have a similarity index of 0, whereas highly overlapping dyes (FITC and BB515) will have a similarity index close to 1. The recommended cut-off to use dyes in combination is 0.98.[9] As demonstrated by the similarity index table, the novel fluorochromes have a similarity index ranging from 0 up to 0.85, indicating that the novel fluorochromes could be combined with existing commercial fluorochromes.

3.5 | An example of a high-dimensional flow cytometry panel incorporating the novel fluorochromes

To demonstrate the utility of the novel fluorochromes, we attempted to design a 34-color panel for broad human immunophenotyping by incorporating six novel reagents along 28 commercially available reagents (Table 2).

To optimize the instrument settings for the novel fluorochromes, the gains for each channel were adjusted by the voltage titration method to maximize resolution (Table S1) as previously described.[10,11] After setting the gains, single color compensation bead controls were acquired and used for spectral data unmixing. In some cases, we recognize that compensation beads may behave differently from cells.[12] Therefore, we fine-tuned the compensation matrix by imposing the compensation bead-derived matrix on single-color stained blood cells and made minor adjustment to the matrix to correct for the differences between cells and compensation beads. The final matrix was applied to the samples. Single staining of each novel antibody-conjugated fluorochrome is shown in Figure S1. The final unmixing matrix was applied to the blood donor samples.

To demonstrate the general utility of the 34-color flow panel, we simply gated major immune cell subsets using 2D dot plots in SpectroFlo—the default analysis software package on the Aurora cytometer (Figure 5). Within single lymphocytes, central memory CD4 and CD8 T cells were gated as $CD4^+CD27^+CD45RA^-$ and $CD8^+CD27^+CD45RA^-$, respectively. Regulatory T cells were gated as

TABLE 2 Thirty-four-color flow cytometry panel for broad immunophenotyping in human. Six novel antibody-conjugated fluorochromes were combined with 28 commercially available antibody reagents—13 channels for violet laser, 13 channels for blue laser, and eight channels for red laser. Novel in-house antibody-conjugated fluorochromes are noted with asterisks.

Violet		Blue		Red	
Fluorochrome	**Antibody**	**Fluorochrome**	**Antibody**	**Fluorochrome**	**Antibody**
BV421	CD33	BB515	DNAM	APC	TCRγδ
SB436	CD22	FITC	IgD	Alexa Fluor 647	CD303
eFluor 450	CD57	Alexa Fluor 532	CD11b	CF680	CCR6
BV480	CD138	PE	CXCR3	APC-R700	CD25
BV510	CD28	PE-CF594	CCR7	APC-750*	CD20
Krome Orange	CD14	BB660	CD4	APC-eFluor780	HLA-DR
BV570	CD27	PE-Cy5	CD11c	APC-800*	CD3
Qdot585	CCR3	PE-Cy5.5	PD1	APC-830*	CD8
BV605	CD127	PerCP-eFluor710	CD38		
BV650	CD123	PE-750*	CD16		
BV711	CXCR5	PE-Vio770	CCR4		
BV750	CD56	PE-800*	CD19		
BV786	CD45RA	PE-830*	CD45		

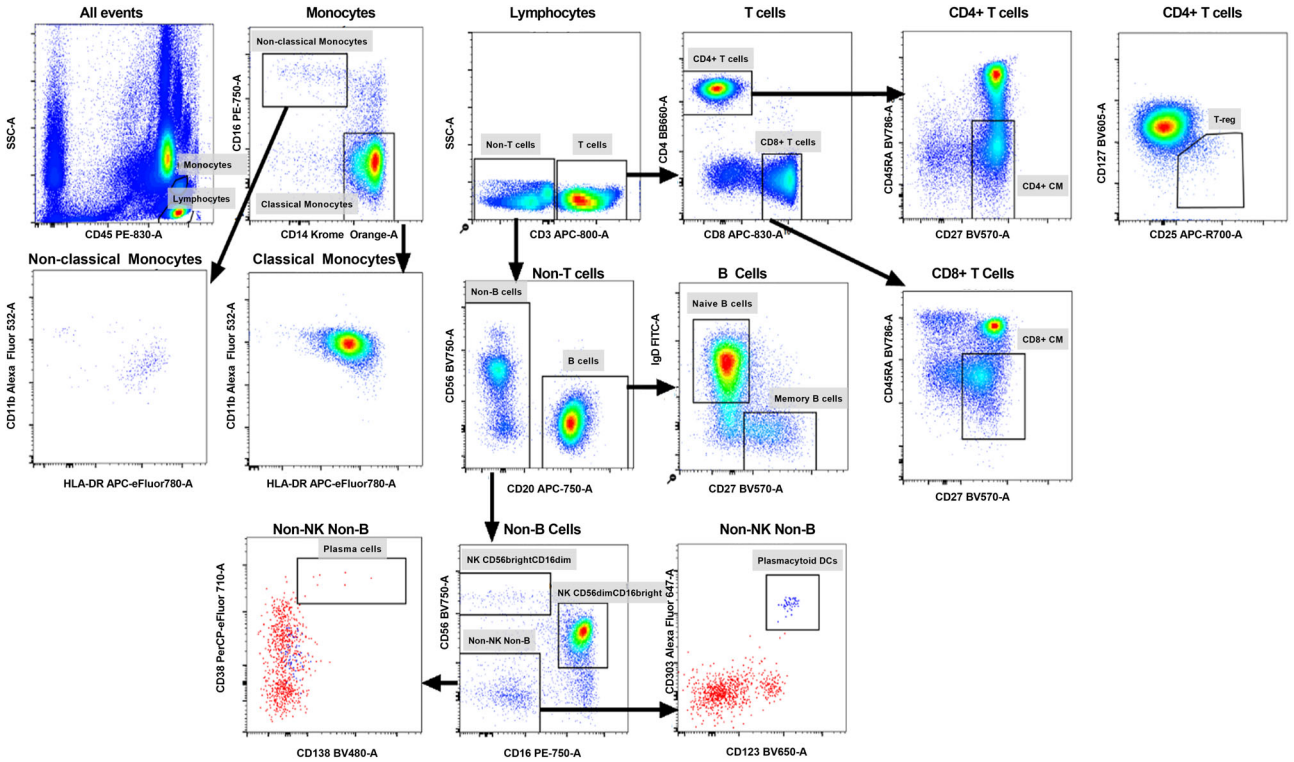

FIGURE 5 2D dot plots for immune cell subsets in human whole blood. Some immune cell subsets were gated by cell-lineage markers. CM, central memory; Treg, regulatory T cells [Color figure can be viewed at wileyonlinelibrary.com]

$CD4^+CD25^+CD127^-$ cells. Within $CD3^-$ lymphocytes, we identified memory B cells as $CD20^+CD27^+IgD^-$ and naïve B cells as $CD20^+CD27^-IgD^+$. Similarly, within the $CD3^-CD20^-$ lymphocytes, NK cells were divided into two groups $CD56^+CD16^-$ and $CD56^-CD16^+$ NK cells. Rare populations such as plasma cells ($CD38^{++}$ and $CD138^+$) and plasmacytoid dendritic cells ($CD303^+$ and $CD123^+$) could be easily identified.

3.6 | High dimensional analysis using viSNE confirmed antibody staining patterns on individual cells was consistent with established immune phenotype expression patterns

A variant of t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis, termed viSNE, was performed on results obtained following antibody cocktail staining of blood from three human donors (Figure S2) using an antibody cocktail that incorporated one of each of the novel fluorochromes as well as 28 additional cell surface antigen-specific fluorescently labeled antibodies. These viSNE graphic visualizations confirmed that staining of each antibody in the cocktail was specific for immune cells known to express each of those antigens. For example, CD3 staining intensities were highest for $CD4^+$ and $CD8^+$ cells, but not detectable for $CD20^+$ cells and PD-1 levels were highest on immune cells with a CD4 effector memory T cell phenotype ($CD4^+CD45RA^-CD27^-$) and lower on cells with a CD4 naïve ($CD4^+CD45RA^+CD27^+$) phenotype. Following viSNE analysis, FlowSOM could be used to generate clusters and provide statistical analysis to identify the most significant population cluster node (Figure S3). These analyses may accelerate novel biomarker discovery using high-content flow cytometry panels in clinical and pre-clinical studies.

4 | DISCUSSION

Recent advances of flow cytometric instrumentation have significantly increased the number of parameters that can be measured simultaneously at the single cell level. Here we aimed to expand the capacity of a common flow cytometer platform by creating fluorochromes for channels where commercially available antibody conjugates are limited. Six combinations of tandem fluorochromes were generated and were successfully conjugated to monoclonal antibodies for characterization. Antibody conjugation to each novel fluorochrome was achieved using simple Click Chemistry reactions.

The SSM and similarity index show that these novel antibody-conjugated fluorochromes can be easily combined with other commercially available fluorochromes. Stability test with fixative buffers showed that they could be used for experiments requiring fixation and permeabilization steps.

Recently various groups have demonstrated examples of 40-color flow cytometry panel run on the 5-laser Aurora with eight UV-laser excited fluorochromes.[9, 13] In this study, by using the six novel fluorochromes, we were able to design and achieve a 34-color panel using only fluorochromes with primary excitation off the blue, red, or violet lasers.

Taken together, these results demonstrate novel fluorochrome development to enable the high sensitivity, simultaneous measurement of high-content flow cytometry. The tandem-dye approach, as demonstrated here, has the potential to provide novel fluorochromes that could expand the number of simultaneous parameters.

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CONFLICT OF INTEREST

Yekyung Seong, Denny Nguyen, Archana Thakur are employees of AbbVie. Yian Wu, Tuan Andrew Nguyen, and Fiona Harding were employees of AbbVie at the time of the study. Authors declare no conflict of interest. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication. This study was performed with IRB approval from AbbVie (IRB# GPRD-11-001). All blood samples used in this study were obtained with informed consent.

AUTHOR CONTRIBUTIONS

Yekyung Seong: Data curation (equal); formal analysis (lead); investigation (lead); methodology (equal); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Denny X Nguyen:** Formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Yian Wu:** Formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Archana B Thakur:** Supervision (equal); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Fiona Harding:** Funding acquisition (supporting); supervision (equal); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Tuan Andrew Nguyen:** Conceptualization (lead); data curation (equal); formal analysis (supporting); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); validation (supporting); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal).

PEER REVIEW

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