Novel PE and APC Tandems: Additional Near-Infrared Fluorochromes for Use in Spectral Flow Cytometry

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ABSTRACT

Flow cytometry is an essential technology to identify and quantify cell populations. To support the need for high content analyses, we developed new fluorochromes designed specifically to enhance our flow cytometry capabilities. A standard Cytek Aurora flow cytometer has 3 excitation lasers (405nm, 488nm, and 640nm) and incorporates the latest Avalanche Diodes Photodetector (ADP) technology, which demonstrates significant improvement in sensitivity for fluorescent emission signals longer than 800nm. However, there are no commercially available fluorochromes capable of being excited by the above lasers with peak emission signal beyond 800nm. To address this gap, we engineered 6 new fluorochromes: PE-750, PE-800, PE-830 for the 488nm laser and APC-750, APC-800, APC-830 for the 640nm laser. These novel fluorescent molecules were created by covalently linking a protein donor dye with an organic small molecule acceptor dye. Via the principle of fluorescence resonance energy transfer (FRET), these conjugates create unique fluorochromes with the excitation properties of the donor and the emission properties of the acceptor. After the fluorochromes were created and validated, they were conjugated to a test antibody. We demonstrated long-term stability of the conjugated antibodies at -20^{o}C with protein stabilizing cocktails. Most importantly, in order to show the utility of these novel fluorochromes, we created and validated a 34-color flow cytometry panel designed to measure broad human immune function with high sensitivity. This panel will be applied for immunophenotyping of multiple different cell types from dissociated tumors and blood samples. In conclusion, this novel 34-color high-content flow cytometry panel will be able to support enhanced analysis and diagnosis in Immuno-oncology and facilitate innovation in biomarker discovery.

INTRODUCTION

An advantage of near-infrared emission (wavelength from 780nm to 3,000nm) is limited interference from cellular autofluorescence compared to those at lower wavelength (400nm-550nm) (1). As a result, the use of fluorochromes with near-infrared emissions may result in higher sensitivity and fluorescent staining indexes. Moreover, in a standard 3-laser flow cytometer system (Violet 405nm, Blue 488nm, and Red 640nm), infrared emission fluorochromes have minimal spillover into shorter wavelength emission spectra.

Despite these advantages, there have been limited application of fluorochromes with near-infrared emission spectra in flow cytometry. The majority of commercial cytometers are commonly manufactured with a 488nm blue laser and 640nm red laser, but not a near-infrared laser. To make fluorochromes with peak excitation of 488nm or 640nm and peak emission within the near-infrared region, manufacturers rely on the fluorescent resonance energy transfer (FRET) principle, 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 a 'tandem dye'. Historically, PE-Cy7 and APC-Cy7 are the most commonly used tandem dyes with peak emission wavelength at 780nm. There are no other commercial PE and APC tandem fluorochromes with emission wavelengths longer than 780nm that could be easily distinguished from PE-Cy7 and APC-Cy7. Very low quantum efficiency of photomultiplier tube (PMT) detector for emissions longer than 800nm (3) could explain the limited commercial availability of fluorochromes with near-infrared emission in flow cytometry. Recent advances in optic technology lead to the commercial adoption of Avalanche photodiodes (e.g. Beckman Coulter Cytoflex and Cytek Aurora), which have much higher quantum efficiency than photomultiplier tubes for fluorescent emission in the infrared region (4).

In this letter, we describe six novel fluorochromes: PE-750, PE-800, PE-830, APC-750, APC-800, and APC-830. These fluorochromes have a peak emission wavelength at 750nm, 800nm, 830nm, respectively and could be easily distinguished from PE-Cy7 and APC-Cy7 on a Cytek Aurora spectral flow cytometer. We also show an example of a 34-parameter panel incorporating these six novel fluorochromes using human whole blood samples.

MATERIALS AND METHODS

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, San José, CA) and then processed for staining on the same day of collection.

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, 240kDa) (Prozyme, Hayward, CA) was extensively dialyzed into phosphate-buffered saline (PBS, pH 7.2) (GE Life Sciences, PA) using the Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the R-PE is dialyzed for 2 hours at room temperature and this process is repeated with fresh PBS for another 2 hours. 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, 105kDa) (AAT Bioquest, Sunnyvale, CA) was resuspended with PBS to a final concentration of 2.5 mg/ml.

Organic small molecule fluorophores such as Dy705, Dy706 (Dyomics, Germany), Dy800-P4 (Thermo Fisher, Carlsbad, CA), and iFluor810 (AAT Bioquest, Sunnyvale, CA) were dissolved with anhydrous DMSO (Thermo Fisher, Carlsbad, CA) to a final concentration of 1,000 nmol/ml. For example, 1mg of Dy800-P4 NHS Ester was dissolved in 952µl of anhydrous

DMSO. Meanwhile, 1M sodium bicarbonate (pH 8.3-8.5) was prepared by resuspending sodium bicarbonate (Sigma-Aldrich, St Louis, MO) in deionized water.

The reaction condition is summarized in Table 1. The reaction was rotated at room temperature for at least 60 minutes. The absorbance spectra of the fluorochromes were preliminarily measured with a spectrophotometer (Marshall Scientific, Hampton, NH) using 1:100 dilution in PBS. Desalting procedure is followed using the Zebra Spin 7K MWCO desalting columns (Thermo Fisher, Carlsbad, CA) according to the manufacturer's instruction.

Characterize the brightness of the tandem fluorochrome molecules

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

Similarly, anti-mouse Ig, κ compensation particles were stained with 1μg 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, Fremont, CA).

Click chemistry to generate novel antibody-conjugated fluorochromes

Conjugation of a monoclonal antibody and tandem fluorochrome was achieved by click chemistry reaction between methyl-tetrazine and trans-cyclooctene-tetrazine (TCO) (Click Chemistry, Scottsdale, AZ). First, TCO was tagged to the antibody. 100 μgs of anti-human monoclonal antibodies were used for conjugation. Each of 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, Fenton, MO), and CD45 (Clone HI30, BioLegend) antibodies were mixed with 5 µl (5% of the total reaction volume) of 1M $NaHCO_3$ 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 1M $NaHCO_3$ 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 minutes. Desalting procedure is followed for both mixtures using spin desalting columns (Thermo Fisher, Carlsbad, CA). 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-tetrazine ester in 1:2 molar ratio. The reaction mixture was stored at 4°C overnight. The next day, protein stabilizing cocktails (Thermo Fisher, Carlsbad, CA) and bovine serum albumin were added. The final products were stored at -20°C.

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, Burlington, MA).

Anti-human CCR6 (Clone G034E3, BioLegend) was conjugated with CF680 (Biotium, Fremont, CA) in 1:10 molar ratio. 1M sodium bicarbonate (pH 8.3-8.5) was added to 10% of total volume. After 2-hour 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, Carlsbad, CA). 2 mg of antibody was mixed with 150 μg of biotin reagent. The mixture was incubated at room temperature for 30 minutes and subsequently desalted.

Anti-human CD11b (Clone C377, Leinco Technologies, Fenton, MO) was conjugated with Alexa Fluor 532 NHS ester according to the manufacturer's instructions. 0.75 nmol of CD11b antibody was added to the pre-mix and 1M sodium bicarbonate (pH 8.3-8.5) was added to 10% of total volume. After 2-hour incubation at room temperature, the mixture went through the desalting spin column.

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 reference controls were stained with each antibody-conjugated fluorochrome. 1.5 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. 0.25 μl of CD4-BB660 per test was used due to its intense brightness.

Antibody-conjugated fluorochromes for the violet 405nm 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 4KB128), 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 488nm 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-AlexaFluor532 (Clone ICRF44), 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) (Miltanyi Biotec, Auburn, CA).

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).

The samples were incubated for 30 minutes in the dark at room temperature. 2ml of 1 × FACS lysing solution (BD, San Jose, CA) were added to the mixture and incubated for another 10 minutes in the dark at room temperature. The tubes were centrifuged at 500 × g for 5 minutes. Qdot 585 VIVID streptavidin conjugate (Thermo Fisher, Carlsbad, CA) was added to the samples and then incubated for 15 minutes. The samples were washed with 2 to 3 ml of FACS buffer (1 × PBS with 1% FCS) and centrifuged at 500 × g for 5 minutes. Washing step was repeated twice.

Flow Cytometry and High-dimension Data Analysis

The samples and reference controls were analyzed on an Aurora spectral flow cytometer (Cytek Biosciences, Fremont, CA) with customized configuration (Supplementary Table 1) at

Abbvie Biotherapeutics (Redwood City, CA). Using reference controls, voltage of each channel was adjusted for optimal sensitivity. Sample QC and unmixing was run on SpectroFlo.

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

RESULTS

Conjugation of protein dye with a small molecule dye

Novel tandem fluorochromes were generated by a principle of Fluorescence Resonance Energy Transfer (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. Also, highly water-soluble fluorescent dyes were selected as an acceptor dye for covalent labeling.

Tandem dyes were synthesized by a reaction between NHS ester on the small molecule dye and amine residue on the protein dye. Various molar ratios of a protein dye and a small molecule dye were tested from 1:10 to 1:80. By comparing relative brightness and emission spectrum at distinctive wavelength, optimal ratio for each tandem dye was determined (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 Dy705 and Dy706 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.

Conjugation of novel-fluorochromes to a monoclonal antibody

The novel fluorochromes are conjugated with antibodies using click chemistry reaction (6). Click chemistry is a simple, robust reaction 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 with mixing two reagents in 1:2 molar ratio. The reaction is completed in 1-2 hours at room temperature or overnight at 4°C.

As described in panel design, we conjugated lineage markers with the novel fluorochromes. Their expression pattern is very predictable, so the quality of the novel antibody-conjugated fluorochromes can be easily evaluated (Figure 2).

Long-term stability of the novel antibody-conjugated fluorochromes

Long-term stability of the tandem fluorochromes and final antibody-conjugated products is crucial for experimental purposes.

We tested various composition of protein storage buffers (data not included). Optimal long-term storage buffer was determined to be phosphate-buffered saline buffer pH 7.2, 0.1% bovine serum albumin, and the Protein Stabilizing Cocktail which is a proprietary formulation of sodium chloride, glycerol, ammonium sulphate. The bottled conjugates were stored at -20 °C for increased shelf life. With this storage condition, the in-house antibody-conjugated fluorochromes exhibited consistent staining intensity when tested by staining of compensation beads and primary cells, for at least 4 months.

High-content flow cytometry panel design for human immunophenotyping

A 34-color flow cytometry panel design for broad human immunophenotyping is described in **Table 2**. Prior to acquiring the donor blood samples, the voltages were adjusted by the voltage titration method to maximize resolution (Supplementary Table 1) (7, 8).

After setting the voltages, reference controls were acquired and used for spectral unmixing to achieve higher sensitivity. Unmixing was additionally validated by cells labeled with each fluorochrome and further adjusted manually. Single staining of each novel antibody-conjugated fluorochrome is shown in Figure 3. The final unmixing was applied to the blood donor samples. Then, resulted FCS files were exported for the subsequent analysis.

To demonstrate the general utility of the 34-color flow panel, we simply gated major immune cell subsets using 2D dot plots in FlowJo (Figure 4). Within lymphocytes, central memory CD4 and CD8 T cells were gated as $CD4^{+}CCR7^{+}CD45RA^{-}$ and $CD8^{+}CCR7^{+}CD45RA^{-}$, respectively. Regulatory T cells were gated as $CD4^{+}CD25^{+}CD127^{-}$ cells. Within $CD3^{-}$ lymphocytes, each subset of natural killer (NK) cells were populated by expression of CD16 and CD56.

Computational analysis of immunophenotyping data at the single cell level

The complexity of the resulting data from the high-content flow cytometry panel requires novel platform of analysis. Cytobank is a web-based cytometry data analysis platform which provides many kinds of analytic tools for high-dimensional datasets. Unsupervised clustering algorithms, such as viSNE and FlowSOM, identify cellular clusters in multidimensional space without prior manual gating. Using these clustering algorithms, we can not only define major

immune cell subsets but also recognize phenotypically novel or rare cell subsets. Combining these unbiased analytic tools provides robust and novel insight.

Three donor samples were combined and analyzed in an unbiased way. viSNE based on t-Distributed Stochastic Neighbor Embedding (t-SNE) reduced the dimensionality. Different cell types and phenotypes were graphed on two-dimensional space maintaining the multi-dimensional relationships. Then, FlowSOM generated clusters using all markers on all cells using a self-organizing map (SOM). It allows quick and consistent clustering and better to reproduce the analysis and t-test across treatment using cluster output information.

This combined analysis comprehensively and reproducibly identifies rare immune cell subsets like regulatory T cells as well as major lineages of T cells, B cells, and natural killer (NK) cells (Figure 5). It allows for broad and non-invasive monitoring of all major immune cell subsets.

DISCUSSION

Recent advances of flow cytometry instrumentation have significantly increased the number of parameters that can be measured simultaneously at the single cell level. Here we aimed to create fluorochromes for channels where commercially available antibody conjugates do not exist in order to enhance the capacity of multi-color flow cytometry panel development. Initial assessment reveals the following available channels: B11, B14, B16 for the blue laser and R5, R8, R10 for the red laser (Supplementary Table 2).

The experiments of this study were designed to evaluate the fluorescence spectrum signatures of the new tandem fluorochromes and their application in multicolor spectral flow cytometry. Six combinations of tandem fluorochromes were generated and they were successfully conjugated to a monoclonal antibody. Considering excitation and emission wavelengths of a donor and an acceptor molecule, this strategy can be applied to create other combination of tandem dyes of interest. Antibody conjugation to the novel fluorochromes was achieved by Click Chemistry reactions: methyl-tetrazine and TCO was the reaction pair of choice. Click Chemistry is required for successful conjugation of the novel tandem dyes with antibodies as the conjugation can occur at near neutral pH condition (pH = 7.2). As described earlier, the tandem dyes were created by coupling a protein donor (PE or APC) with an infrared acceptor dye. These infrared acceptor dyes are relatively hydrophobic due to bulky aromatic ring core, and thus making the tandem dye complex susceptible to aggregation. Traditional cysteine antibody conjugation chemistry takes place at an acidic condition (pH = 6.0), which would trigger aggregation of the tandem dyes and result in conjugation failure.

One of the tandem dyes, APC-Dy706, was not stable upon storage, so Dy706 was replaced by Dy705 for APC-750. Another way to circumvent the stability issue would be to

directly conjugate Dy706 to an antibody, not as a tandem dye. However, Dy706-conjugated antibody was not efficiently excited by the Aurora 633nm red laser (data not shown).

Most importantly, we successfully demonstrated the utility of our 34-color high content panel using the 3-laser system of spectral flow cytometry. With the additional UV laser, the capacity of potential flow cytometry panels will be easily enhanced to more than 40-50 parameters. As with other high content data technologies, deconvolution algorithms for the high-dimensional data will need to improve to support the general use of these tools.

CyTOF is another emerging technology for high-dimensional immunophenotyping. Antibodies are labeled with heavy metal ions and samples are acquired by time-of-flight mass spectrometry. Compared to CyTOF, flow cytometry can provide high sensitivity, high speed (4,000 cells/sec), high-throughput sample acquisition by plate-reader, which are key advantages for clinical and pharmaceutical settings. Furthermore, our advanced system can be utilized for personalized immunotherapy where high-content immunophenotyping panels on larger cohorts of patients are required for execution and analysis.

Recently Cytek and Phitonex demonstrated 40-color flow cytometry panels using the 5-laser system. We designed and demonstrated a 34-color panel using the 3-laser system, so this technology will provide researchers additional flexibility on panel design and larger capacity of cell-lineage markers and targets of interest.

Here we demonstrated how to analyze the high-content flow cytometry data using viSNE and FlowSOM which are built-in tools in Cytobank. These algorithms allowed us to define major immune cell subsets and rare immune cell populations. Even though computational methods for biological data analysis have been evolving rapidly, there is no gold-standard pipeline for immunophenotyping and supporting immuno-oncology drug development. On the other hand,

traditional methods of gating immune cell populations limits data accessibility for non-immunologists since users need to know a priori the complex cell lineage markers. This work showcases a simple yet robust way of immunophenotyping that can be widely applied and lower the entry barrier of computational analysis.

Taken together, these results demonstrate novel fluorochrome development to enable the high sensitivity, simultaneous measurement of 34 parameters. The tandem-dye approach utilized has the potential to provide novel fluorochromes that could expand the number of simultaneous parameters to 40-50 different markers with installation of additional lasers.

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DISCLOSURES

YS, TN, DN, AT, FH are employees of AbbVie. YW was an employee of AbbVie at the time of the study. 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.

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Channel	Fluorochrome	Donor Protein Conc. (mg/ml)	Mixture Volume (µl)				Molar ratio (Protein:Dye)	1M $NaHCO_3$ (µl)
			Donor		Acceptor (1,000 nmol/ml)			
B11	PE-750	6.095	PE	19.7	Dy706	25	1:50	10
B14	PE-800	5.54	PE	21.7	Dy800-P4	30	1:60	10
B16	PE-830	12.2	PE	9.8	iFluor810	20	1:80	10
R5	APC-750	2.5	APC	41.6	Dy705	20	1:20	10
R8	APC-800	2.5	APC	41.6	Dy800-P4	15	1:15	10
R10	APC-830	2.5	APC	41.6	iFluor810	30	1:30	10

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

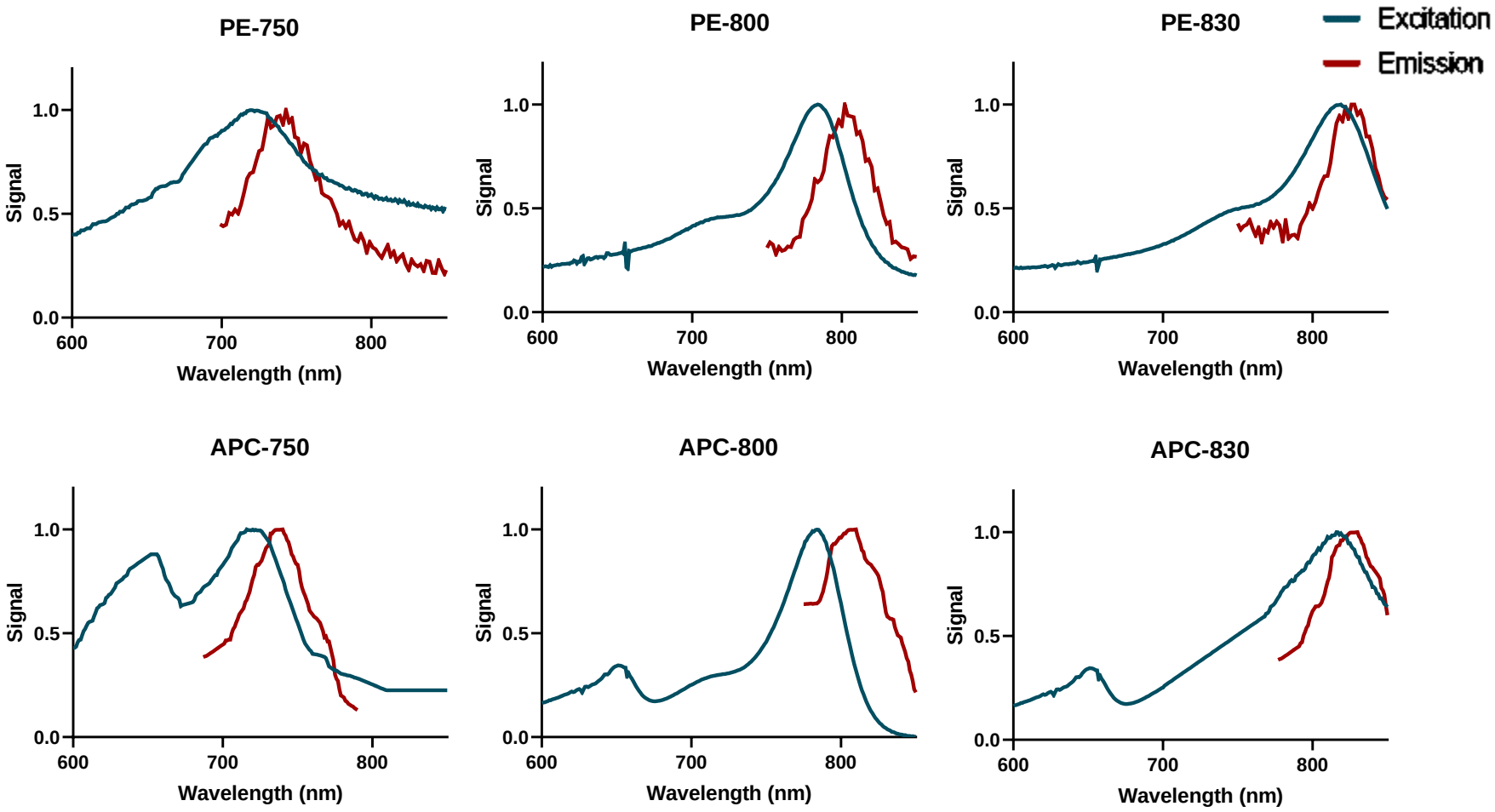
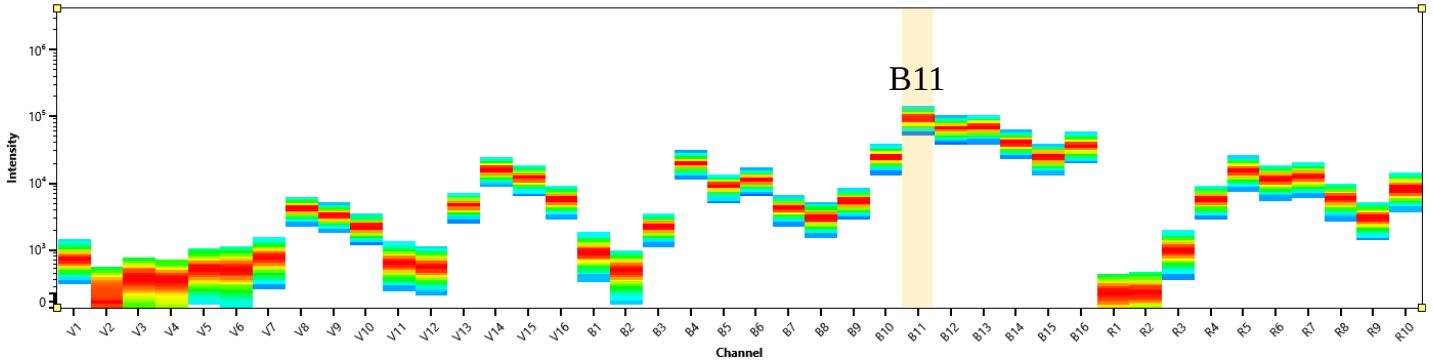


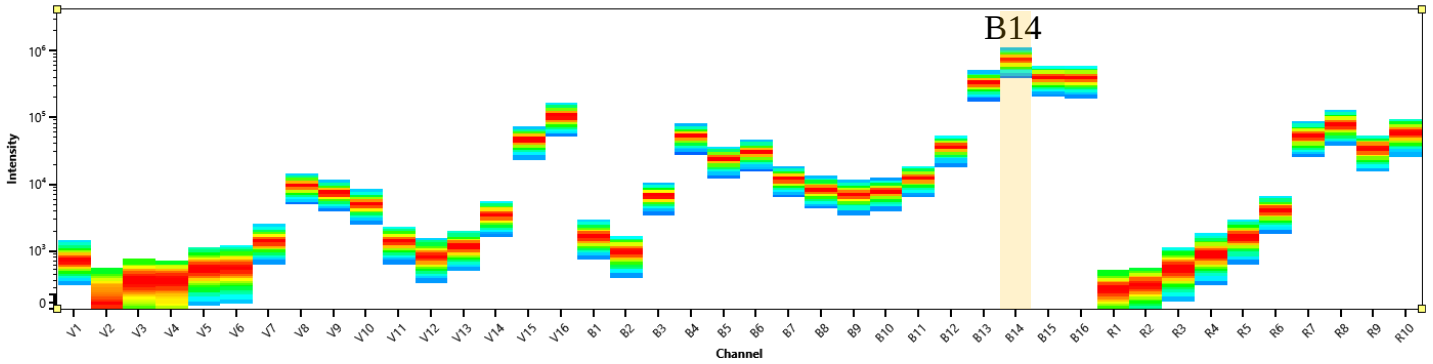
Figure 1. Excitation and emission curves of the novel fluorochromes.

Figure 2.

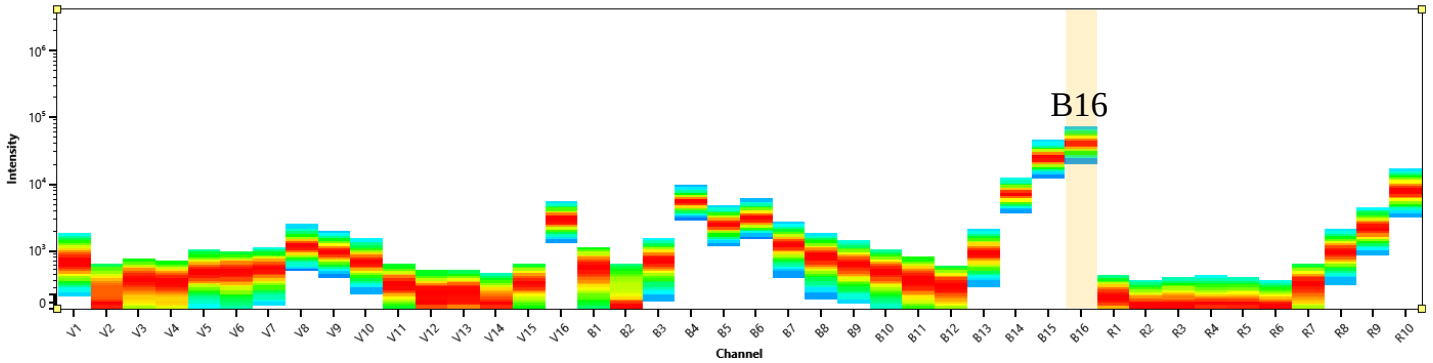
CD16-PE-750



CD19-PE-800



CD45-PE-830



CD20-APC-750

CD3-APC-800

CD8a-APC-830

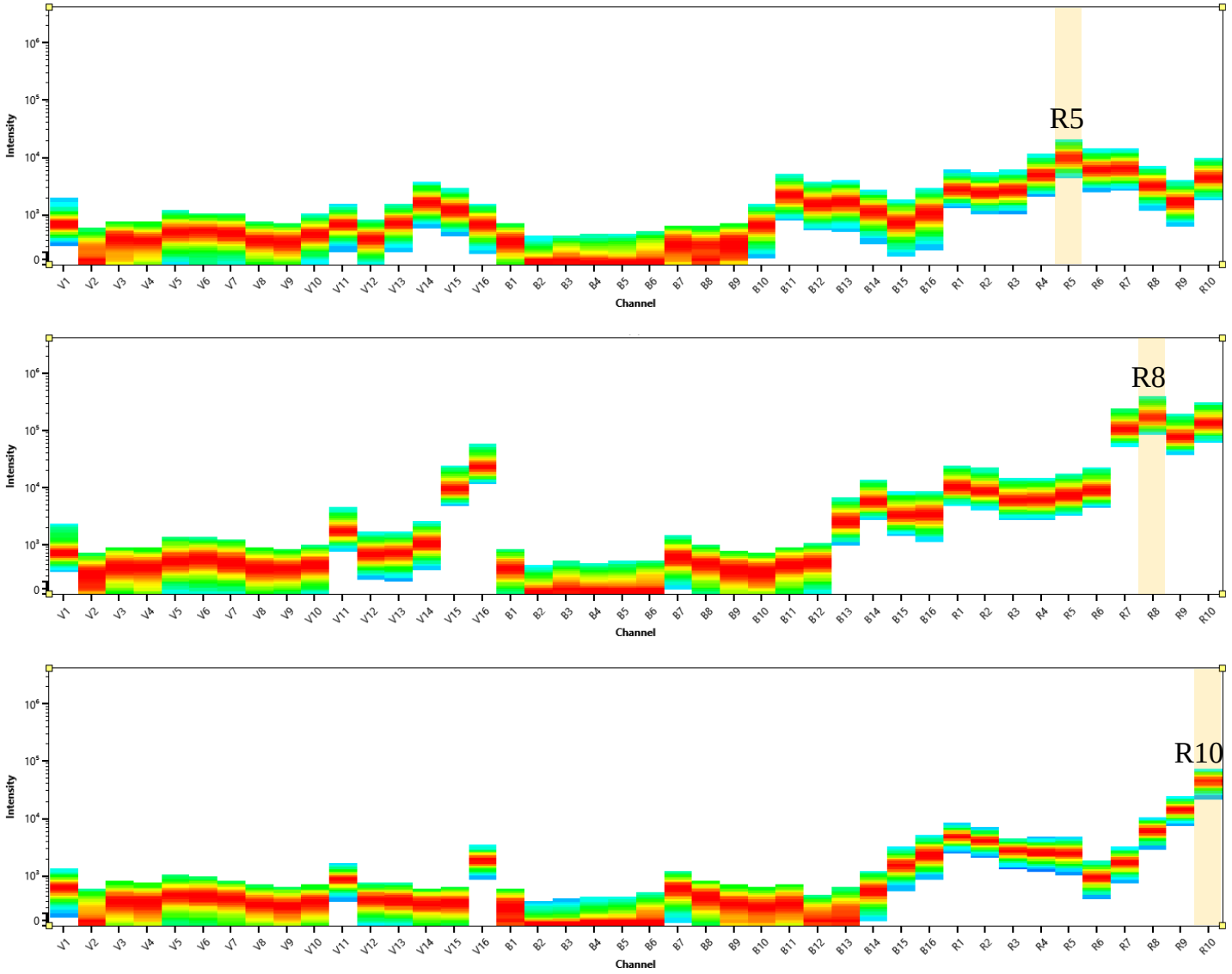


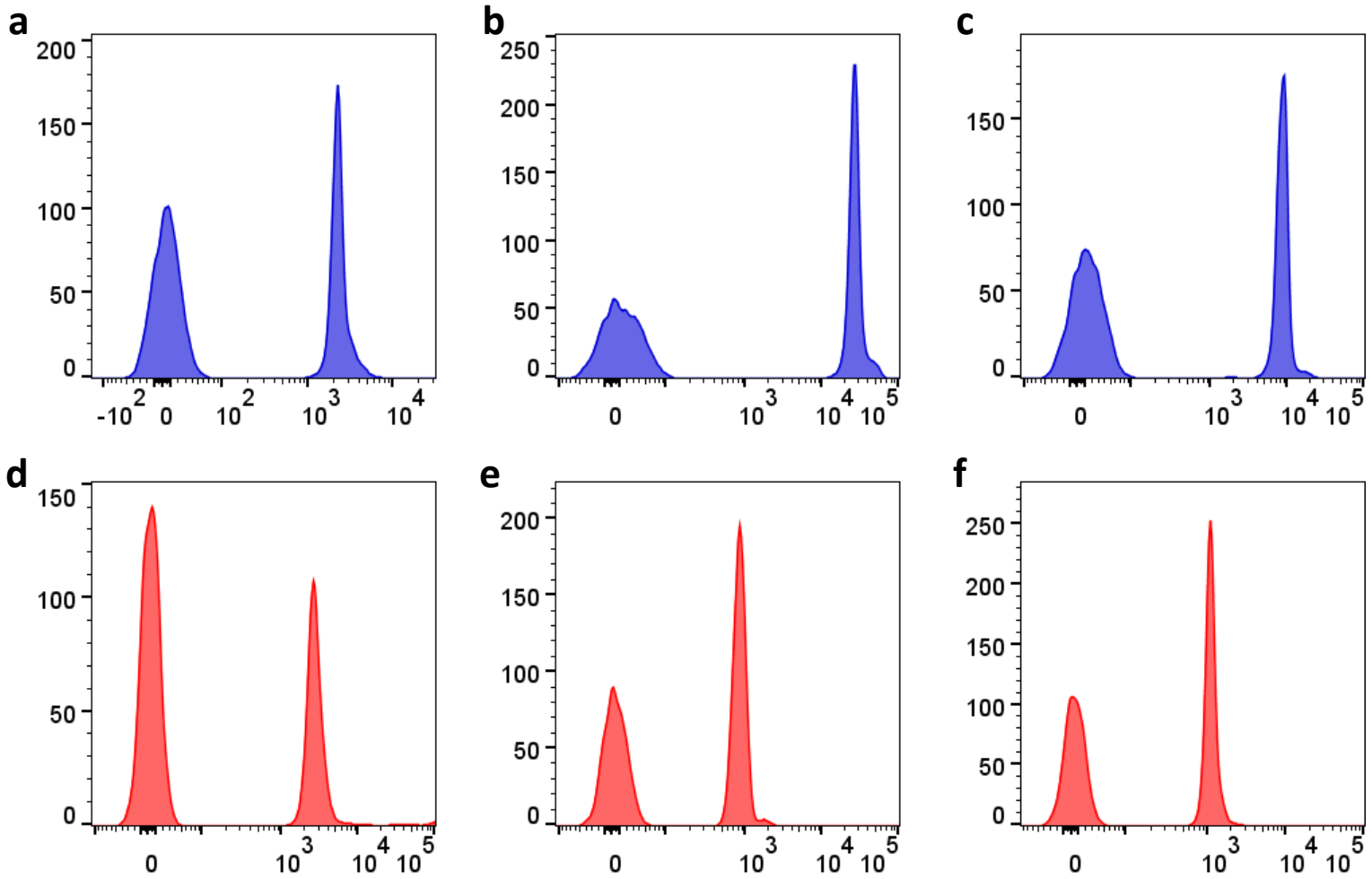
Figure 2. Spectral plots of the novel antibody-conjugated fluorochromes. Each spectral plot shows peak emission and spectrum signatures throughout violet, blue, and red lasers.

Violet		Blue		Red	
Fluorochrome	Antibody	Fluorochrome	Antibody	Fluorochrome	Antiobody
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		

In-house Ab-conjugated fluorochromes

Table 2. 34-color flow cytometry panel for broad immunophenotyping in human. 6 novel antibody-conjugated fluorochromes were combined with 28 commercially available ones – 13 channels for violet laser, 13 channels for blue laser, and 8 channels for red laser.

Figure 3.



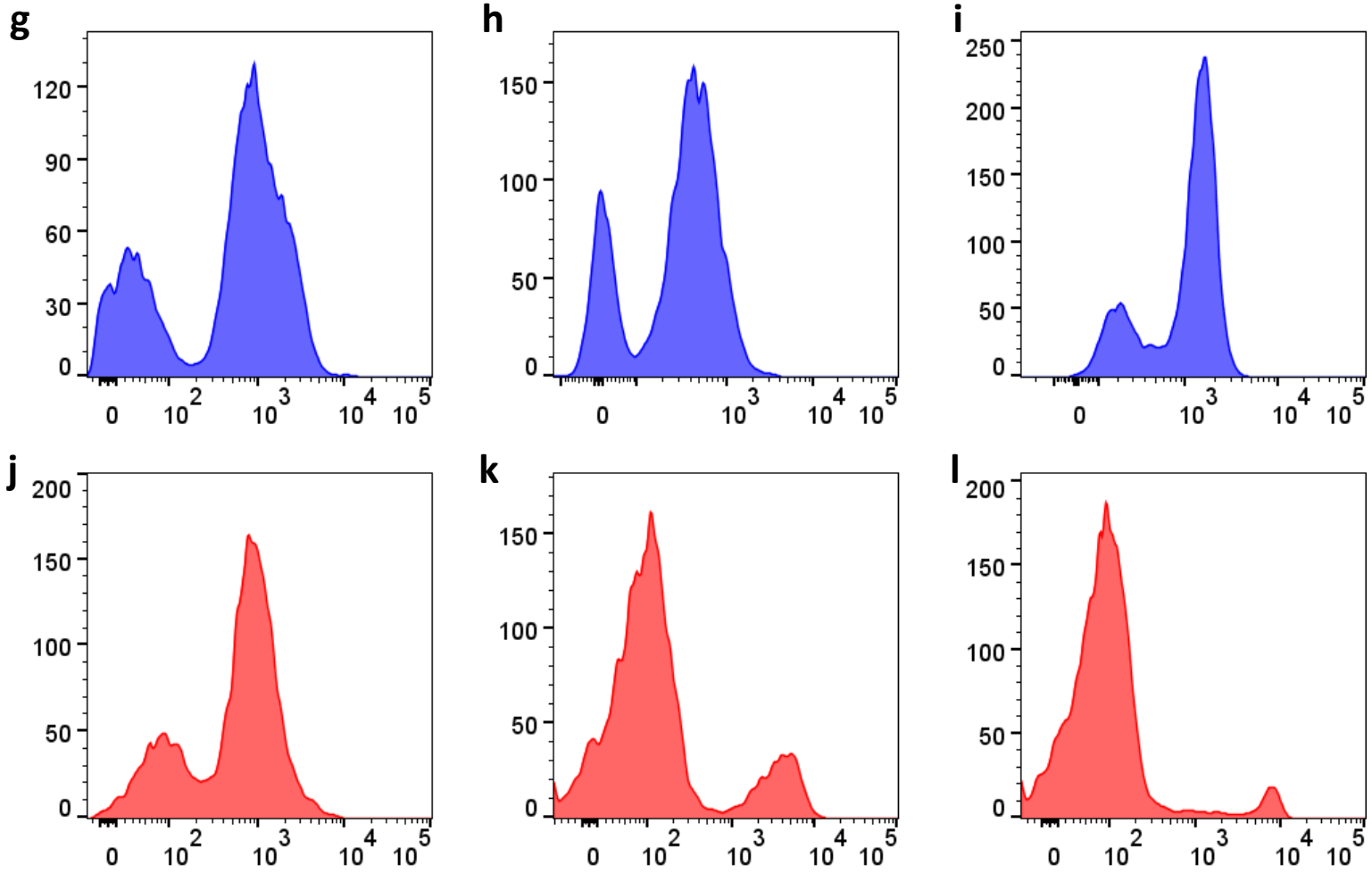


Figure 3. Single staining of the novel antibody-conjugated fluorochromes. (a-f) Compensation beads and (g-l) cells isolated from whole blood were stained with each antibody-conjugated fluorochromes and acquired by the spectral flow cytometer. The histograms were generated on FlowJo, a, g. PE-750; b, h. PE-800; c, i. PE-830; d, j. APC-750; e, k. APC-810; f, l. APC-830.

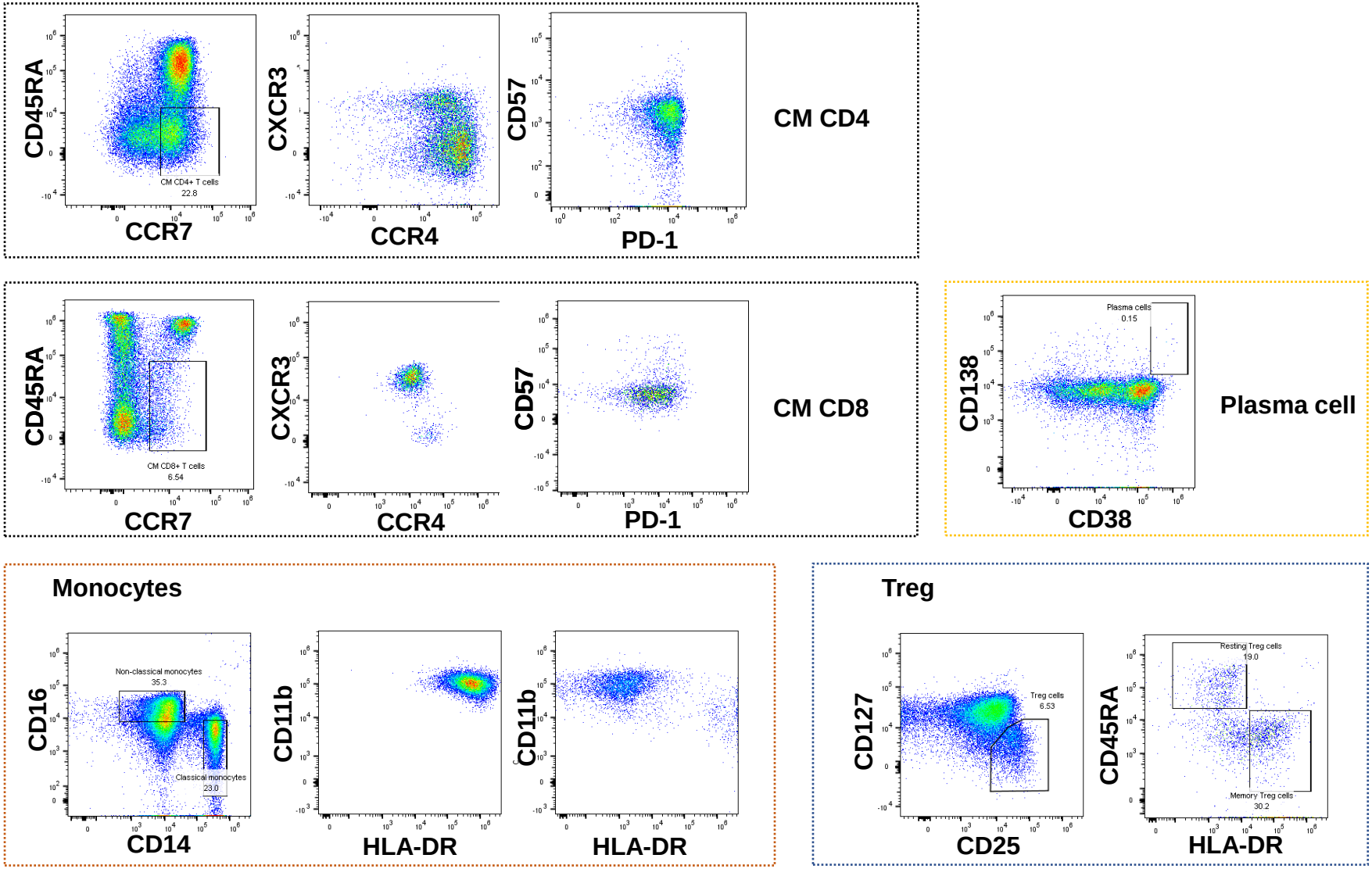


Figure 4. 2D dot plots for immune cell subsets in human whole blood. Some immune cell subsets were gated by cell-lineage markers. CM: central memory; Treg: regulatory T cells.

Figure 5.

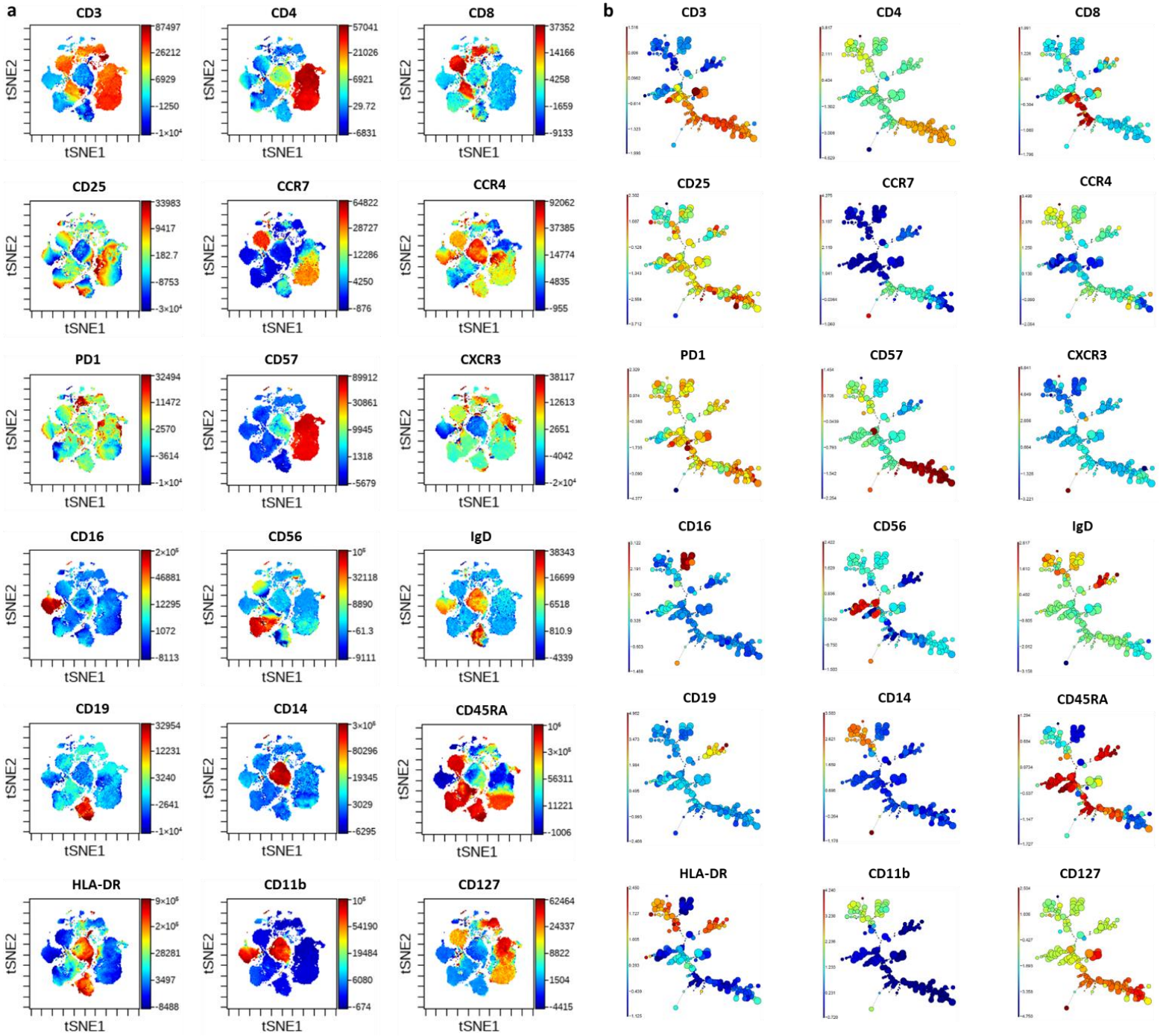

a
CD3
CD4
CD8
CD25
CCR7
CCR4
PD1
CD57
CXCR3
CD16
CD56
IgD
CD19
CD14
CD45RA
HLA-DR
CD11b
CD127
tSNE1
tSNE2
b
CD3
CD4
CD8
CD25
CCR7
CCR4
PD1
CD57
CXCR3
CD16
CD56
IgD
CD19
CD14
CD45RA
HLA-DR
CD11b
CD127

Figure 5. Dimension reduction algorithms visualize expression of each cell type-specific marker. Both viSNE and FlowSOM were performed on Cytobank. (a) viSNE takes high dimensional data and reduces it to a low-dimensional graph while retaining original information. Perplexity of 100 was used to generate viSNE plots. (b) FlowSOM generated 15 meta clusters using 3 donor blood samples.