Supplementary Materials

Small Molecular Fluorescence Dyes for Immuno-Cell Analysis

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Experimental Materials

The following materials and tools are used for antibody conjugation to dyes

- Immunoglobulin G (IgG): Biolegend CD19 (HIB19), CD45 (HI30), CD3 (SK7), and CD4 (SK3)
- Anhydrous DMSO
- 1M sodium bicarbonate (pH ~ 8.3)
- Biotium CF633 dyes
- Biotium CF405L dyes
- Biolegend PE-Cy7 dye
- Biolegend FITC dye
- Phosphate buffered saline (PBS) buffer (pH ~7.4)
- Sodium azide
- Bovine serum albumin (BSA)
- 2 small vials
- 10 pack ultrafiltration vial, 10K MWCO
- Lab pipettes
- Centrifuge
- Vortex
- Rocker
- Spectrometer
- Infrared light

The following materials and tools are used for stain lyse no wash and characterization

- CD19-CF633 and CD45-CF405L
- Antibody dilution buffer
- CD3-FITC and CD4-PE-Cy7
- Whole blood
- Lysis Buffer
- 8 test tubes
- Lab pipettes
- Test tube rack
- Foil (optional)
- Vortex
- Northern Lights flow cytometer, NL3000.

Experimental Methods

Antibody Conjugation (See Figure 2)

1. Pipette 20 microliters of anti-human CD19 antibody into a small vial. Discard the pipette tip and then add 2 microliters of 1 M sodium bicarbonate buffer to the CD19 antibody solution. Vortex to mix.
2. Using the ratio of 15 moles of dye to 1 mole of antibody, calculate the amount of CF633 dye needed to add into the CD19 solution. Pipette 0.843 microliters of CF633 into the small vial containing CD19. Vortex to mix.

a. To prepare a 10 mM dye stock solution, add 100 microliters of anhydrous DMSO into a vial for a 1 micromole dye or 25 microliters of anhydrous DMSO for a 0.25 micromolar dye. Vortex to fully dissolve the dye, followed by a brief centrifugation to collect the dye at the bottom of the vial.

3. Take the small vial containing CD19 antibody and CF633 dye and set it on a rocker at room temperature for 1 hour protected from light.
4. After 1 hour, take the small vial out from the rocker. Pipette the contents of the small vial into an ultrafiltration vial. Then, pipette 100 microliters of 1x PBS into the ultrafiltration vial and put it in a centrifuge. Spin at 1550 rpm for 4 minutes. Once done, take out the membrane and pipette the spun down solution into another tube. Then, put the membrane back into the ultrafiltration vial and pipette 100 microliters of 1x PBS into the vial. Centrifuge again. Repeat these steps until the filtrate is clear (about 3 spins in centrifuge).
5. Once the filtrate is clear, re-dissolve the left-over antibody and dye in 150 microliters of 1xPBS.
6. Before testing in the spectrometer, dilute the CD19 CF633 solution even further to approximately 0.1 mg/mL (10 mL of CD19 CF633 solution + 90 mL 1xPBS)
7. Taking the now conjugated antibody solution, bring it to a spectrometer to measure the concentration of protein. Follow instructions to the spectrometer. Once the graph of the solution is shown, look for the absorbance readings at two wavelengths, Amax at the absorption maximum wavelength, which is associated with the dye peak absorption wavelength, and A280 at 280 nm, which is associated with conjugated antibody. Record data. Then, using the formula:
    - DOL = (Amax*MWT*dilution factor)/(E*[conjugate]), and
    - [conjugate]=((A280-(Amax*Cf))/1.4)*dilution factor

    to find the optimal degree of labeling (DOL) and the concentration of the conjugated antibody [conjugate]. If it is within the bounds of the optimal DOL for the dye as shown in Table 1, the antibody conjugation is successfully completed.
    a. DOL is Degree of labeling, the average number of dye molecules conjugated to each antibody molecule.
    b. MWT is the molecular weight of the antibodies (~150,000)
    c. E is molar extinction coefficient of dye as shown in Table 1
    d. Dilution factor = (10 mL CD19 CF633 + 90 mL PBS)/10 mL CD19 CF633 = (10+90)/10 = 10
    e. Cf is the absorbance correction factor (See Table 1)
    f. The value of 1.4 is the extinction coefficient of IgG in mL/mg
    g. Testing results:
        i. Amax absorption reading for CD19 CF633 conjugation = 0.2853
        ii. A280 absorption reading for CD19 CF633 conjugation = 0.2317
        iii. Calculated DOL = 6.3 (in range)
8. Moving on to the anti human CD45 antibody, pipette 13 microliters of CD45 into a small vial. Then, pipette 1.3 microliters of 1M sodium bicarbonate into the vial. Vortex to mix.
9. Using the ratio of 25 moles of dye to 1 mole of antibody solution, calculate the amount of CF405L dye needed to add into the dissolved CD45. Pipette 1.72 microliters of CF405L into the small vial. Vortex to mix. The ratio for CF405L is different from that for CF633 due to the requirement of aiming for higher DOL, which corresponds to the numbers of dyes for each protein .
10. Set vial on a rocker for 1 hour at room temperature.

11. Repeat step 4 and wash the solution with 1xPBS until the filtrate is clear (around 4 times).
    a. Since CF405L is hard to see with the naked eye, use an infrared light to see if the filtrate is clear.
12. Repeat steps 5-7 for the CD45 CF405L solution
    a. Testing results:
        i. Amax absorption reading for CD45 CF405L conjugation = 0.1434
        ii. A280 absorption reading for CD45 CF405L conjugation = 0.1628
        iii. Calculated DOL = 13.8 (slightly higher than the optimal)
13. With the optimal measurements, prepare antibody CD4 to PE-Cy7 and CD3 to FITC, referencing steps, 1-6.

Stain Lyse No Wash

1. Taking the prepared antibody conjugations, dilute CD19-CF633 and CD45-CF405L according to table 2.
    a. Pipette 0.8 microliters of CD19-CF633 into a test tube.
    b. Discard the pipette and pipette 49.2 microliters of antibody dilution buffer to the tube with CD19-CF633.
    c. Repeat steps 1a and b with the CD45-CF405L antibody conjugation.
    d. Vortex all tubes to mix thoroughly
2. Taking two more test tubes, add 50 microliters of CD3 FITC antibody conjugation into one and 50 microliters of CD4 PE-Cy7 antibody conjugation into the other. There are now a total of 4 test tubes.
3. Now, pipette 50 microliters of diluted CD19 CF633 (0.8 microliters of CD19 CF633 antibody conjugation and 49.2 microliters of antibody dilution buffer), CD45 CF405L (0.8 microliters of CD45 CF405L antibody conjugation and 49.2 microliters of antibody dilution buffer), CD3 FITC, and CD4 PE-Cy7 (each) into three different test tubes
    a. Each tube must have 50 microliters each of CD19 CF633, CD45 CF405L, CD3 FITC, and CD4 PE-Cy7.
4. Add 50 microliters of whole blood to each of the 7 tubes. Vortex tubes to mix. Then, take a clean test tube and pipette 50 microliters of whole blood into the tube. This will be the control experiment.
5. Set all of the tubes in a test tube rack and all for 15 mins at room temperature in the dark. Cover the test tube rack with a piece of foil so that light will not activate the dye molecules in the antibody conjugation.
6. After 15 minutes, take out the test tubes. Add 450 microliters of lysis buffer into each tube. Vortex all of the tubes to mix.
7. Incubate all tubes for 15 min at room temperature in the dark. (Again, wrap the test tube rack with foil).
8. While the tubes are incubating, operate the flow cytometer (Northern Lights 3000).
9. Load the samples onto the flow cytometer one by one. Run samples, and collect data of 10,000 lymphocytes.

Supplement Tables

Table 1: CF Dye Technical Data

Dye	Amax (nm)	Cf (protein)	Extinction coefficient (E)	Optimal DOL (IgG)
CF405L	395	0.5	24,000	8-12
CF633	630	0.48	100,000	4-7

Table 2: Antibody Dilution Measurements

Marker	Clone	Color	Conc. mg/ml	ng/ul	Titer (ng)	Target [Ab] ng/ul	Dilution Factor	Stock [Ab] ul	Buffer ul	Total Volume
CD19	HIB19	CF633	0.677	677	31.25	10.42	65	0.8	49.2	50.0
CD45	HI30	CF405L	0.6507	650.7	120	40.00	16	3.1	46.9	50.0

Supplement Figures

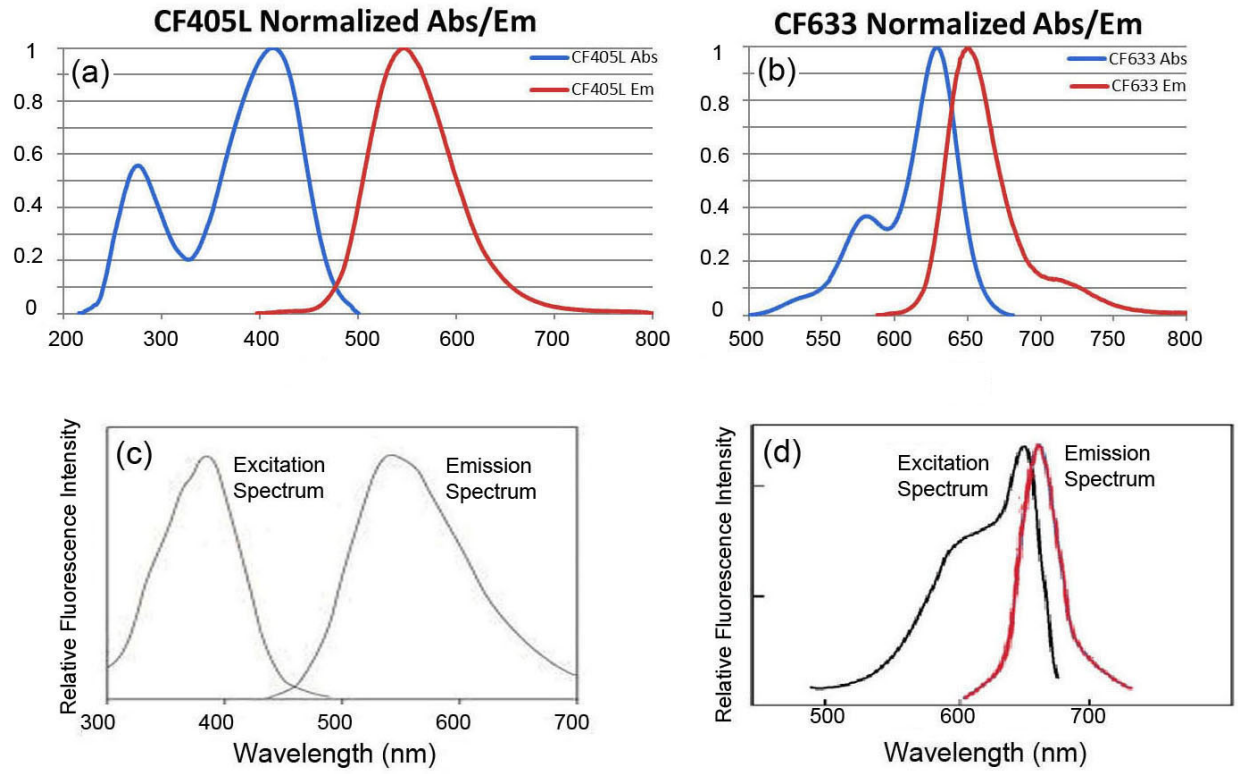


Figure S1

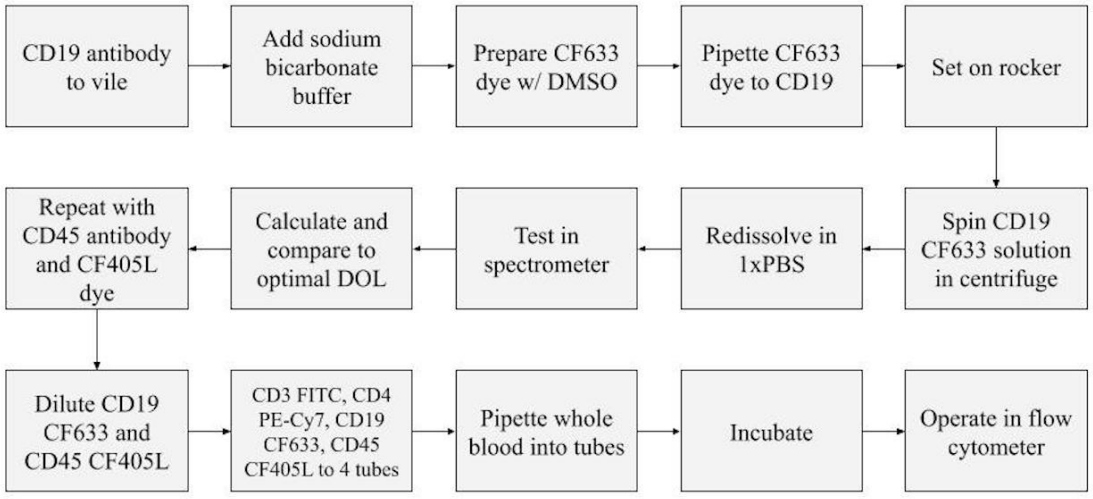
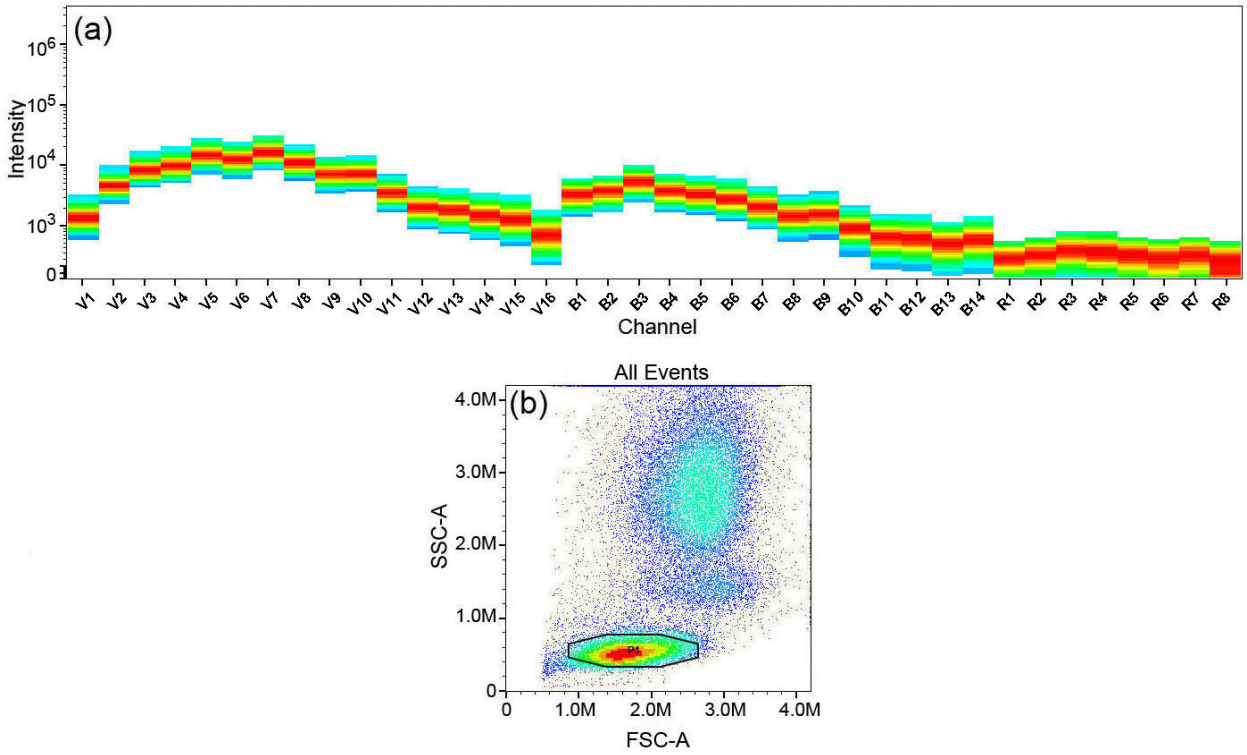


Figure S2



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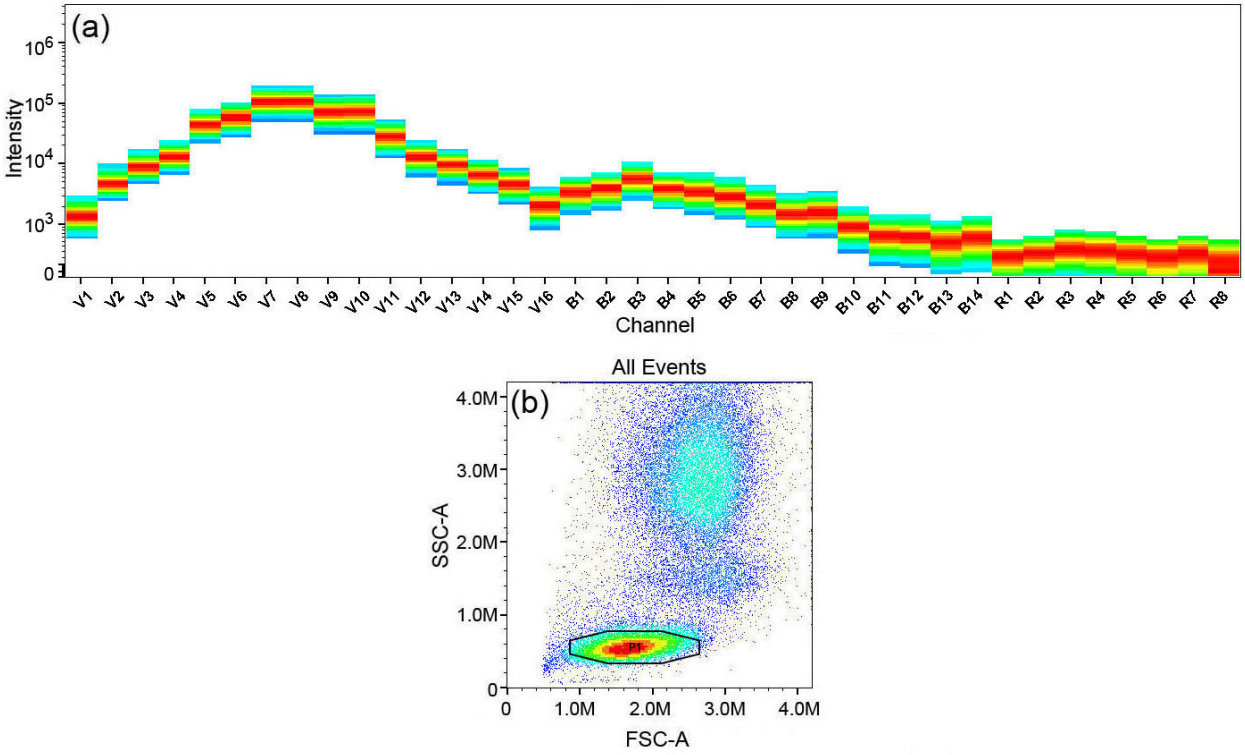

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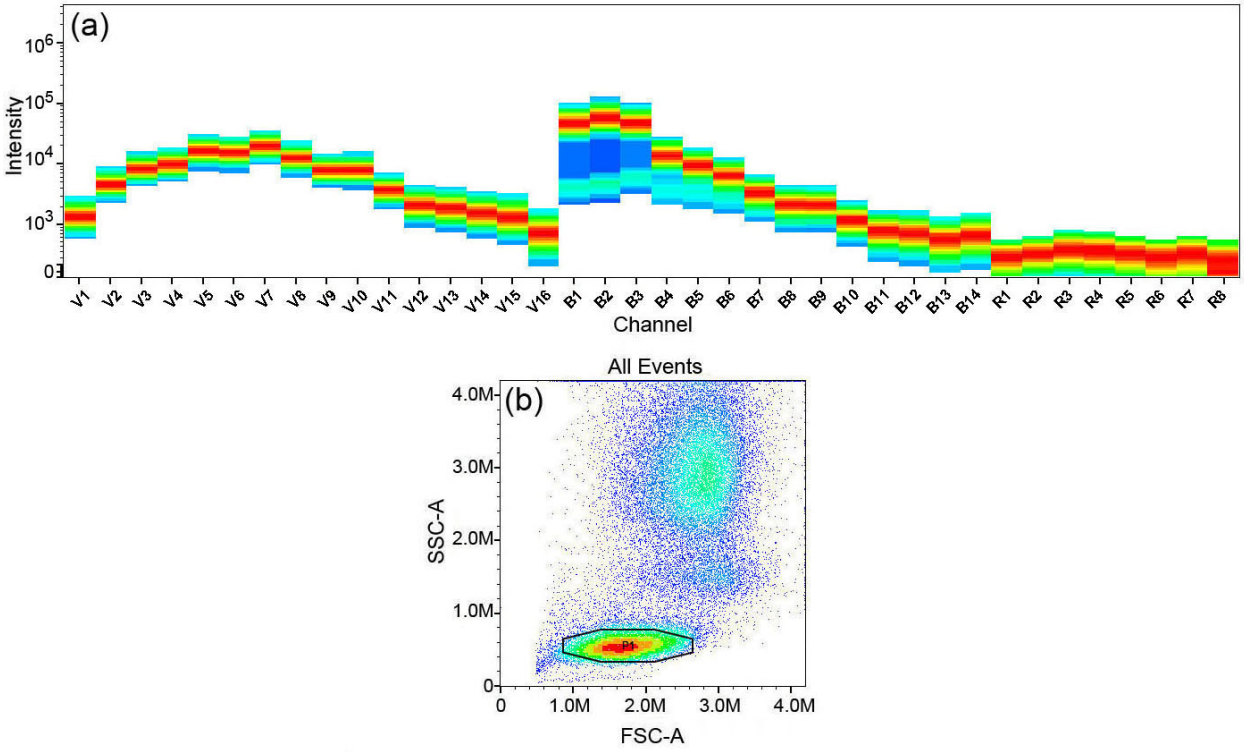

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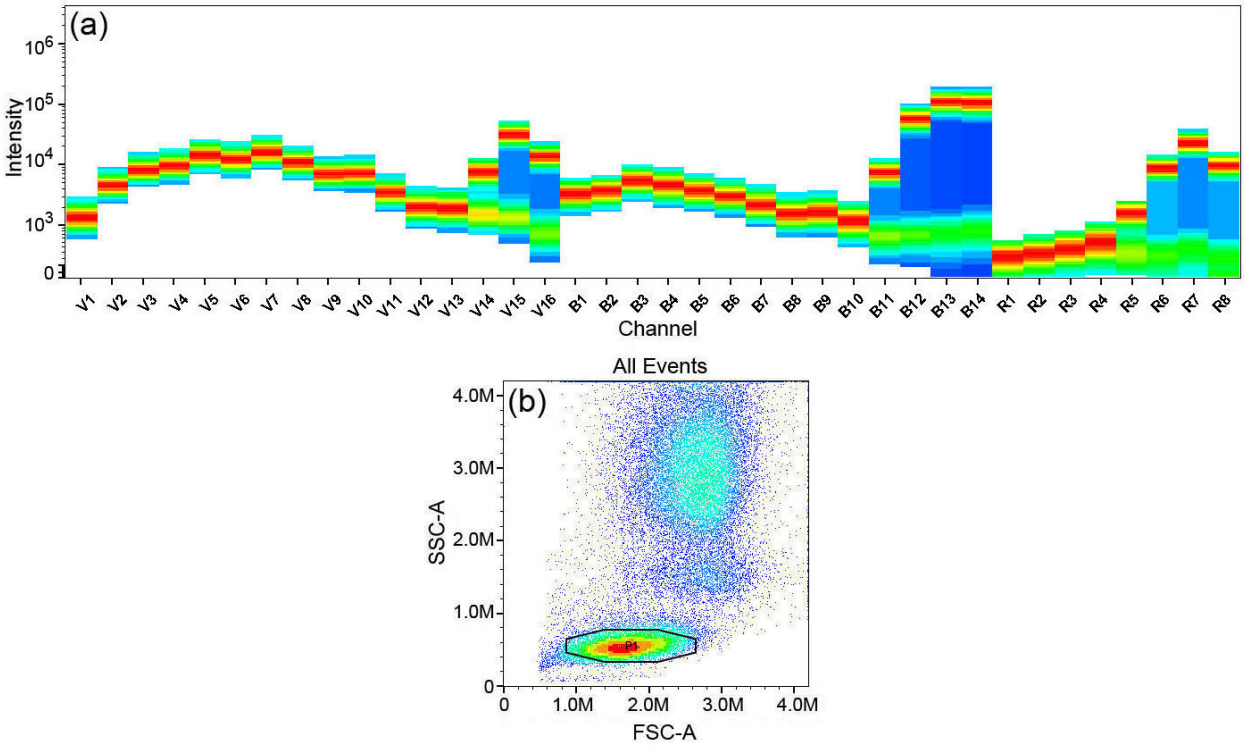

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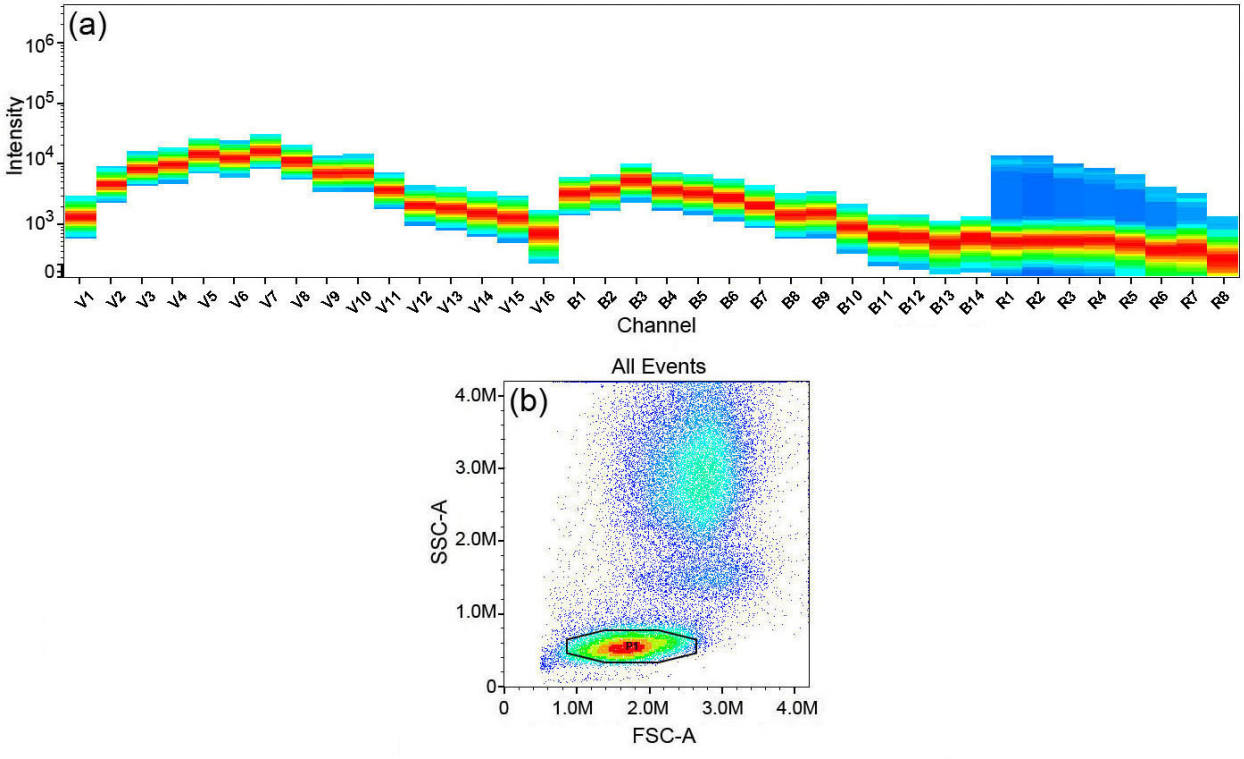
(a)
Intensity
Channel
V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 R1 R2 R3 R4 R5 R6 R7 R8
All Events
(b)
SSC-A
FSC-A
P1

Figure S7

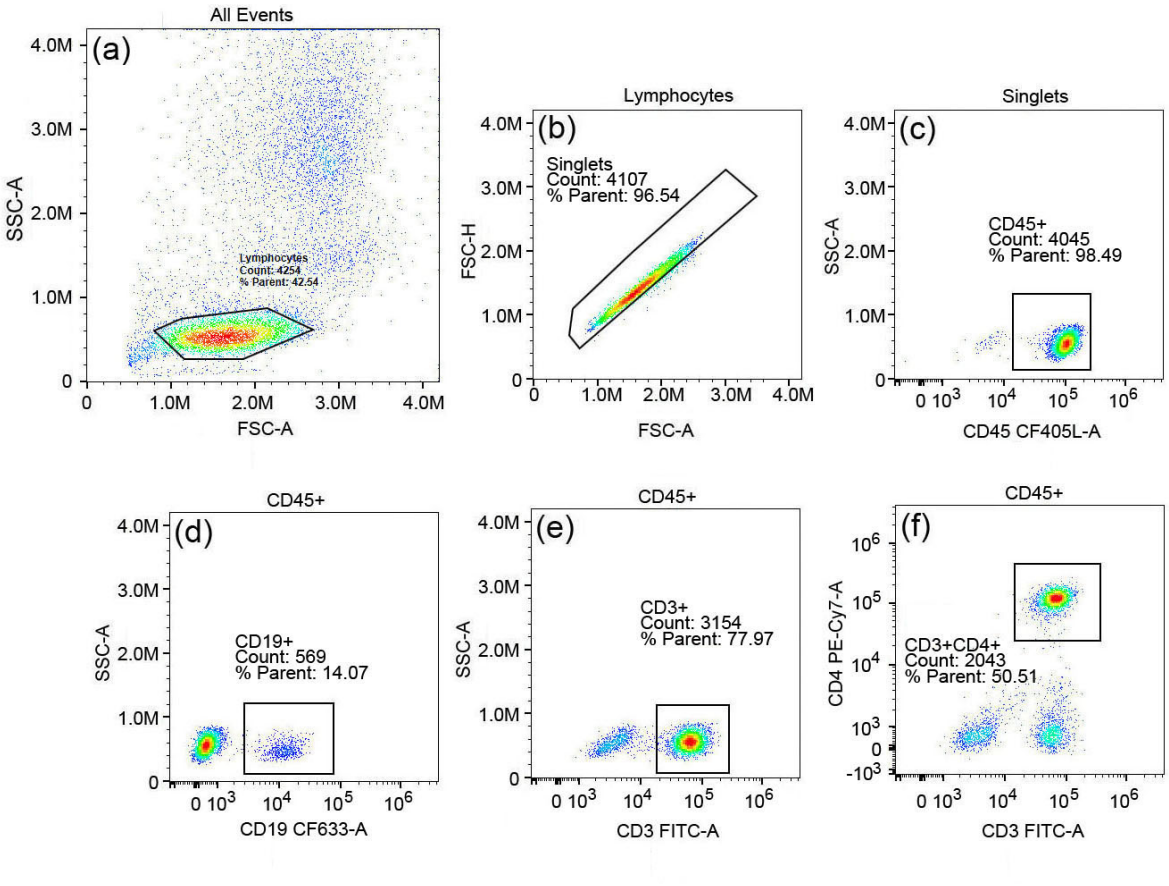
All Events
(a)
SSC-A
FSC-A
Lymphocytes
Count: 4254
% Parent: 42.54
Lymphocytes
(b)
FSC-H
FSC-A
Singlets
Count: 4107
% Parent: 96.54
Singlets
(c)
SSC-A
CD45 CF405L-A
CD45+
Count: 4045
% Parent: 98.49
CD45+
(d)
SSC-A
CD19 CF633-A
CD19+
Count: 569
% Parent: 14.07
CD45+
(e)
SSC-A
CD3 FITC-A
CD3+
Count: 3154
% Parent: 77.97
CD45+
(f)
CD4 PE-Cy7-A
CD3 FITC-A
CD3+CD4+
Count: 2043
% Parent: 50.51

Figure S8

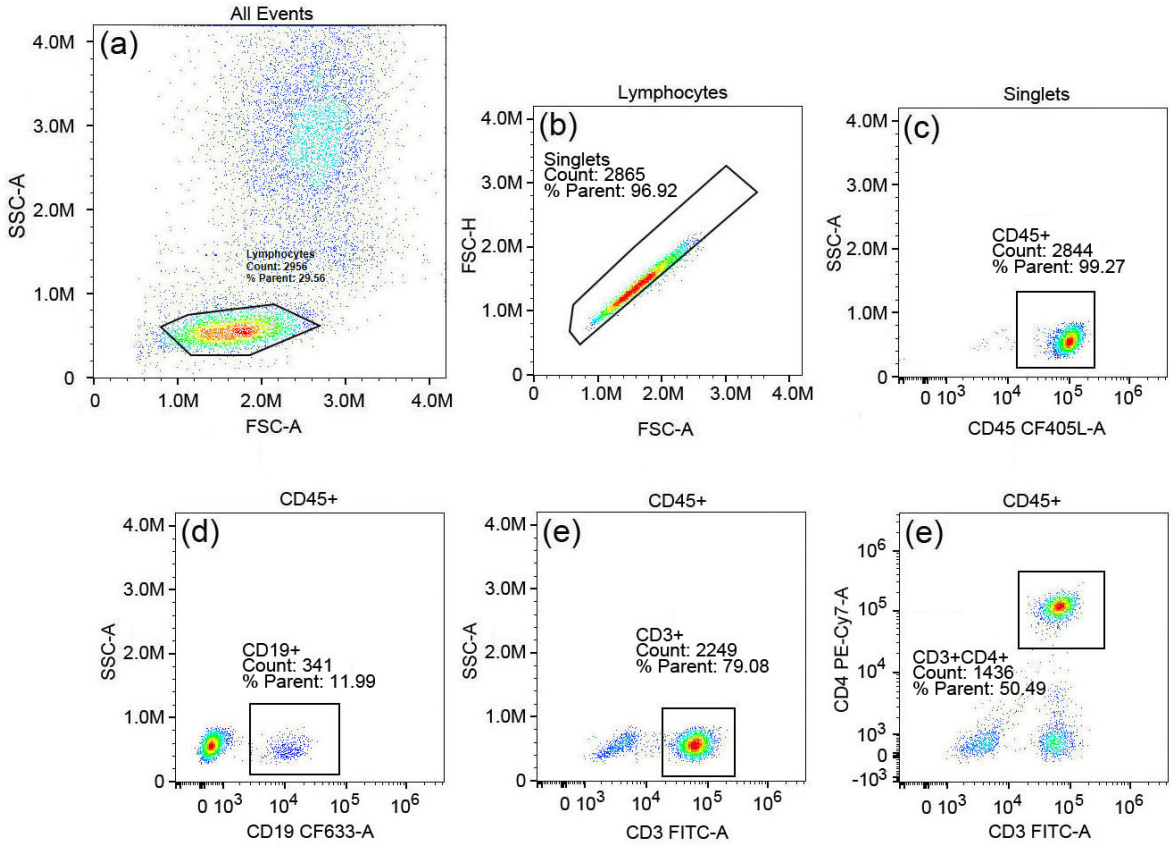
All Events
(a)
SSC-A
FSC-A
Lymphocytes
Count: 2956
% Parent: 29.56
Lymphocytes
(b)
Singlets
Count: 2865
% Parent: 96.92
FSC-H
FSC-A
Singlets
(c)
CD45+
Count: 2844
% Parent: 99.27
SSC-A
CD45 CF405L-A
CD45+
(d)
CD19+
Count: 341
% Parent: 11.99
SSC-A
CD19 CF633-A
CD45+
(e)
CD3+
Count: 2249
% Parent: 79.08
SSC-A
CD3 FITC-A
CD45+
(e)
CD3+CD4+
Count: 1436
% Parent: 50.49
CD4 PE-Cy7-A
CD3 FITC-A

Figure S9

Captions of the Supplement Figures

Figure S1 Absorption (Abs) and emission (Em) spectra of (a) CF405L dye, (b) CF633 dye, (c) Pacific Orange dye, and (d) APC dye.

Figure S2 Experimental Flow Chart.

Figure S3. (a) Fluorescence full spectrum of lymphocytes in unstained whole blood sample data by NL3000 flow cytometer; (b) all events dot plot between side scattering light area (SSC-A) vs. forward scattering light area (FSC-A) with lymphocytes gated.

Figure S4 (a) Fluorescence full spectrum of lymphocytes stained by CD45-CF405L for reference calibration; (b) all events dot plot between side scattering light area (SSC-A) vs. forward scattering light area (FSC-A) with lymphocytes gated.

Figure S5 (a) Fluorescence full spectrum of lymphocytes stained by CD3-FITC for reference calibration; (b) all events dot plot between side scattering light area (SSC-A) vs. forward scattering light area (FSC-A) with lymphocytes gated.

Figure S6 (a) Fluorescence full spectrum of lymphocytes stained by CD4-PeCy7 for reference calibration; (b) all events dot plot between side scattering light area (SSC-A) vs. forward scattering light area (FSC-A) with lymphocytes gated.

Figure S7 (a) Fluorescence full spectrum of lymphocytes stained by CD19-CF633 for reference calibration; (b) all events dot plot between side scattering light area (SSC-A) vs. forward scattering light area (FSC-A) with lymphocytes gated.

Figure S8 The first repeated 4-color panel test with (a) the 4 color panel for all cells between side scattering signal area (SSC-A) vs. forward scattering signal area (FSC-A), showing three groups of cells and gated for lymphocytes with count at 43% of the total cells; (b) forward scattering signal height (FSC-H) vs. forward scattering signal area (FSC-A) for lymphocytes and gated for singlet count at 97% of the total lymphocytes; (c) side scattering light vs. CD45-CF405L fluorescent light, showing CD45+ leukocyte count at 98% of the total singlet lymphocytes; (d) side scattering light vs. CD19-CF633 fluorescence light, showing CD19+ B-cell count at 14% of the singlet leukocytes; (e) side scattering light vs. CD3-FITC fluorescence light, showing CD3+ T-cell co-receptor count at 78% of the singlet leukocytes; and (f) CD4 PE-Cy7 fluorescence light vs. CD3-FITC fluorescence light, showing CD4+ T-helper cell count at 51% of the singlet leukocytes.

Figure S9 The second repeated 4-color panel test with (a) the 4 color panel for all cells between side scattering signal area (SSC-A) vs. forward scattering signal area (FSC-A), showing three groups of cells and gated for lymphocytes with count at 30% of the total cells; (b) forward scattering signal height (FSC-H) vs. forward scattering signal area (FSC-A) for lymphocytes and gated for singlet count at 97% of the total lymphocytes; (c) side scattering light vs. CD45-CF405L fluorescent light, showing CD45+ leukocyte count at 99% of the total singlet lymphocytes; (d) side scattering light vs. CD19-CF633 fluorescence light,

showing CD19+ B-cell count at 12% of the singlet leukocytes; (e) side scattering light vs. CD3-FITC fluorescence light, showing CD3+ T-cell co-receptor count at 79% of the singlet leukocytes; and (f) CD4 PE-Cy7 fluorescence light vs. CD3-FITC fluorescence light, showing CD4+ T-helper cell count at 50% of the singlet leukocytes.