

Establishment of Flow Cytometry Detection Method for Immunophenotyping and Quantification of Human Whole Blood Lymphocyte

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Introduction

In clinical trials, changes in immune cell phenotype and proportion play an important role in elucidating the mechanism of action and evaluating the efficacy and adverse reactions of a drug. Accordingly, the purpose of this study is to develop a method for the comprehensive analyses of lymphocyte immunophenotype, proportion and absolute count in the whole blood samples.

Methods

Using the whole blood samples collected from healthy donors in EDTA-K2 tubes, a 10-color flow cytometry panel shown in Table 1 (Live/Dead dye, CD45, CD3, CD4, CD8, CD19, CD56, CD25, CD127, CD14) is employed for analysis of T, B, and NK cells. The analytical procedure entails the lysis of red blood cells, followed by washing, staining with live/dead dye, incubation with mixed antibodies, and the addition of absolute counting microspheres for detection. The method is optimized by evaluating the antibody dosage, detection voltage, compensation, and so on. A stringent data analysis process is applied, encompassing gating strategies (Figure 1) and statistical methods.

Table 1. 10-color detection panel

Laser	Optical filter	Fluorescence	Marker
405 nm	450/45	BV421	CD25
	525/40	BV510	CD19
	610/20	BV605	CD56
	660/10	BV650	CD3
488nm	780/60	BV785	CD14
	585/42	PE	CD127
	610/20	PE-Texas Red	Live_Dead
638 nm	690/50	Percp	CD8
	660/10	APC	CD4
	712/25	Alexa Fluor® 700	CD45

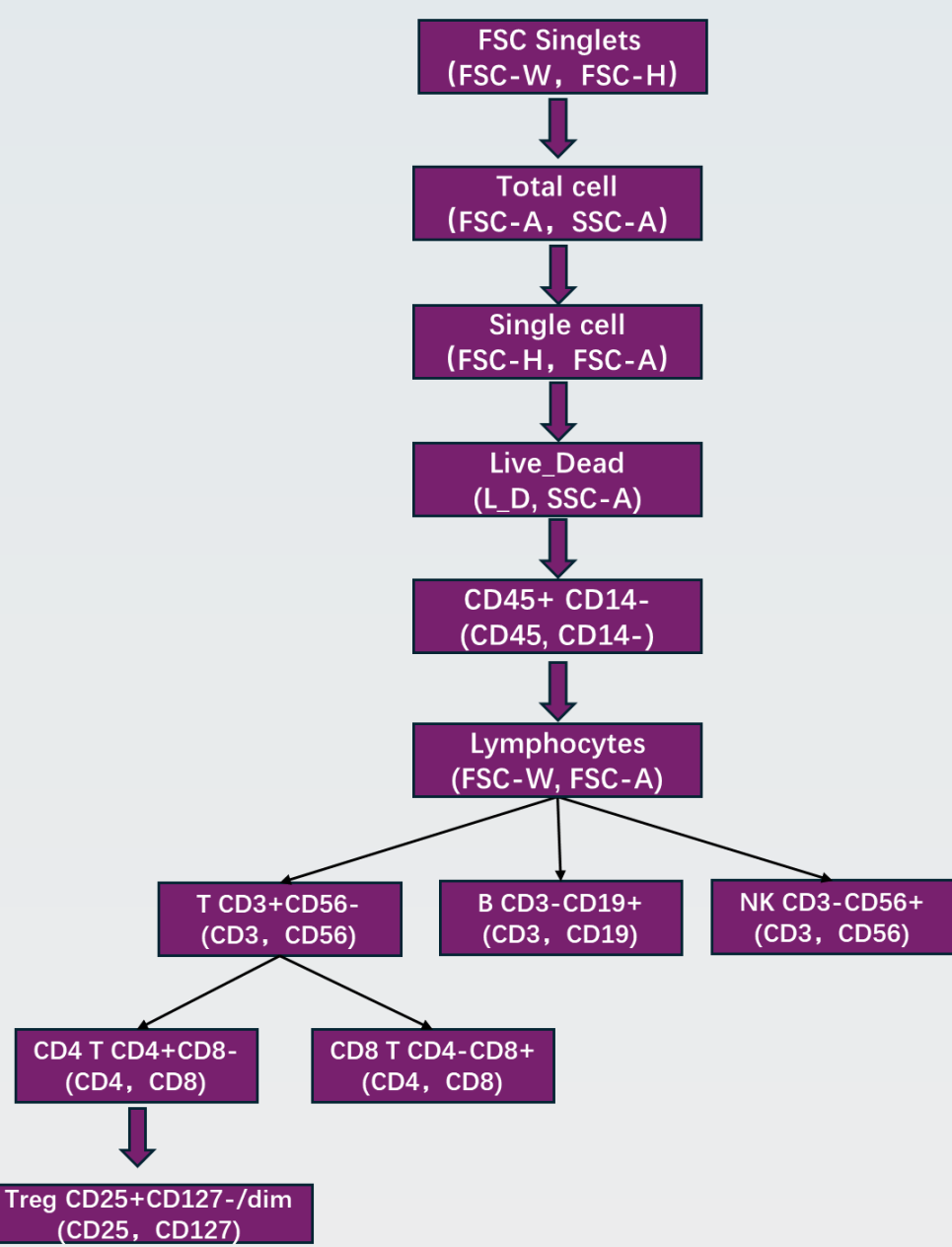


Figure 1. Analysis logic of lymphocyte population

Assay Results

Gating Strategy

As illustrated in Figure 2, lymphocyte subsets are analyzed in accordance with the gating logic depicted in Figure 1.

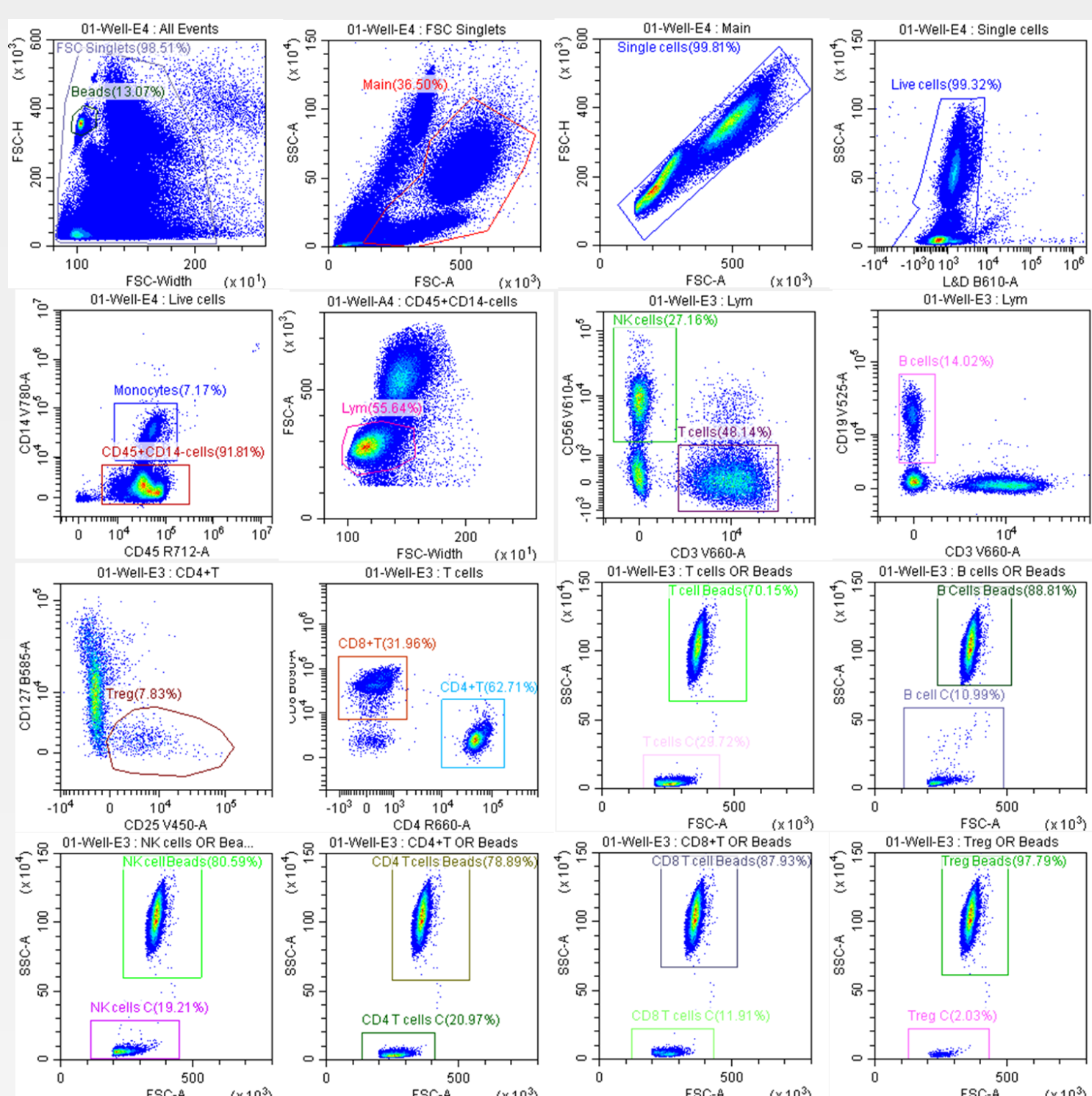


Figure 2. Gating strategy for the 10-color lymphocyte panel

Specificity

The specificity of the assay was evaluated using three individual human whole blood samples. The results indicated that the proportion of positive cell populations in the CD25 FMO and

CD127 FMO groups was less than 1%, as shown in Table 2. This finding is in accordance with the specificity acceptance criterion.

Table 2. CD25 and CD127 specificity

	Donor 1	Donor 2	Donor 3
CD127 Specificity	0.35%	0.06%	0.57%
	0.47%	0.08%	0.18%
	0.34%	0.08%	0.11%
CD25 Specificity	0.01%	0.05%	0.17%
	0.05%	0.10%	0.23%
	0.07%	0.05%	0.20%

Precision

The inter-batch precision was evaluated with 3 individual human whole blood samples and the results indicate that both the inter-batch precision of cell proportion and cell count meet the acceptance criterion that more than two-thirds of the samples have a coefficient of variation (%CV) ≤ 25 (Table 3&4).

Table 3. The inter-batch precision of lymphocyte proportion.

Cell subset	Donor 1	Donor 2	Donor 3
T	4.9	10.6	15.6
B	21.5	29.7	8.8
NK	33.1	11.1	17.2
CD4 T	13.4	16.2	23.3
CD8 T	7.1	12.3	18.2
Treg	2.3	9.0	10.3

Table 4. The inter-batch precision of lymphocyte absolute count.

Cell subset	Donor 1	Donor 2	Donor 3
T	5.9	8.2	5.1
B	15.4	28.9	10.5
NK	4.1	8.6	12.2
CD4 T	2.6	2.5	6.7
CD8 T	3.0	2.3	3.7
Treg	7.0	6.2	8.3

Stability

The stability was evaluated with 3 individual human whole blood sample at 2-8 °C and the results indicate that the stability of lymphocyte proportion detection can reach up to 96 hours, and for lymphocyte count stability, except for Treg which is 48 hours, the stability of other cell subsets can reach 72 hours (Table 5&6). The acceptance criterion for stability is that %RE of ≥ 2/3 of the samples is not greater than 30.

Table 5 Stability of lymphocyte proportion.

Cell subset	Sample	%RE 48 h	%RE 72 h	%RE 96 h
T	Donor 1	-6.8	-8.3	-13.2
	Donor 2	-10.0	-11.3	-14.4
	Donor 3	-6.4	-10.6	-13.1
B	Donor 1	13.7	6.7	-32.4
	Donor 2	1.4	-10.3	-25.9
	Donor 3	5.1	-6.4	-21.3
NK	Donor 1	11.5	7.6	13.3
	Donor 2	18.0	13.8	16.1
	Donor 3	-1.7	6.2	0.8
CD4 T	Donor 1	-5.3	-4.5	-3.9
	Donor 2	-9.7	-13.1	-14.6
	Donor 3	-9.9	-21.0	-24.4
CD8 T	Donor 1	6.4	9.6	12.2
	Donor 2	9.1	13.8	20.7
	Donor 3	5.4	14.0	18.2
Treg	Donor 1	7.3	-1.7	5.9
	Donor 2	30.0	13.4	19.3
	Donor 3	26.8	6.7	5.0

Table 6. Stability of lymphocyte absolute count.

Cell subset	Sample	%RE 48 h	%RE 72 h
T	Donor 1	-15.6	-19.5
	Donor 2	-29.1	-44.5
	Donor 3	-15.9	-9.7
B	Donor 1	27.7	19.8
	Donor 2	3.3	-11.8
	Donor 3	-14.9	-16.1
NK	Donor 1	1.0	-6.0
	Donor 2	-7.7	-28.7
	Donor 3	-10.8	-11.1
CD4 T	Donor 1	-20.0	-23.1
	Donor 2	-35.9	-51.6
	Donor 3	-24.1	-18.2
CD8 T	Donor 1	-10.4	-12.1
	Donor 2	-22.9	-36.9
	Donor 3	-11.3	-14.6
Treg	Donor 1	-13.8	-22.3
	Donor 2	-18.4	-43.7
	Donor 3	-2.5	-37.2

Conclusion

A multi-color flow cytometry method for lymphocytes immunophenotyping in whole blood samples has been developed and qualified. Given the satisfactory performance of the assay and the stability profiles of the samples, the method is suitable for analysis of samples in multi-center clinical trials.