Observational Study

Medicine

OPEN

Comparison of different combinations of antibodies and labeled fluorescein in the detection of lymphocyte subsets by flow cytometry

Yumei Ban, MD[a], Ming Zhao, MD[b], Meng Zhao, MD[c,*]

Abstract

Flow cytometry is a classical method for analyzing human peripheral blood lymphocyte subsets. This study aims to explore a new combination of antibody and labeled fluorescein for detecting lymphocyte subsets by comparing the effects of different combinations of antibody and labeled fluorescein in flow cytometry. We conducted a prospective study and enrolled 362 healthy patients undergoing physical examination in the medical examination center of the third hospital of hebei medical university. Venous blood was drawn from volunteers at the same time in the morning and divided into 3 tubes (Tube A, Tube B and Tube C). T lymphocytes were detected by 3-colors method (CD4-FITC/CD8-PE/CD3-PC5) in Tube A, B lymphocytes were detected by 2-colors method (CD19-FITC/CD3-PE) in Tube B, and T lymphocytes and B lymphocytes were detected by 4-colors method (CD4-FITC/CD8-PE/CD3-PC5/CD19-FITC) in Tube C. The repeatability and accuracy of the test scheme for Tube C shall not be inferior to that of Tube A and Tube B. There were no significant difference in the results of CD3 + and $CD4^{+}/CD8$ + between Tube A and C, as well as in the results of CD3 + and $CD19^{+}$ between Tube B and C. Pearson correlation analysis showed that the test results of a and C and B and C were highly correlated. The 4-colors method (CD4-FITC/CD8-PE/CD3-PC5/CD19-FITC) can detect T lymphocytes and B lymphocytes at the same time, reduce the use of fluorescence channels and save the detection cost, which is worthy of recommendation.

Abbreviations: FCM = Flow cytometry.

Keywords: combination, flow cytometry, lymphocyte subsets

1. Introduction

Flow cytometry (FCM) is a modern cell analysis technology integrating high and new technologies and methods such as computer technology, laser technology, electronic technology, hydrodynamics, cytochemistry and cellular immunology.[1,2] It has the advantages of simplicity, rapidity, many cell counts and high accuracy.[3] In recent years, with the popularization of flow cytometry, flow cytometry technology has attracted more and more clinical attention, especially lymphocyte subsets is the most common FCM test item in clinic.[4,5]

Lymphocytes are a very important group of cells in the immune system. According to their functions, sources and surface markers, they are mainly divided into T lymphocytes, B lymphocytes and natural killer cells (NK cells).[6] FCM analysis of lymphocyte subsets is a relatively new and complex technique.[7] During the experiment, there are many factors affecting the results of FCM analysis.[8] FCM started early and developed rapidly in developed countries. The Centers for Disease Control and Prevention and the Institute of clinical and laboratory standards introduced the standardized scheme of FCM lymphocyte subgroup analysis in the 1990s.[9] Since then, with the continuous upgrading of FCM instruments and reagents, these standardization schemes have been further improved. At present, there is no recognized standardized scheme for FCM lymphocyte subgroup analysis in China, and FCM analysis is still in the stage of learning, exploration and integration in China.

With the rapid development of FCM detection technology, the matching schemes of antibody and fluorescent dye emerge 1 after another and are becoming more and more perfect, however, the cost of consumables is an important constraint to the

Scientific Research Foundation of Hebei Health Commission (No. 20181101).

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

This study was approved by the institutional review board of the the third hospital of Hebei Medical University in compliance with the Helsinki and declaration and consent were waived for its retrospective nature.

[a] Shijiazhuang Railway Institute for Disease Control and Prevention, China Railway Beijing Group Co.,Ltd., Shijiazhuang, Hebei, China, [b] Department of Bone Oncology, The Third Hospital of Hebei Medical University, Shijiazhuang, Hebei, China, [c] Department of General Laboratory,The third hospital of Hebei Medical University, Shijiazhuang, Hebei, China.

** Correspondence: Meng Zhao, Department of General Laboratory, the Third Hospital of Hebei Medical University, NO.139, Ziqiang Road, Shijiazhuang City, Hebei Province 050051, China (e-mail: sunnyday2277@sina.com).*

How to cite this article: Ban Y, Zhao M, Zhao M. Comparison of different combinations of antibodies and labeled fluorescein in the detection of lymphocyte subsets by flow cytometry. Medicine 2022;101:48(e31550).

Received: 19 July 2022 / Received in final form: 2 October 2022 / Accepted: 5 October 2022

http://dx.doi.org/10.1097/MD.0000000000031550

popularity of FCM.[10] At present, the standardized detection schemes in clinical application are 1 antibody combined with 1 fluorescent dye for detection, that is, each detection index should monopolize a fluorescent dye detection channel of flow cytometry. In lymphocyte detection, B lymphocytes and T lymphocytes need to be detected separately with their own antibodies. However, there is no report on the scheme of simultaneously labeling antibodies on different lymphocyte subsets with the same dye, so as to simultaneously detect 2 indicators in a fluorescent dye detection channel.

Given that, based on the structure and principle of flow cytometers and the distribution characteristics of lymphocyte antigens on the surface of lymphocyte subsets based on the structure and principle of flow cytometers and the distribution characteristics of lymphocyte antigens on the surface of lymphocyte subsets, the purpose of this study is to explore a new scheme for flow cytometers to identify lymphocyte subsets, leading to a reduction in the number of operations, a reduction in the error rate of operations, and a reduction in the cost of performing these operations.

2. Materials and methods

This prospective study was designed in accordance with the principles outlined in the declaration of Helsinki. This study has been approved by the institutional review board of the third hospital of Hebei medical university and all participants have signed informed consent forms.

2.1. Inclusion and exclusion criteria

Between June 2018 and June 2021, healthy people from the medical examination center of the Shijiazhuang Railway Institute for Disease Control and Prevention were collected. The inclusion criteria were as follows: Participants were asked to fasting before blood drawing; Participants have signed informed consent and are willing to participate in this clinical study. The exclusion criteria were as follows: The participants had severe hepatic and renal insufficiency; Participants suffer from infectious diseases such as colds, pneumonia, etc.; Participants have diseases that affect lymphocytes, such as malignant tumors.

2.2. Quality control

The detection system was optimized according to the method suggested by Porwit MacDonald A[11]: A double-distilled water was used to prepare all buffer solutions, 1 solution, erythrocyte lysate, and paraformaldehyde fixative. The flow cytometer's sheath tank should be cleaned with distilled water. Each measurement was performed using the particulate free cleaning solution (0.07moL/L Nacl, 0.05moL/L NAOH) filtered by the 0.2M filter and the particulate free sheath solution for 20 minutes before the measurements. With distilled water that has been filtered and is not particulate, run it for 30 minutes. As a next step, use filtered particle free PBS as the sample to detect background particles until no particles are detected above the detection threshold, so as to remove the cells and particles left in the previous experiment. With the sample without fluorescent label, you can remove spontaneous fluorescence influence, while the fluorescent labeled antibody (CD4-FITC/CD8-PE/CD3-PC5 or CD19-FITC/ CD3-PE or CD4-FITC/CD8-PE/CD3-PC5/ CD19-FITC) to exclude the influence of nonspecific fluorescence staining.

2.3. Inspection process

At 8 o'clock every morning, 6 ml of peripheral venous blood was drawn from the participants and divided into 6 tubes (Tube A, Tube A1, Tube B, Tube B1, Tube C, Tube C1) on average. It was quickly stored in sodium citrate anticoagulant tube and sent for examination within 1 hour. Tube A and A1 were tested for T lymphocytes by conventional 3-colors method (CD4-FITC/ CD8-PE/CD3-PC5), Tube B and B1 were tested for B lymphocytes by conventional 2-color method (CD19-FITC/ CD3-PE), Tube C and C1 were tested for T lymphocytes and B lymphocytes by 4-colors method (CD4-FITC/CD8-PE/CD3-PC5/ CD19-FITC), and blood samples were tested by flow cytometry (Epics XL, Beckman Couler, USA). All anti-bodies were purchased from Beckman Couler.

Take heparin anticoagulant bone marrow fluid 50 to 100 μl. According to the above antibody combination scheme, add 20μl of each of the 3 fluorescent labeled antibodies, incubate in the dark at room temperature for 15 minutes, and then add 5ml of red blood cell lysate [NH4HCO3 (1moL/L) and 50ml of Nh4CL (1.44moL/L) to 500mL] to incubate in a dark environment for 5 to 10 minutes. Then, wash it twice with phosphate buffer solution (PBS-BSA) containing 0.2% fetal bovine serum, and add 0.5mL of 1% paraformaldehyde fixative (Batch NO.30525-89-4, MACKLIN Inc.) for detection.

Set gates with FS and SS, and test Tube A, B and C respectively with Tube A1, B1 and C1 as the same type control. The test result of Tube A is the data of T lymphocyte subsets (Fig. 1A-D), the test result of Tube B is the data of B lymphocytes (Fig. 2A-C), and the test result of Tube C is the results of T lymphocyte subsets and B lymphocytes obtained simultaneously after merging tubes A and B (Fig. 3). The C-gate C2 quadrant in Figure 3C obtains the results of CD3 + CD4 + T lymphocyte subsets, and the C1 quadrant obtains the results of B lymphocytes. The results of CD3 + lymphocyte, CD4 + and CD8 + lymphocyte test and gate setting were shown in Figure 3A, B, D.

2.4. Repeatability and accuracy test

The repeatability of the 3 schemes was investigated by coefficient of variation (CV); Lymphocyte subpopulation standardization protocol; The average value of lymphocyte subsets detected many times by standardized scheme was used as the target value, and the accuracy was evaluated by relative average deviation. Take a sample at random and operate it 20 times according to the above steps. Test the data on the computer for statistical processing to obtain CV value and relative average deviation. If CV value is < 10%, it is considered that the repeatability is good; The relative average deviation is used to evaluate the accuracy. If the relative average deviation is < 10%, it is considered that the accuracy verification is passed.

2.5. Statistical analysis

All statistical analysis was performed with the Statistical Package for Social Sciences software (version 23.0; SPSS Inc., Chicago, IL). The continuous data were expressed as mean ± standard deviation (SD) or median (interquartile range, IQR). Group *t* test was used for comparison between groups. P values lower than 0.05 were interpreted as statistically significant in all the statistical analysis model. The correlation of the 2 groups of data was analyzed by Pearson correlation analysis. The correlation coefficient *R* value > 0.8 was considered to be highly correlated; The consistency of the 2 methods was analyzed by intraclass correlation coefficient (ICC) of single factor random effect model and Bland Altman analysis. The ICC value above 0.8 was considered to have good consistency. The ICC value between 0.61 and 0.80 was medium, between 0.41 and 0.60 was general, between 0.1 and 0.40 was low, and below 0.1 was no consistency.

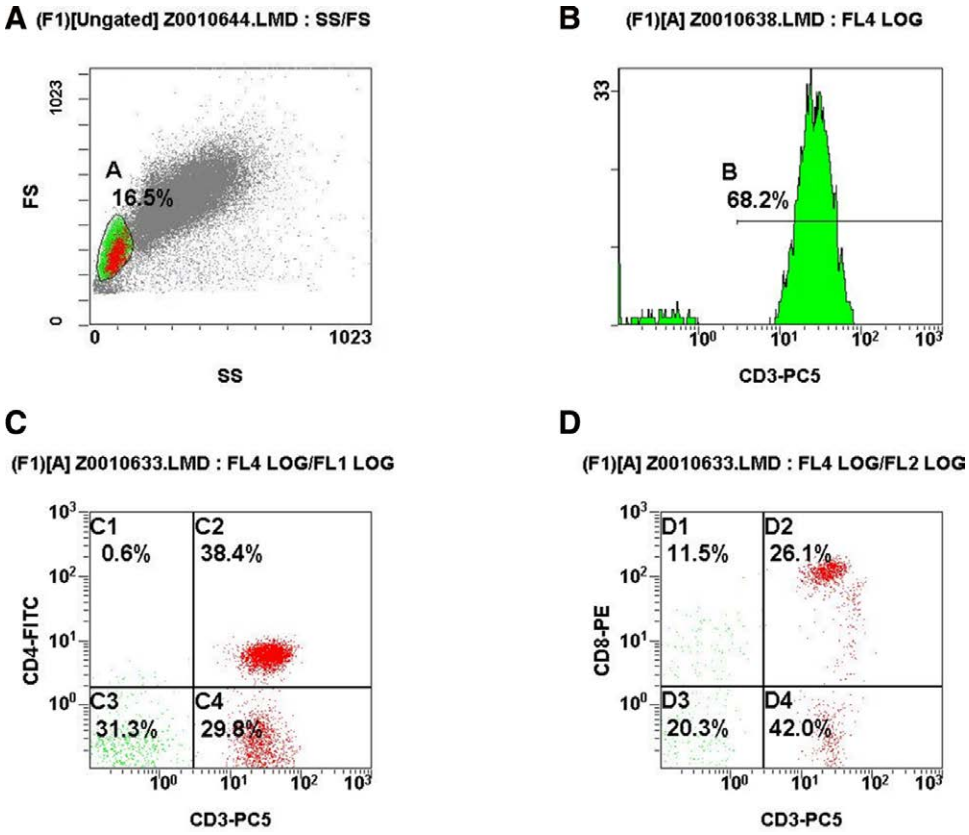

Figure 1. CD4-FITC/CD8-PE/CD3-PC5 staining flow cytometry results. (A) FS/SS gated lymphocytes; (B-D) T lymphocyte subsets test results.

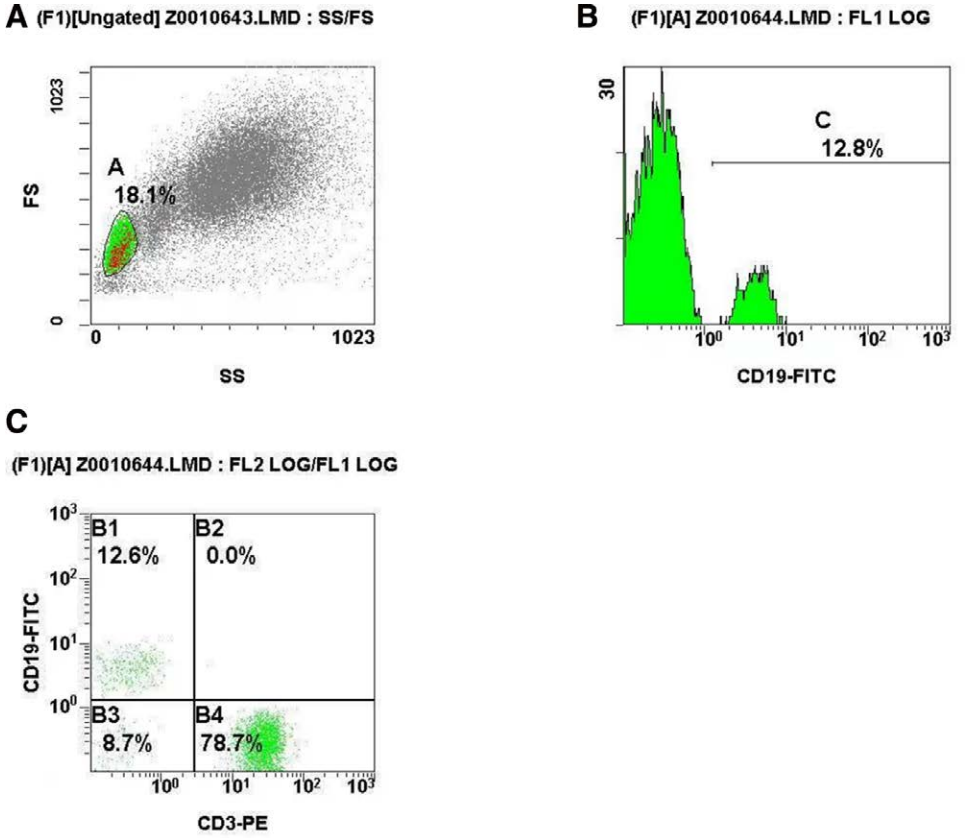

Figure 2. CD19-FITC staining flow cytometry results. (A) FS/SS gated lymphocytes; (B-C) CD19$^+$ lymphocyte test results.

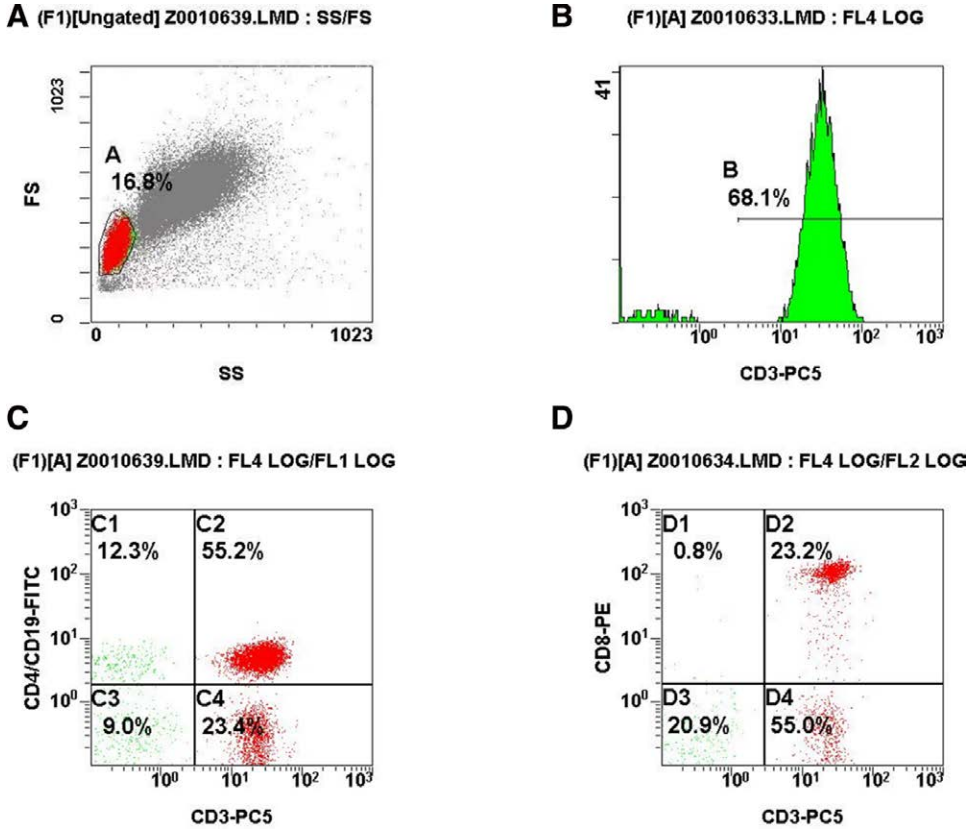

Figure 3. Co-staining flow cytometry results of CD4-FITC/CD8-PE/CD3-PC5 and CD19-FITC. (A) FS/SS gated lymphocytes; (B) CD3 + lymphocyte test results; (C) $CD4^+$, $CD3^+$ and $CD19^+$ lymphocyte test results; (D) $CD4^+$ and $CD8^+$ lymphocyte test results.

3. Results

A total of 362 patients were enrolled in this study. There were 178 males and 184 females, with a mean age of 41.6 ± 9.9 years (range 25–63 years) and a mean Body mass index of 26.9 ± 3.2 kg/m^2 (range 23.2–33.7 years).

A sample is divided into 20 pieces and dyed on the computer. The CV value and the relative average deviation are calculated from the results. The results showed that the CV value and relative average deviation of each index from test method of tube A, B and C are < 10%, and the difference is small, suggesting that the repeatability of the test method of tube C is not worse than that of tube A and tube B and the repeatability of the 3 schemes meets the expectation and the accuracy meets the laboratory requirements. The detailed information is presented in Table 1-2.

There was no significant difference in T lymphocyte count between tube A and tube C [CD3 + (tube A:73.06 ± 9.97, tube C:72.64 ± 10.11, t = 0.606, P = .579); $CD4^+/CD8^+$ (tube A:1.69 ± 0.83, tube C:1.73 ± 0.89, t = 0.581, P = .613)] and there was no significant difference in B lymphocyte count between tube B and tube C [CD3 + (tube B:73.06 ± 9.97, tube C:72.64 ± 10.11, t = 0.622, P = .694); CD19 + (tube B:9.97 ± 5.11, tube C:10.02 ± 5.43, t = 0.703, P = .756)].

Pearson correlation analysis was used to analyze the correlation of CD3 + and $CD4^+/CD8^+$ between tube A and C, and the correlation of CD3 + and CD19 + between tube B and C. The results showed that the correlation coefficients were > 0.95 and the P values were < 0.01. The slopes of linear regression equations were all in the range of 1.00 ± 0.03. The detailed information is presented in Table 3-4. As shown in Figure 4, Pearson correlation analysis scatter diagram shows that the indexes of tube A and tube C and tube B and tube C are highly correlated and positively correlated.

4. Discussion

Currently, with the rapid development of flow cytometry technology, combination of antibodies and fluorescent dyes matching scheme were also emerging and improving. However, in each scheme, 1 antibody and fluorescent dye was established. When lymphocyte flow was conducted, it is often necessary to detect B and T lymphocyte separately, or different fluorescent dyes were consumed in 1 detection. Scheme of labeling the

Table 1

Comparison of repeatability results for T lymphocytes between tube A and C (n = 362).

	Tube A			Tube C		
	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^+$	$CD4^+$	$CD8^+$
mean value(%)	71.95	37.61	21.82	72.09	36.88	21.46
standard deviation(%)	0.54	1.27	0.88	0.82	1.20	0.73
CV value(%)	0.79	3.77	3.98	1.16	3.55	3.43
Relative mean deviation	0.67	2.83	2.97	0.91	2.64	2.48

Table 2

Comparison of repeatability results for B lymphocytes between tube B and C (n = 362).

	Tube B		Tube C	
	$CD3^+$	$CD19^+$	$CD3^+$	$CD19^+$
mean value(%)	71.94	10.72	72.09	11.03
standard deviation(%)	0.76	0.66	0.82	0.58
CV value(%)	1.02	5.47	1.16	4.96
Relative mean deviation	0.83	5.28	0.91	4.91

Table 3

Comparison of detection result for correlation analysis between tube A and C (n = 362, $\chi \pm s$).

	r-value	P-value	Linear regression equation
$CD3^+$	0.971	<0.001	Y = 0.9874X + 0.0084
$CD4^+/CD8^+$	0.986	<0.001	Y = 1.0002X-0.0049

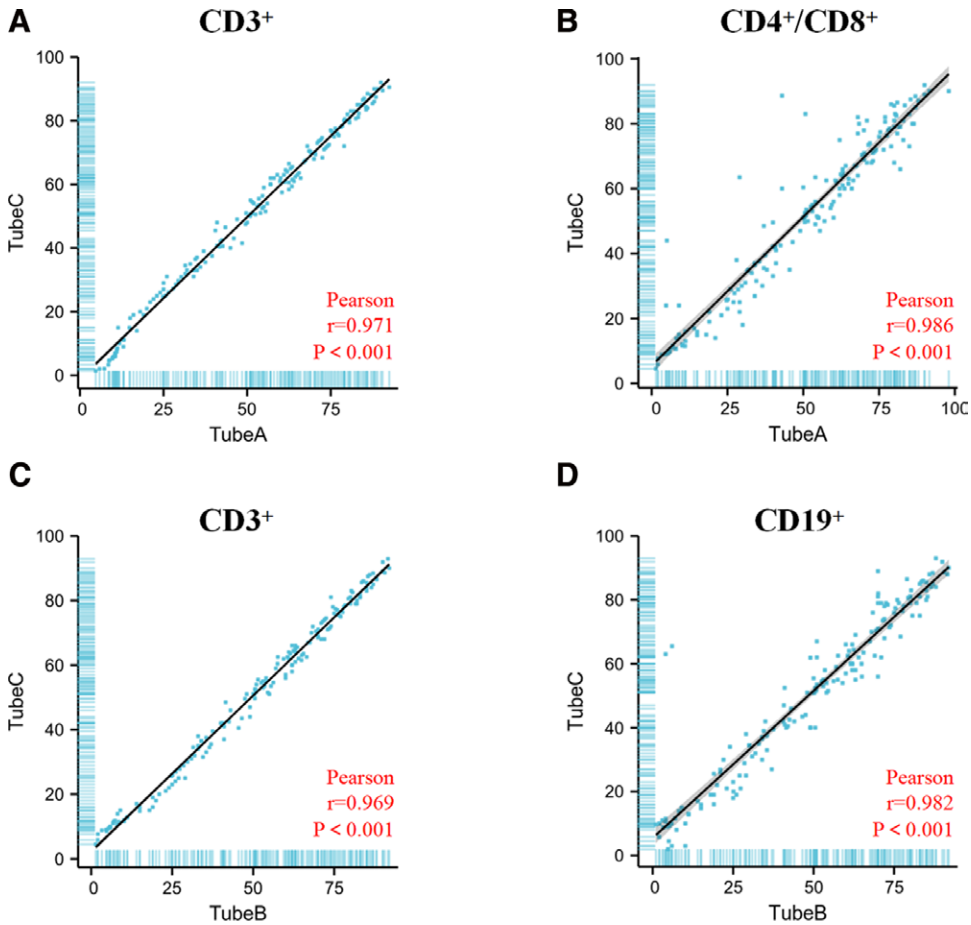

Figure 4. Pearson correlation analysis scatter diagram. Pearson correlation analysis scatter diagram between Tube A and Tube C for $CD3^+$ (A) and $CD4^+/CD8^+$ (B), and between Tube B and Tube C for $CD3^+$ (C) and $CD19^+$ (D). Tube A: CD4-FITC/CD8-PE/CD3-PC5; Tube B: CD19-FITC/ CD3-PE; Tube C:CD4-FITC/ CD8-PE/CD3-PC5/CD19-FITC.

Table 4

Comparison of detection result for correlation analysis between tube B and C (n = 362, $\chi \pm s$).

	r-value	P-value	Linear regression equation
$CD3^+$	0.969	<0.001	Y = 1.0029X-0.0113
$CD19^+$	0.982	<0.001	Y = 0.9972X + 0.0022

same fluorescent dye with antibodies of different cells has not been reported widely. The purpose of this study is to design a new detection scheme based on the distribution characteristics of lymphocyte surface antigens, analyze lymphocyte subsets with different combinations of fluorescein and antibody reagents of FCM, and observe the influence of the results, in order to provide strong evidence for the establishment of a new scheme for FCM lymphocyte subsets analysis. The introduction of multi-color flow cytometry for the identification and quantification of distinct cell lineages, commonly known as immunophenotyping, has proven to be a powerful tool in both clinical and research settings. Our results showed that the 4-colors method can detect T lymphocytes and B lymphocytes at the same time. Compared with the traditional antibody combination, the results obtained by the new antibody combination method (CD4-FITC/CD8-PE/CD3-PC5/CD19-FITC) have no significant difference.

At present, there are many FCM antibody combinations for detecting lymphocyte subsets, and the results are different.[12] However, whether this difference is statistically significant has not been reported. In this paper, 2 different antibody combinations were selected to detect B and T lymphocyte subsets respectively. In order to verify the reliability of the results, T lymphocyte subsets were also detected with 4-colors labeled antibodies. Results of this present study shows that it is feasible to assign a fluorescent dye detection channel to 2 specific antibodies in the detection of lymphocyte subsets.

Three-colors method adopts the antibody combination form of CD4-FITC/CD8-PE/CD3-PC5, this method can make use of the technical advantages of FCM to analyze the expression of 3 differentiation antigens of single cells at the same time, so that people can study the expression of differentiation antigens on the cell surface and cytoplasm more finely and deeply. It is a classic antibody combination for detecting the content of T lymphocytes by FCM.[13]

CD3 molecules are expressed on the surface of mature T lymphocytes, that is, lymphocytes expressing CD3 molecules are T lymphocytes, and T lymphocytes do not express CD19 molecules.[14] However, CD4 and CD8 cannot be expressed on the surface of mature T lymphocytes at the same time, so mature T lymphocytes can be divided into CD4 + T cells and CD8 + T cells.[15] CD19 was expressed on the surface of mature B lymphocytes, but CD3 was not expressed. Who defines CD3 negative CD16 or CD56 positive lymphocytes as NK cells. Because there are very few CD3-CD4 + cells in the population, and B cells do not express CD3, cd19-fitc is added to the reagent CD4-FITC/CD8-PE/CD3-PC5, so that both PC5 fluorescent dye and FITC fluorescent dye positive cells are CD3 + CD4 + cells, PC5 fluorescent dye negative and FITC fluorescent dye positive cells are B lymphocytes, so as to achieve the purpose of simultaneous detection of T cells and B cells. Similarly, add CD (16 + 56) - FITC to reagent CD4-FITC/CD8-PE/CD3-PC5, so that PC5 and FITC positive cells are CD3 + and CD4 + cells, PC5 negative and FITC positive cells are NK lymphocytes, which can also achieve the purpose of simultaneous detection of T lymphocytes and NK cells.

It is undeniable that there are still some limitations in this study. First of all, sample size of our investigation was related small and selection bias was inherited; at the same time, random error was inevitable owing to the manual experiment operation during conduction of the present study. Secondly, the typing of lymphocyte subsets would be influenced by a variety of technical influences which including the duration and temperature of sample storage and the method applied for staining samples, therefore, the finding of this study may compromised in clinical application however, our novel method still has the characteristics of broad applicability under the conventional experimental conditions.

5. Conclusion

In summary, our data suggest 4-colors method (CD4-FITC/CD8-PE/CD3-PC5/CD19-FITC) can detect T lymphocytes and B lymphocytes at the same time, reduce the use of fluorescence channels and save the detection cost, which is worthy of recommendation.

Acknowledgements

The authors would like to thank all the members of medical examination center and department of clinical Laboratory the third hospital of Hebei Medical University for their great help and support.

Author contributions

Conceptualization: Yumei Ban.
Data curation: Yumei Ban.
Formal analysis: Yumei Ban.
Funding acquisition: Meng Zhao.
Investigation: Yumei Ban.
Methodology: Ming Zhao.
Project administration: Ming Zhao.
Resources: Ming Zhao.
Software: Ming Zhao.
Supervision: Ming Zhao.
Validation: Meng Zhao.
Visualization: Meng Zhao.
Writing – original draft: Meng Zhao.
Writing – review & editing: Meng Zhao.

Reference

[1] McKinnon KM. Flow cytometry: an overview. Curr Protoc Immunol. 2018;120:5.1.1–5.1.11.
[2] Maciorowski Z, Chattopadhyay PK, Jain P, et al. Basic multicolor flow cytometry. Curr Protoc Immunol. 2017;117:5.4.1–5.4.38.
[3] Lambert C, Sack U. Monocytes and macrophages in flow cytometry. Cytometry B Clin Cytom. 2017;92:178–9.
[4] Louati N, Rekik T, Menif H, et al. Blood lymphocyte T subsets reference values in blood donors by flow cytometry. Tunis Med. 2019;97:327–34.
[5] Njemini R, Onyema OO, Renmans W, et al. Shortcomings in the application of multicolour flow cytometry in lymphocyte subsets enumeration. Scand J Immunol. 2014;79:75–89.
[6] Larosa DF, Orange JS. 1. Lymphocytes. J Allergy Clin Immunol. 2008;121(2 Suppl):S364–9; quiz S412. quizS412.
[7] Gascue A, Merino J, Paiva B, et al. Flow cytometry. Hematol Oncol Clin North Am. 2018;32:765–75.
[8] Givan AL. Flow cytometry: an introduction. Methods Mol Biol. 2004;263:1–32.
[9] Schwartz A, Fernández-Repollet E, et al. Development of clinical standards for flow cytometry. Ann N Y Acad Sci. 1993;677:28–39.
[10] Makowski EK, Wu L, Desai AA, et al. Highly sensitive detection of antibody nonspecific interactions using flow cytometry. MAbs. 2021;13:1951426.
[11] Porwit-MacDonald A, Björklund E, Lucio P, et al. BIOMED-1 concerted action report: flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of T cell acute lymphoblastic leukemia (T-ALL). Leukemia. 2000;14:816–25.
[12] Autissier P, Soulas C, Burdo TH, et al. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A. 2010;77:410–9.
[13] Park J, Han K. Single-color multitarget flow cytometry using monoclonal antibodies labeled with different intensities of the same fluorochrome. Ann Lab Med. 2012;32:171–6.
[14] Mateus J, Lasso P, González JM, et al. Design of a multicolor panel to assess intracellular and surface molecules by flow cytometry. Biomedica. 2013;33:660–72.
[15] Ginaldi L, Farahat N, Matutes E, et al. Differential expression of T cell antigens in normal peripheral blood lymphocytes: a quantitative analysis by flow cytometry. J Clin Pathol. 1996;49:539–44.