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Three-Color Supplement to the NIAID DAIDS Guideline for Flow Cytometric Immunophenotyping

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Since the publication of the ''NIAID DAIDS Guideline for Flow Cytometric Immunophenotyping'' (1) in 1993, significant scientific and technological advances in the development and production of reagents, instrumentation, and software have permitted widespread access to multicolor flow cytometry in both research and clinical laboratories. As is often the case with complex new technologies, each advance, while opening up exciting new capabilities, can also bring new sources of variability and a commensurate increase in the requirements for quality assurance at all levels. The purpose of this document is to update the 1993 NIAID DAIDS Guideline with new recommendations designed to help standardize the methodology and minimize measurement variability in determining patients' CD4 and CD8 T-cell counts using 3-color flow cytometry. Issues pertaining to specimen collection and handling, problematic specimens, hematology, and laboratory performance assessment were addressed in the previous guideline.[1] In assembling this supplement, factors such as reduced technician time, decreased need for isotype controls, fewer assay tubes, and the potential for diminished cost were considered advantages of 3-color (vs. 2-color) flow cytometry. In contrast, the increased complexity of factors such as spectral compensation, instrument set up, and data collection/analysis were considered disadvantages. In addition, some antibody combinations are not commercially available, and certain third-color fluorochromes may not be optimal with all flow cytometers. The specifications and recommendations contained herein were developed for use in laboratories that support clinical trials and epidemiologic studies done under the auspices of the National Institute of Allergy and Infectious Diseases, Division of AIDS (NIAID DAIDS). Efforts currently underway by the Centers for Disease Control and Prevention and by the National Committee for Clinical Laboratory Standards (NCCLS) will likely provide additional guidance in this rapidly changing arena.

5.[1] 3-COLOR IMMUNOPHENOTYPING

5.01. 3-Color Monoclonal Antibody Panel

There are several commercially available combinations of antibodies (premixed) and several theoretical combinations of antibodies. When not using commercially premixed combinations of antibodies, the laboratory is advised to institute proper quality control for the reagent combinations in use. Several 3-color panels have been proposed for the routine measurement of lymphocyte subsets, each with its own advantages and disadvantages in gating, internal quality control, and efficiency. Any 3-color panel chosen by a laboratory should include a CD3/CD4 combination and a CD3/CD8 combination, so that the dual positive $CD3^+CD4^+$ and $CD3^+CD8^+$ cell percentages can be measured and reported.

A CD3/CD4/CD8 tube alone is not recommended (see Section 5.02).

The NIAID DAIDS Flow Cytometry Advisory Committee recommends the following minimum 3-color panel:

Monoclonal antibody combination	Cell type enumerated
1. CD3/CD4/CD45	T cells, $CD4^+$ T cells
2. CD3/CD8/CD45	T cells, $CD8^+$ T cells
3. CD3/CD19/CD45[a]	T cells, B cells

[a]See 5.01b.

The advantages of this panel are:

a. lymphocytes are easily distinguished based on CD45 fluorescence and 90° (side) scatter;
b. replicate CD3 determinations ensure reproducibility between tubes;
c. an isotype control is not needed (see Section 5.03a);
d. gating on ''bright'' CD45 for each tube helps ensure

[1]Section numbering system is designed to maintain continuity with the 1993 ''NIAID DAIDS Guidelines for Flow Cytometric Immunotyping'' (1).

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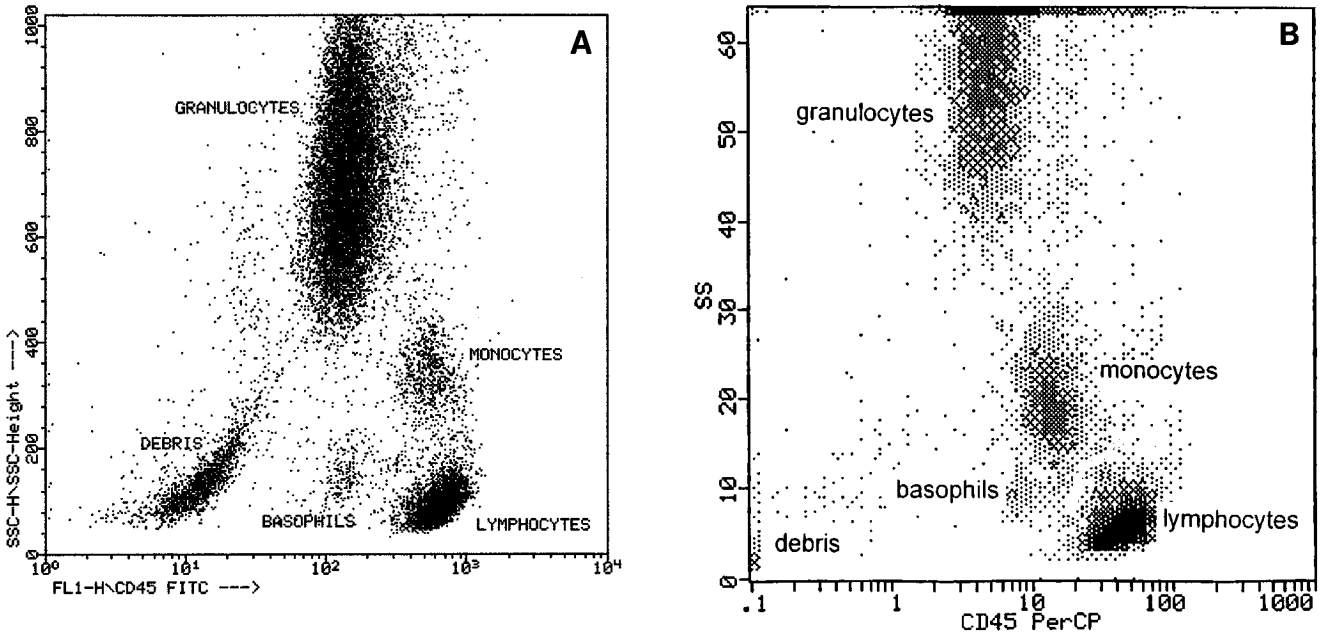

FIG. 1. **A:** FACScan™ Flow Cytometer (BDIS, San Jose, CA). **B:** EPICS® XL Flow Cytometer (Coulter Corporation, Hialeah, FL). Dual-parameter histograms of side scatter and CD45 fluorescence. Ungated displays show lymphocytes, granulocytes, monocytes, basophils, and debris.

inclusion of only lymphocytes, thus eliminating the need to correct for gate purity (see Sections 5.02b, 5.04); and

e. many nonlymphocyte contaminants of a light scatter gate (e.g., unlysed red blood cells) can be easily excluded from a CD45/side-scatter gate.

The disadvantages of this panel are:

a. in drawing the gate, distinction among natural killer (NK) cells, monocytes, B cells, and debris may not always be clear-cut; and
b. lymphocyte recovery cannot be determined (see Section 5.02a).

5.01a. For all NIAID DAIDS *adult* studies, it is mandatory to report $CD4^+$ and $CD8^+$ T cells, and it is mandatory that these be measured as $CD3^+CD4^+$ cells and $CD3^+CD8^+$ cells, respectively.

5.01b. For all NIAID DAIDS *pediatric* studies, in addition to reporting $CD4^+$ and $CD8^+$ T cells as above, it is also mandatory to report total B cells, measured as $CD19^+$ cells.

5.01c. Except for pediatric studies, NIAID DAIDS studies do not require inclusion of markers for B cells and for NK cells. However, these markers can be useful in accounting for additional cell populations for quality control purposes. For 3-color NK analysis, it is recommended that laboratories stain with CD56 and CD16 by using the same fluorochrome for both combined with CD3 and CD45.

5.02. Lymphocyte Gating

Lymphocyte gates are set suing by linear 90° side-scatter and log CD45 fluorescence. Lymphocytes are defined as $CD45^{bright}$ with low side scatter (Fig. 1) (2).

Other lymphocyte/fluorescent gating strategies have been proposed and may prove to be equally valid.

5.02a. Lymphocyte recovery. The proportion of all lymphocytes (T, B, and NK cells) present in the specimen and contained within the boundaries of the lymphocyte gate cannot be determined by using the panel outlined above. To determine lymphocyte recovery (T, B, and NK cells), a panel including tubes for B and NK cell determinations is needed (3).

5.02b. Lymphocyte purity. The presence of nonlymphocytes within the boundaries of the lymphocyte gate is assumed to be negligible. By using low side scatter and $CD45^{bright}$ fluorescence for identification of lymphocytes, an assumption is made that the only cells meeting this criteria are lymphocytes, therefore the lymphocyte purity of the gate is close to 100%.

5.03. Enumeration of Lymphocyte Subsets

Fluorescence data should be displayed as a histogram of log fluorescence, as outlined in the guidelines, Section 3.09a(1).

5.03a. Cursor settings for data analysis. An isotype control is not included in this monoclonal antibody panel. The unlabeled (negative) populations in each tube serve in

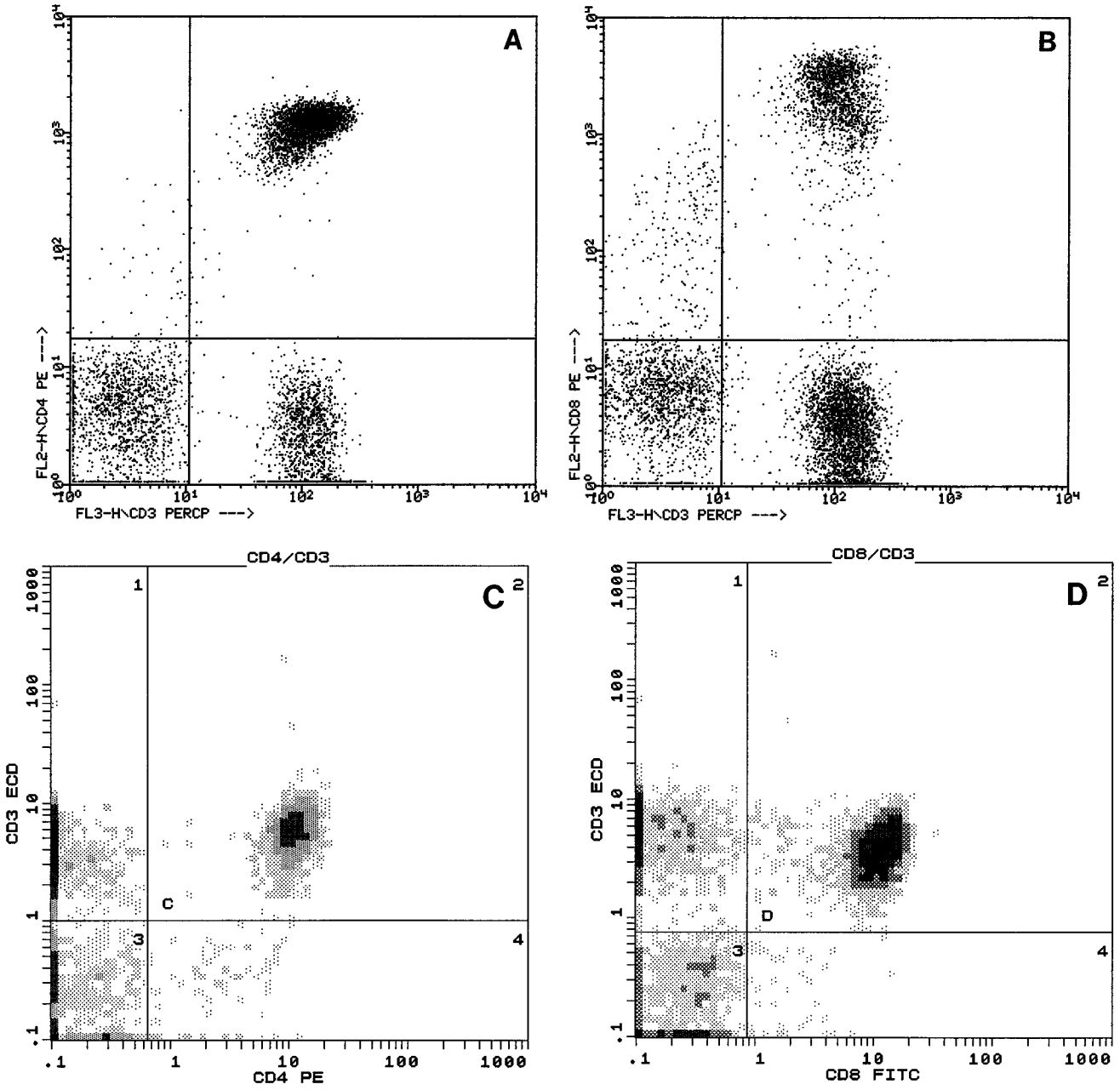

FIG. 2. Dual-parameter histograms generated from a CD45-side scatter gate, showing the fluorescence distributions of specimens stained with the CD3 and CD4 (**A** and **C**); CD3 and CD8 (**B** and **D**). Cursors were positioned based on the unlabeled cells in the histograms. (Note: CD3 displayed on X-axis in FACScan™ histograms; Y-axis in EPICS™ XL histograms.) A and B: FACScan™ Flow Cytometer (BDIS, San Jose, CA). C and D: EPICS™ XL Flow Cytometer (Coulter Corporation, Hialeah, FL).

the same capacity as an isotype control. Cursor settings are determined by the fluorescence patterns from the negative and positive populations for CD3, CD4, and CD8. Because both CD3 and CD4 label cells brightly, the cutoff between the negative and positive populations is easily determined. The cursor placement from the CD3/CD4 tube may be used for the CD3/CD8 tube (Fig. 2).

5.03b. Color compensation. A standard subtraction/compensation protocol to correct the spectral overlap of one fluorochrome into the fluorescence spectrum of another should be established for 3-color analysis (4, 5).

5.04. Corrections of Lymphocyte Subset Percentages

Because an assumption is made that 100% of the cells within the gate are lymphocytes, no correction is needed for the lymphocyte subset percentages.

5.05. Reporting of Values

Cell type enumerated	Value reported
CD4 T cells	$CD3^+CD4^+$ percent
CD8 T cells	$CD3^+CD8^+$ percent
CD3 T cells	Mean percentage obtained for $CD3^+$ cells (i.e., single- and dual-positive) from all tubes that include CD3

5.06. Switching From 2-Color to 3-Color Immunophenotyping

As stated in the introduction, there are many reasons for switching from 2-color to 3-color immunophenotyping. When a laboratory considers a change in procedures, there is need to document the effect (or lack of effect) that this will have on laboratory values. NIAID DAIDS laboratories seeking approval for implementation of any 3-color panel must demonstrate equivalence of $CD3^+CD4^+$ and $CD3^+CD8^+$ values measured by the proposed 3-color method versus those measured by the laboratory's current 2-color method. Laboratories are required to evaluate a minimum of 60 different, sequential patient specimens with $CD3^+CD4^+ \leq 30\%$ (by 2-color). The design for a 2-color/3-color comparison is available from the corresponding author.

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