

Fluorescence-Intensity Multiplexing: Simultaneous Seven-Marker, Two-Color Immunophenotyping Using Flow Cytometry

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Background: Conventional immuno-based multiparameter flow cytometric analysis has been limited by the requirement of a dedicated detection channel for each antibody-fluorophore set. To address the need to resolve multiple biological targets simultaneously, flow cytometers with as many as 10–15 detection channels have been developed. In this study, a new Zenon immunolabeling technology is developed that allows for multiple antigen detection per detection channel using a single fluorophore, through a unique method of fluorescence-intensity multiplexing. By varying the Zenon labeling reagent-to-antibody molar ratio, the fluorescence intensity of the antibody-labeled cellular targets can be used as a unique identifier. Although demonstrated in the present study with lymphocyte immunophenotyping, this approach is broadly applicable for any immuno-based multiplexed flow cytometry assay.

Methods: Lymphocyte immunophenotyping of 38 clinical blood specimens using CD3, CD4, CD8, CD16, CD56, CD19, and CD20 antibodies was performed using conventional flow cytometric analysis and fluorescence-intensity multiplexing analysis. Conventional analysis measures a single antibody-fluorophore per photomultiplier tube (PMT). Fluorescence-intensity multiplex analysis simultaneously measures seven markers with two PMTs, using Zenon labeling reagent-antibody complexes in a single tube: CD19, CD4, CD8, and CD16 antibodies labeled with

Zenon Alexa Fluor[®] 488 Mouse IgG₁ labeling reagent and CD56, CD3, and CD20 antibodies labeled with Zenon R-Phycoerythrin (R-PE) Mouse IgG₁ or IgG_{2b} labeling reagents.

Results: The lymphocyte immunophenotyping results from fluorescence-intensity multiplexing using Zenon labeling reagents in a single tube were comparable to results from conventional flow cytometric analysis.

Conclusions: Simultaneous evaluation of multiple antigens using a single fluorophore can be performed using antibodies labeled with varying ratios of a Zenon labeling reagent. Labeling two sets of antibodies with different Zenon labeling reagents can generate characteristic and distinguishable multivariate patterns. Combining multiple antibodies and fluorescent labels with fluorescence intensity multiplexing enables the resolution of more cellular targets than detection-channels, allowing sophisticated multiparameter flow cytometric studies to be performed on less complex 2- or 3-detection-channel flow cytometers. For typical biological samples, approximately 2–4 cellular targets per detection channel can be resolved using this technique. © 2004 Wiley-Liss, Inc.

Key terms: fluorescence-intensity multiplexing; molar ratio; Zenon reagent labeling; fluorophore; multivariate pattern

The trend in multicolor flow cytometric analysis is to minimize the number of sample tubes while maximizing the number of antibodies (1–5), with new applications incorporating two-dimensional pattern recognition analysis (4–7). A new and simple method for enabling multiparameter analysis from a single-tube uses Zenon labeling technology. The Zenon labeling reagents (Molecular Probes, Eugene, OR) used in this study are covalently labeled Fab fragments of goat IgG antibody raised against the Fc domain of the isotypes of mouse IgG antibody. Mixing of the labeled Fab fragment with the desired primary antibody rapidly forms a

Fab-primary antibody complex. The fluorescence intensity and utility of this complex is similar to that of a directly conjugated primary antibody. Unlike directly labeled primary antibodies, the ratio of Fab fragments to primary antibody can be easily modified, permitting

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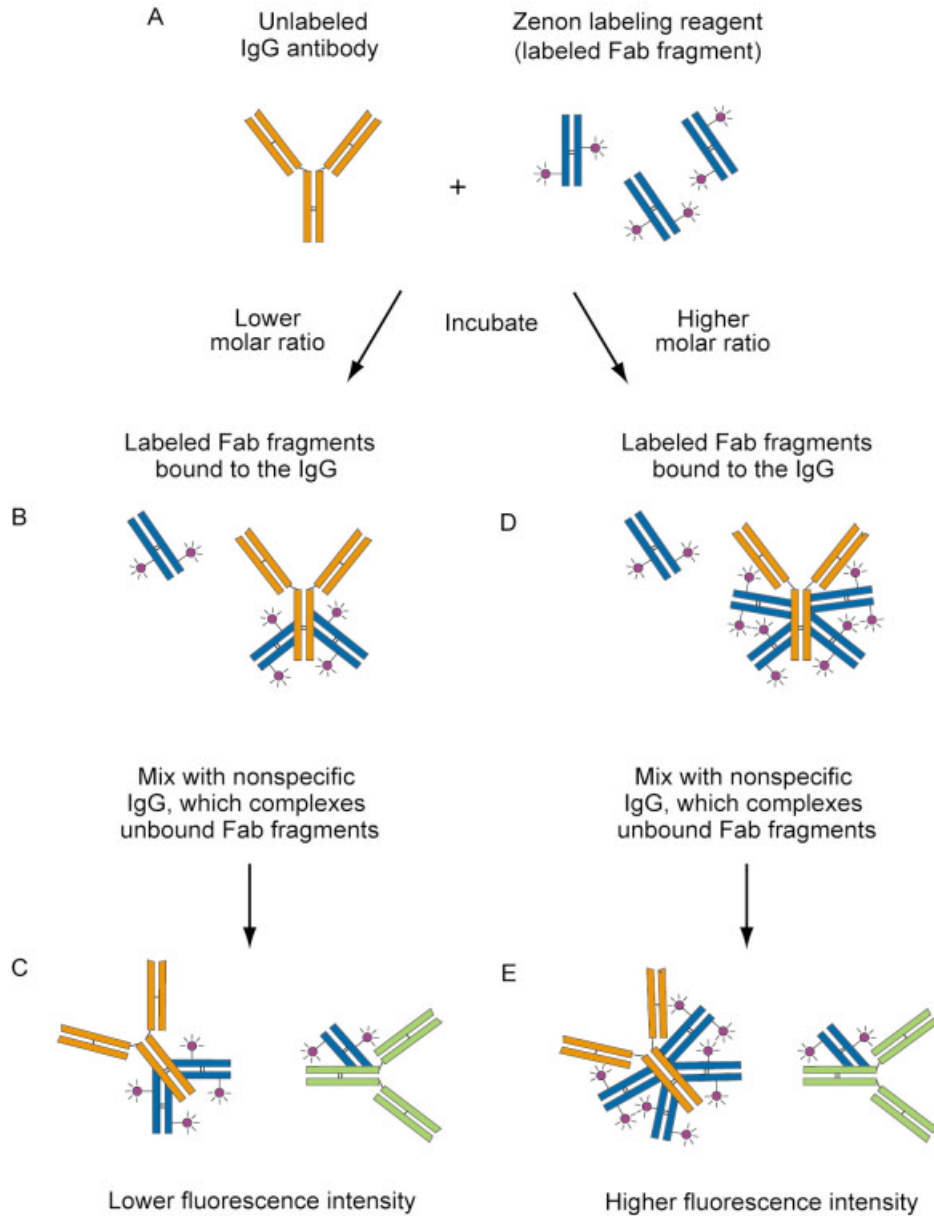


FIG. 1. Zenon reagent antibody labeling scheme. An unlabeled IgG is incubated with the Zenon labeling reagent, which contains a fluorophore-labeled Fab fragment (A). The labeled Fab fragment binds to the Fc portion of the IgG antibody at a lower molar ratio (B) and at a higher molar ratio (D), and excess Fab fragment is bound by the addition of a nonspecific IgG (C) and (E). The addition of nonspecific IgG prevents cross-labeling of the Fab fragment in experiments in which multiple primary antibodies of the same isotype are present.

some control over the fluorescence intensity of the labeled antibody (Fig. 1). By generating a set of differentially labeled antibody complexes using one fluorophore and combining this with a second set of differentially labeled antibody complexes using a second fluorophore, a multiplexed array based on fluorescent color and intensity can be formed. Applied to an immunophenotyping panel, the resulting multivariate plot for normal samples has a characteristic and recognizable

pattern, and deviations from the normal pattern are easily identified.

MATERIALS AND METHODS

Study Population

Peripheral blood samples were collected from 38 clinical patients in heparin, tripotassium ethylenediamine tetra-acetate (EDTA), or acid citrate dextrose (ACD), main-

tained at room temperature (RT) and processed within 24 h of collection (8).

Conventional Testing: Reagents, Instrumentation, and Methods

Flow cytometric enumeration of lymphocyte subsets (CD3, CD4, CD8, CD19, CD20, CD16, and CD56) was performed at Oregon Medical Laboratories (OML, Eugene, OR), a clinical reference laboratory, using an EPICS-XL flow cytometer. All instrumentation, software, reagents, and monoclonal antibodies used by OML were obtained from Beckman Coulter (Miami, FL). Conventional four-color analysis using four fluorophores, fluorescein isothiocyanate (FITC), phycoerythrin (R-PE), phycoerythrin-Texas Red[®]-X dye (ECD) and phycoerythrin-cyanin 5.1 (PC5) was performed in a four-tube panel using CYTOSTAT/COULTER CLONE directly labeled monoclonal antibodies in the following antibody combinations:

- Tube 1: CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5
- Tube 2: CD45-FITC/CD56-PE/CD19-ECD/CD3-PC5
- Tube 3: CD19-FITC/CD20-PE/CD45-PC5
- Tube 4: CD16-FITC/CD56-PE/CD45-PC5

For each tube, monoclonal antibodies were added to 100 μ l of whole blood. These tubes were vortexed, incubated for 10 min at room temperature, then lysed with the TQ Prep Workstation and ImmunoPrep Reagent System. Data were acquired on the EPICS-XL, equipped with a 488-nm argon-ion laser with 525-, 575-, 620-, and 675-nm band-pass filters. At least 5,000 lymphocyte events per tube were accumulated. Tubes 1 and 2 were evaluated using the tetraONE software system, which employs automated CD45/Side Scatter (SS) lymphocyte gating to derive percentages for lymphocytes expressing CD3, CD4, CD8, CD19, and CD56. Tubes 3 and 4 were evaluated with CD45/SS gating on lymphocytes to determine the percentage of lymphocytes positive for the FITC- and PE-conjugated markers (9). Instrument alignment was checked daily using Flow-Check Fluorospheres, instrument compensation was performed weekly using CYTO-COMP Reagent and Cell Kits, and normal control cells were run daily using Immuno-Trol Control Cells. OML has demonstrated good agreement with other laboratories for parameters in this study in proficiency testing via both the College of American Pathology and Center for Disease Control.

Fluorescence-Intensity Multiplexed Zenon Reagent Labeling Testing: Reagents, Instrumentation and Methods

Whole blood lysis using ammonium chloride was performed and cell concentrations were adjusted to 1×10^7 cells/ml in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (8,10). Mouse anti-human monoclonal antibodies (BD Pharmingen, San Diego, CA) used: IgG₁ isotype (clone MOPC-21), IgG_{2b} isotype (clone 2-35), CD3 (clone UCHT1, IgG₁), CD4 (clone RPA-T4, IgG₁), CD8 (clone HIT8a, IgG₁), CD16 (clone 3G8, IgG₁), CD19

(clone HIB19, IgG₁), CD20 (clone 2H7, IgG_{2b}), CD56 (clone B159, IgG₁), and CD45 (clone HI30, IgG₁). Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent, Zenon R-PE Mouse IgG₁ Labeling Reagent, Zenon R-PE Mouse IgG_{2b} Labeling Reagent, and Zenon Alexa Fluor 647-R-PE Mouse IgG₁ Labeling Reagent (Molecular Probes, Inc.) were used. Testing was performed using an Elite Flow Cytometer (Beckman Coulter) equipped with a 488 nm argon-ion laser and 525-, 575-, and 675-nm bandpass emission filters. Acquisition was performed using Expo32 version 1.2 software (Beckman Coulter), collecting both compensated data and uncompensated data. Analysis of compensated data was performed using Expo32 version 1.2 software. Uncompensated data analysis was performed using Win List 5.0 3D software (Verity Software House, Topsham, ME).

In forming a complex between Zenon labeling reagents and antibodies (Fig. 1), the molar ratio (MR) of the Zenon labeling reagent to the primary antibody must be selected. A MR of 3, i.e., 3 Fab fragments ($\sim 50,000$ MW each) to 1 primary antibody ($\sim 150,000$ MW), is equivalent to a mass ratio of 1:1 (Fab fragment to IgG target). Each complex is formed by adding 1 μ g primary antibody to the Zenon labeling reagent of the appropriate IgG type (i.e., 5 μ l (1 μ g) of the Fab fragment at 200 μ g/ml for MR3, or 8.5 μ l for MR5).

To determine the optimal MR for combined antibody testing, assays were performed with Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent complexed with 1 μ g CD4 and CD8 antibodies at molar ratios of 2 through 10. Each complex was tested individually, and the resulting histograms were overlaid.

Initial testing for differentiating targets based on fluorescence intensity combining two colors was performed using the following complexes and molar ratios: CD4 at MR2 and CD8 at MR8 complexed with the Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent, and CD3 at MR3 complexed with the Zenon R-PE Mouse IgG₁ Labeling Reagent. Complexes were tested in one, two, and three antibody combinations per tube.

For fluorescence-intensity multiplex testing, complexes were made using 1 μ g monoclonal antibody combined with Zenon labeling reagents at various molar ratios, staining cells in one tube for simultaneous testing: Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent with CD19 at MR3, CD4 at MR4, CD8 at MR4, and CD16 at MR14; Zenon R-PE Mouse IgG₁ Labeling Reagent with CD56 at MR6 and CD3 at MR3; Zenon R-PE Mouse IgG_{2b} Labeling Reagent with CD20 at MR14; Zenon Alexa Fluor 647-R-PE Labeling Reagent with CD45 at MR3. CD45/SS gating on lymphocytes was used.

The Zenon labeling reagent and 1 μ g of the primary antibody were combined at the desired MR. Complexes were incubated for 5 min at RT. Zenon blocking reagent (mouse IgG) was then added to each complex in the same volume as Zenon mouse IgG labeling reagent used, and incubated 5 min at RT. All Zenon reagent-labeled complexes were added to 100 μ l of cell suspension, incubated 30 min at RT, washed once in PBS, and resuspended in 1%

formaldehyde/PBS (8). A lymphocyte gate was made on CD45/SS and 50,000 events were collected (10–13). The IgG₁ isotype complexed with Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent at MR14 and the IgG_{2b} isotype complexed with Zenon R-PE Mouse IgG_{2b} Labeling Reagent at MR14 served as negative controls (8,10,11). Single-color compensation controls were generated using the primary antibody complexed with the Zenon labeling reagent at the highest MR for each fluorophore used; standard compensation procedures were followed. (8,10,11). Data analysis using Expo32 used simple regions around distinct multivariate cell populations. Win List data analysis used regions around distinct multivariate cell populations to define subsets, with individual marker values then determined using Boolean Gate Analysis. In this analysis, multivariate populations were gated and Boolean expressions were applied to define populations of interest (14). Subsequent color gating was used to assign an individual color to each discrete population. Statistical interpretation was performed using Microsoft Excel 2000 and the Analysis Toolpack Add-in.

RESULTS

Increasing Signal-to-Noise Ratio

Increasing the MR by adding more Zenon labeling reagent to a constant amount of primary antibody results in an increase of signal-to-noise ratio. A MR titration can be performed to see how a particular primary antibody responds. Results from MR titration demonstrate a constant background signal while the fluorescence of the positive signal steadily increases (Fig. 2A,B). This shift provides an increase of signal-to-noise ratio as the MR of the labeling complex increases, while the percentage of positive cells remains constant over the titration range (Fig. 2C,D). Performing this study for all antibodies in a particular multiplexing construct establishes MR combinations to use. Once established for specific reagents, a given MR has been found to provide reproducible staining patterns over multiple reagent preparations (data not shown).

Concept of Fluorescence Intensity Multiplexing

CD4 complexed with Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent at MR2 results in a single-positive peak (Fig. 3A). CD8 complexed with the same Zenon labeling reagent at MR8 results in a bright-positive CD8 peak at higher fluorescent intensity (Fig. 3B). The two complexes prepared individually and added simultaneously to cells result in two distinct peaks using one fluorescence detector (Fig. 3C,E), although the CD4⁺ cells overlay some dim-positive CD8 cells with this reagent combination. CD3 complexed with a second fluorophore, Zenon R-PE Mouse IgG₁ Labeling Reagent at MR3, results in a single-positive peak in a second fluorescence detector (Fig. 3D). When all three antibody complexes are added simultaneously to stain cells, distinct dual-positive populations for T-helper CD3⁺ CD4⁺ and T-suppressor/cytotoxic CD3⁺ CD8⁺ cell populations are identified and a multivariate pattern is produced (Fig. 3F).

Seven-Marker, Two-Color Lymphocyte Immunophenotyping

For simultaneous seven-marker, two-color evaluation, a single tube can be used when employing Zenon reagent labeling. Four antibodies (CD19, CD4, CD8, and CD16) are combined at various MRs with Zenon Alexa Fluor 488 IgG₁ Labeling Reagent to give a histogram with four peaks using one fluorescent detector (Fig. 4A,B). Three antibodies (CD56, CD3, CD20) are combined with Zenon R-PE IgG₁ or IgG_{2b} Labeling Reagent to give a histogram with three peaks in a second fluorescent detector (Fig. 4C,D). A dual-color plot combines the seven antibodies to shape four distinct cell populations: CD19⁺ CD20⁺ B cells, CD3⁺ CD4⁺ T-helper cells, CD3⁺ CD8⁺ T-suppressor/cytotoxic cells, and CD16⁺ CD56⁺ natural killer (NK) cells (Fig. 4E,F). The resultant multivariate plot pattern is characteristic and reproducible. Regions can be created around the distinct cell populations for enumeration. Boolean expressions can be applied to two-color regions to obtain values for individual markers. Patient samples show some variability in distinguishing multiple peaks in single color histograms.

Characteristic Patterns

Lymphocyte immunophenotyping using fluorescence-intensity multiplexing with Zenon reagent labeling produces a characteristic normal multivariate pattern in healthy individuals (Fig. 5A,B), while deviations from this pattern indicate an abnormal lymphocyte phenotype. Abnormal patterns identified in this study include increase and decrease of T-helper (Fig. 5C,D), T-suppressor/cytotoxic, B-cell, and NK cell populations. Double-negative (DN) T cells (phenotype CD3⁺ CD4⁻ CD8⁻, putative $\gamma\delta$ -receptor T cells) can be identified as a distinct population (Fig. 5E). B-cell chronic lymphocytic leukemia (CLL) produces a characteristic pattern (Fig. 5F).

Statistical Analysis

The results from conventional flow cytometric and fluorescence-intensity multiplexing were compared for all measured parameters. Data are displayed as the mean $\pm$ SEM. Using least-squares regression analysis, r^2 values of ≥ 0.95 for the dual positive populations and ≥ 0.90 for single parameters are seen. Bias scatterplots showed no bias present for all measured parameters except CD56 and CD16, which showed slight systematic bias. The correlation coefficient is ≥ 0.96 for all dual-positive parameters and ≥ 0.93 for single parameters. Data are displayed for lymphosum (T+B+NK cells) and percentage DN T cells (Fig. 6). Preparing and analyzing ten replicates of the same specimen using the fluorescence-intensity multiplexing method gave $\leq 6\%$ coefficient of variation for all measured parameters.

DISCUSSION

In flow cytometry, reagent panels are often limited by the need to assign one fluorescence channel to a marker. Several groups have previously developed methods for

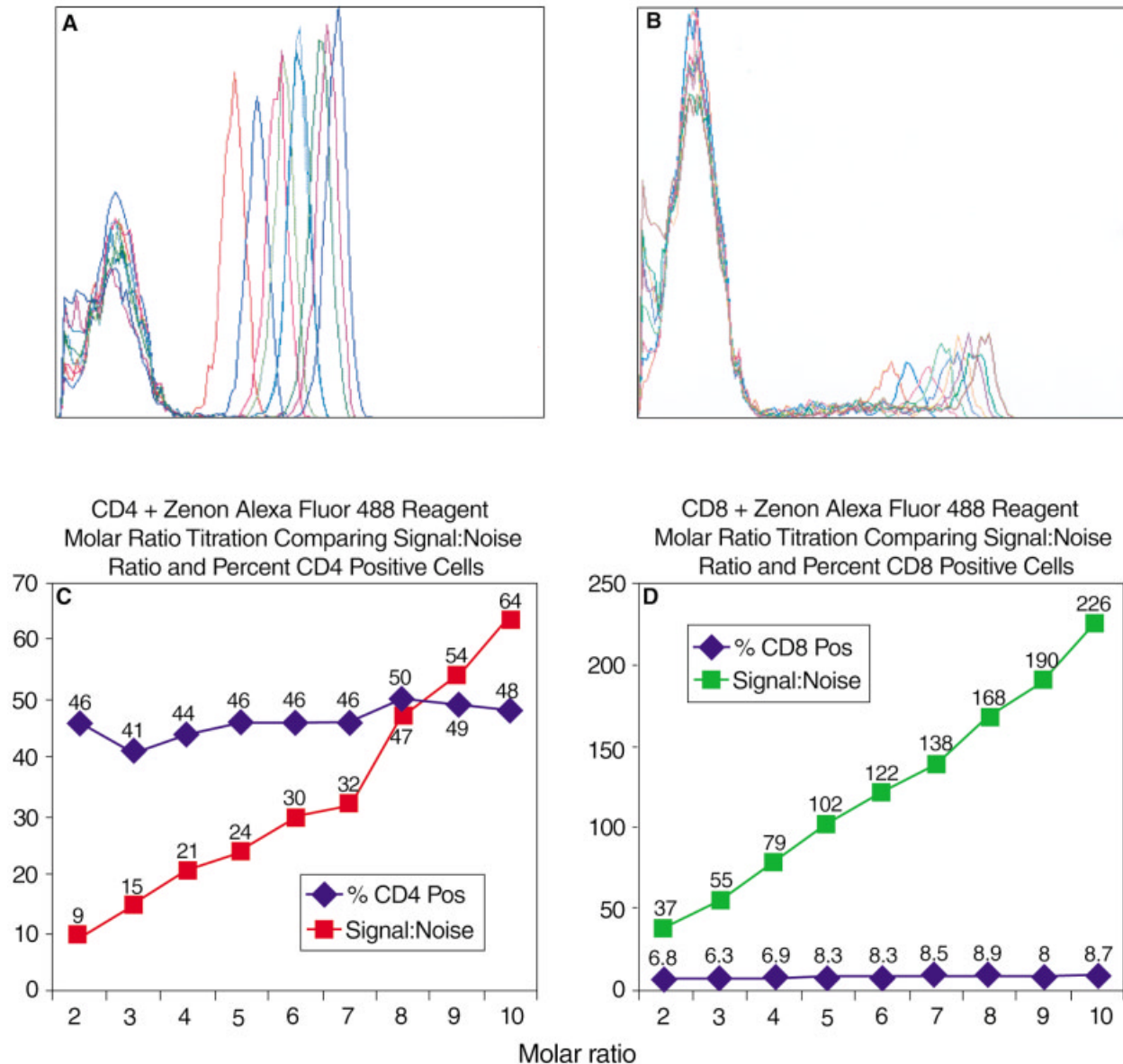


FIG. 2. Representation of molar ratio titration to increase fluorescence. Overlay histograms show CD4 (A) and CD8 (B) antibody labeling of lymphocytes with Zenon reagent-labeled complexes made at molar ratios ranging from 2 to 10. The CD8 data shows dim-positive and bright-positive populations. CD4 labeling (C) and CD8 labeling (D) show an increase of fluorescence signal while background signal remains constant (increase of signal-to-noise ratio) with increasing molar ratio, while percentage positive remains constant.

multiplexed immunophenotyping using fluorescence intensity (1,14-16). Zenon labeling technology builds on this concept by allowing the user to easily incorporate multiplexed measurements into their experimental design. The ability to regulate the fluorescent intensity of a labeled complex is a feature of Zenon labeling technology that is not practical with direct antibody labeling. This technique improves on traditional secondary detection methods, which do not allow controlled differentiation of multiple mouse primary antibodies of the same isotype. Zenon labeling technology provides a straightforward system in which a customized set of antibodies can generate

a characteristic pattern, limited only by availability of appropriate primary antibodies.

Lymphocyte subset testing is well defined, and for this reason was chosen in the present study to demonstrate multiplexing with Zenon labeling reagents. Traditionally, lymphocyte populations have been distinguished in peripheral blood by simultaneously measuring several different surface antigens (1,3). This has necessitated the development of flow cytometric technologies and experimental designs capable of detecting and analyzing increasingly more parameters. The fluorescence-intensity multiplexing technique described here offers clear advantages for lymphocyte subset testing.

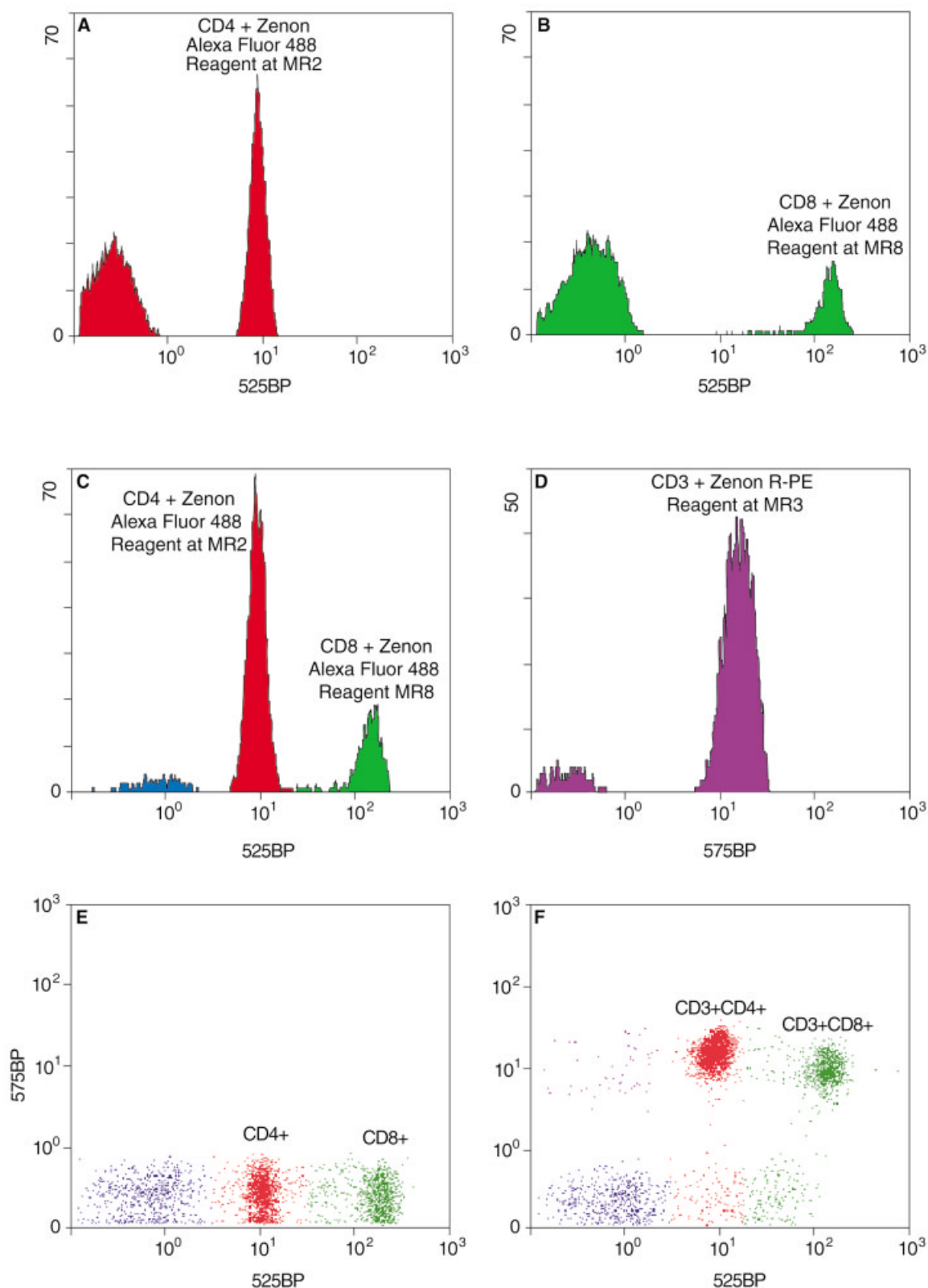


FIG. 3. Concept of fluorescence-intensity multiplexed Zenon reagent labeling using human lymphocytes. Histogram (A) shows single-marker labeling with a complex of CD4 and Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent at MR2. Histogram (B) shows single-marker labeling with a complex of CD8 and Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent at MR8. Histogram (C) and dot plot (E) show two-marker labeling combining CD4 at MR2 and CD8 at MR8 with Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent. Histogram (D) shows single-marker labeling with a complex of CD3 and Zenon R-PE Mouse IgG₁ Labeling Reagent at MR3. Dual-parameter plot (F) shows triple-marker labeling in two colors combining CD4 at MR2 and CD8 at MR8 with Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent and CD3 with Zenon R-PE Mouse IgG₁ Labeling Reagent at MR3. Two distinct dual-positive populations of CD3⁺ CD4⁺ T helper cells and CD3⁺ CD8⁺ T suppressor/cytotoxic cells are demonstrated.

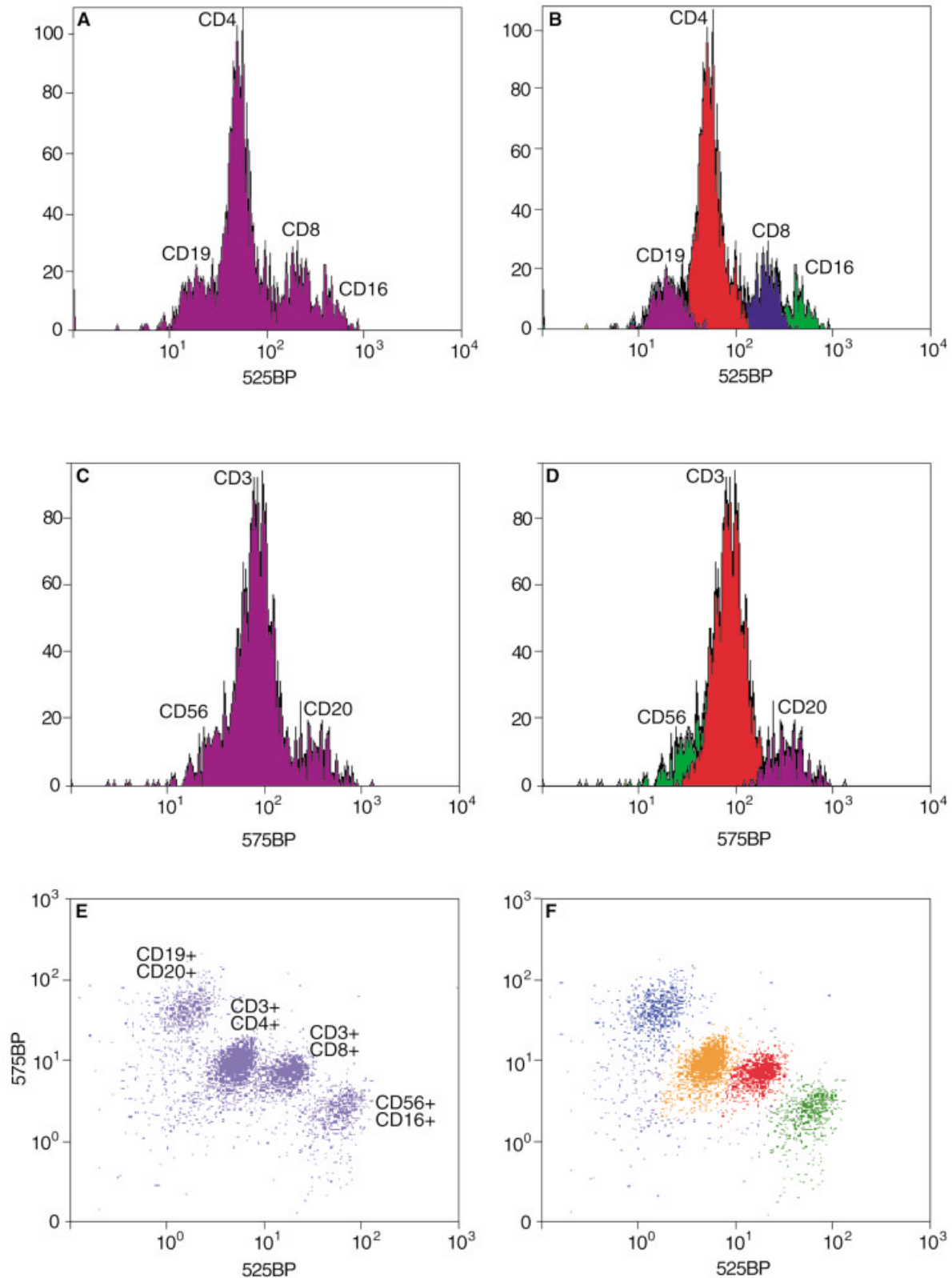


FIG. 4. Fluorescence-intensity multiplexed Zenon reagent labeling of lymphocytes. Four-marker single color labeling using Zenon Alexa Fluor 488 Mouse IgG₁ Labeling Reagent with CD19, CD4, CD8, and CD16: **A:** Histogram as acquired. **B:** Same histogram with WinList software modeling based on dual-positive populations. Three-marker one-color labeling using the Zenon R-PE Mouse IgG₁ or IgG_{2a} Labeling Reagent with CD56, CD3, and CD20: **C:** Histogram as acquired. **D:** Same histogram with WinList software modeling based on dual-positive populations. Two-parameter plot combining all seven markers, presenting four distinct cell populations: **E:** Data as acquired. **F:** Same plot with color gating on the four populations: CD19⁺ CD20⁺ B cells (blue), CD3⁺ CD4⁺ T-helper cells (orange), CD3⁺ CD8⁺ T-suppressor/cytotoxic cells (red), CD56⁺ CD16⁺ NK cells (green).

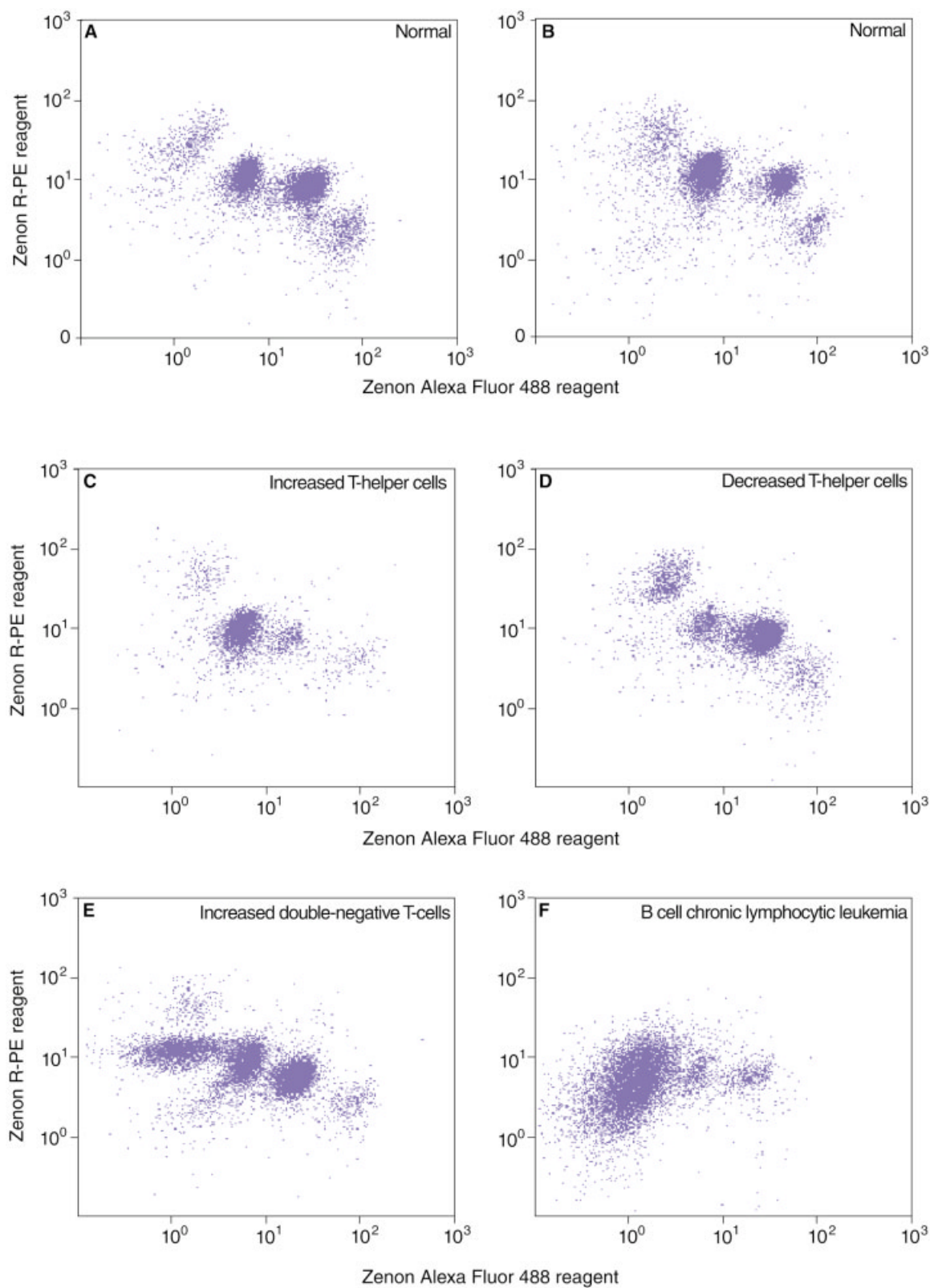


FIG. 5. Dual-parameter plots with seven markers, two-color, single-tube labeling of six different patient blood samples using fluorescence-intensity multiplexed Zenon reagent labeling of lymphocytes. **A,B:** Plots showing a normal pattern. **C:** Plot with a pattern showing increased T-Helper cells. **D:** Plot with a pattern showing decreased T-helper cells. **E:** Plot with a pattern showing increased double-negative T cells. **F:** Plot with a pattern showing increased B cells in chronic lymphocytic leukemia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

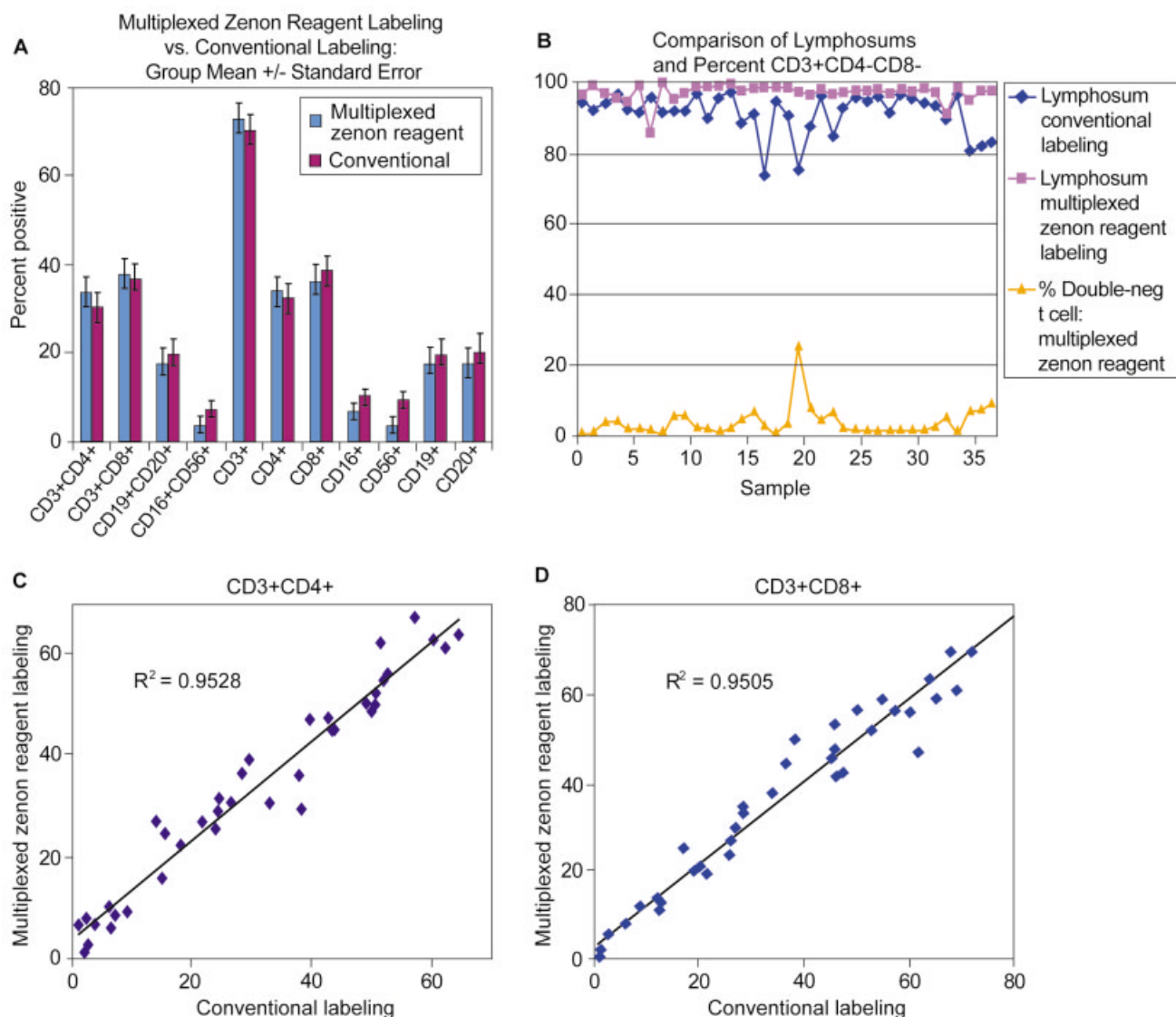


FIG. 6. Mean and standard error analysis of conventional vs fluorescence-intensity multiplexed Zenon reagent labeling (A). Lymphosum of conventional and fluorescence-intensity multiplexed Zenon reagent labeling, with percentage double-negative T cells (B). Linear regression plots of CD3⁺ CD4⁺ population (C) and CD3⁺ CD8⁺ population (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

phocyte immunophenotyping with a limited number of detectors. A key strength is the ability to recognize a characteristic normal multivariate pattern as well as deviations from the normal pattern. The results based on pattern recognition challenge conventional quality control procedures. The inclusion of blood from a normal donor serves as a positive methodologic control, with the characteristic normal multivariate pattern being the expected result. A built-in check for lymphocyte phenotyping, the lymphosum (T+B+ NK cells), serves as an internal control, with expected results being with $100 \pm 5\%$ of total lymphocytes (3,4). Single-tube testing eliminates the need for a common factor in multiple tubes as a control for tube-to-tube variability. CD45/SS gating strategy is now the preferred method for identifying lymphocytes (10–

13), and can also be used with Zenon reagent-labeled fluorescence-intensity multiplexing, as in this study.

Fluorescence-intensity multiplexing offers the capability to expand the analytic potential of a flow cytometer significantly without increasing the complexity of the instrument. For example, only one excitation source is required for multiparameter detection and small sample volumes can be used. Reducing the number of tubes tested per profile offers cost savings when considering analysis time and consumables, while providing higher sample throughput.

Overall, the correlation established between the two methods tested here is acceptable. While there exists no gold standard for lymphocyte phenotyping (8), the use of statistical tools can help validate new methods as new

technology is introduced. Mean and standard error show the data to be similarly distributed between the two methods. Linear regression analysis shows an acceptable correlation between the two sets of data. The values for the dual-positive populations display better agreement than the single-positive populations. This is expected, since the single-parameter data are phenotypically preselected from the multivariate plot for analysis using Boolean Gate Analysis. Bias scatterplots show slight systematic bias with CD16 and CD56. This can be expected since these antigens are dimly expressed and distinguishing a positive peak is often difficult with conventional flow cytometry. Using both CD16 and CD56 in combination with using a higher MR of Zenon labeling reagent to primary antibody improves the identification of the positive population. It is expected to see differences in fluorescence intensity in samples from specific disease states, i.e., dim CD20 expression in CLL (7). Even though antigen densities may change, specific immunophenotyping patterns characteristic of an abnormality or disease state may be observed.

Studying normal and abnormal patterns in a particular testing format can identify unique cell populations. This identification may be of primary importance, while quantification may be less significant or unnecessary depending on the application (5). Most conventional phenotyping uses multiparameter testing to define multiple populations, analyzing a progression of univariate and bivariate plots to obtain results (7). As more parameters are analyzed and complexity increases, the interactions between the measured parameters become more difficult to visualize. Multiplexing, as with Zenon labeling reagents, improves the presentation of multiparameter data by displaying results in a simplified data space. Although there may exist some ambiguity in the setting of regions and markers around equivalent populations between samples, changes in data patterns are readily discerned. The increased complexity of data analysis resulting from advancing technology may require more subjective approaches to the interpretation of flow data, such as pattern recognition (7). As such, multiplexed labeling with Zenon labeling reagents in flow cytometry should prove useful in research applications as well as screening procedures.

Fluorescence-intensity multiplexing using Zenon labeling technology provides a powerful tool for multiparameter flow cytometry. The key factor that limits the number of cellular targets that can be multiplexed in a single detection channel is the intrinsic width of the target distribution(s). With 4-logs of dynamic range per detection channel, quantitation of as many as 6 or 7 targets per channel are possible. For biological systems, we have found that quantitation of 2–4 cellular targets per detection channel represents a reasonable goal.

This study has concentrated on developing fluorescence-intensity multiplexing using immunophenotyping as an example, because it allowed a rigorous estimation of errors and bias of this approach, which were found to be minimal. Perhaps one of the key uses of this fluorescence-intensity multiplexing approach will be for flow cytomet-

ric studies designed to resolve complex cell-signaling pathways (20), or cell phenotype/function/diversity questions (2). In these types of studies, signals from >10 cellular targets are needed, requiring state-of-the-art custom multi-channel instrumentation. Using fluorescence-intensity multiplexing, high numbers of cellular targets can be quantitated using only 3 or 4 detection channels, greatly decreasing the complexity of the instrumentation required.

This technique offers the capability to expand the analytic potential of any flow cytometer, is readily customized, and does not require the use of labeled beads. With pattern recognition emerging as a significant tool in flow cytometric analysis, Zenon labeling technology provides a flexible means for greatly expanding the number of simultaneous cellular targets that can be quantified.

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