Cytometry (Communications in Clinical Cytometry) 30:151–156 (1997)

Technical Innovations

Reducing Cellular Autofluorescence in Flow Cytometry: An In Situ Method

Victoria L. Mosiman,[1]* Bruce K. Patterson,[2] Luis Canterero,[3] and Charles L. Goolsby[1]

[1]Department of Pathology, Northwestern University Medical School, Chicago, IL

[2]Department of Obstetrics and Gynecology, Northwestern University Medical School/Department of Medicine, Division of Infectious Diseases, Northwestern University Medical School, Chicago, Illinois

[3]Aprogenex, Inc., Houston, Texas

Cellular autofluorescence affects the sensitivity of flow cytometric assays by interfering with detection of low level specific fluorescence. These detection limits increase with use of protocols, such as thermocycling and fluorescent in-situ hybridization (FISH), that can increase intrinsic cellular fluorescence to 5,000–20,000 fluorescein isothiocyanate (FITC) equivalents. In order to improve signal to noise ratios when using FITC labeled probes in these procedures, we employed a method using the polyanionic azo dye, trypan blue, to reduce intracellular autofluorescence. Dyes such as these are commonly used in immunofluorescent microscopy to reduce background fluorescence. By using this method, we realized an approximately 5-fold increase in signal to noise ratio (S/N) in the direct detection of RNA target probes using flow cytometry. Trypan blue aided in the resolution of dim surface antibodies, internal markers and probes, and functions to reduce background autofluorescence after thermocycling and hybridization. This technique is rapid and easily applicable for reducing intracellular autofluorescence, and can be used in single and dual color applications. Cytometry 30:151–156, 1997. © 1997 Wiley-Liss, Inc.

Key terms: flow cytometry; cellular autofluorescence; trypan blue

The techniques of flow cytometry rely on the ability of the instrumentation to distinguish and detect specifically bound fluorescent molecules and resolve them above other intrinsic and nonspecific background signals. A critical aspect of this detection involves achieving levels of sensitivity that allow one to quantify low levels of antibody binding or dim fluorescence on highly autofluorescent cells. All cells have some intrinsic level of autofluorescence, which is most commonly caused by NADH, riboflavins, and flavin coenzymes (2,3). These molecules excite over a broad range of wavelengths including the blue region of the spectra. The emission wavelengths of these autofluorescent molecules when excited in the blue is broad (500–700 nm) and overlaps emission spectra of commonly used fluorescent dyes. The peak autofluorescence emission after 488 nm excitation is in the green region of the spectra (13), heavily overlapping with the FITC fluorescence detection region.

Past methodologies that have attempted to correct for this phenomenon include use of dyes emitting in spectral regions of reduced autofluorescence output. Others utilize measurement of autofluorescence in alternative excitation and emission regions by dual laser (15) or dual PMT (1,12) approaches. Another relatively new and promising method utilizes phase sensitive detection (14) to potentially separate autofluorescence from specific fluorochrome emissions based on differences in fluorescence half-lives. Integration of these approaches into current techniques requires considerable alterations in either hardware or software configurations. This plethora of techniques however, accentuates the need for a convenient method to reduce cellular autofluorescence in flow cytometric analysis.

The approach described in this work reduces nonspecific background fluorescence while maintaining the ability to use readily available fluorochromes, and a standard cytometer set-up. The technique employs the addition of a dye, Trypan blue (TB), to fixed and permeabilized cells as a final step before flow cytometric analysis (5). Trypan blue has been used routinely for years as a counterstain in tissue and slide preparations for fluorescent microscopy (8). In flow cytometry, dyes such as methylene blue have previously been used to reduce intracellular autofluorescence background in glutaraldehyde-fixed cells (6). We have further expanded this application to include a variety of cytometric staining protocols, all with the central focus of reducing internal background autofluorescence of indi-

*Correspondence to: Victoria L. Mosiman, 250 East Superior Street, 536 Wesley Pavilion, Chicago, IL 60611.
E-mail: VLM646@nwu.edu
Received 30 January 1996; Accepted 31 March 1997

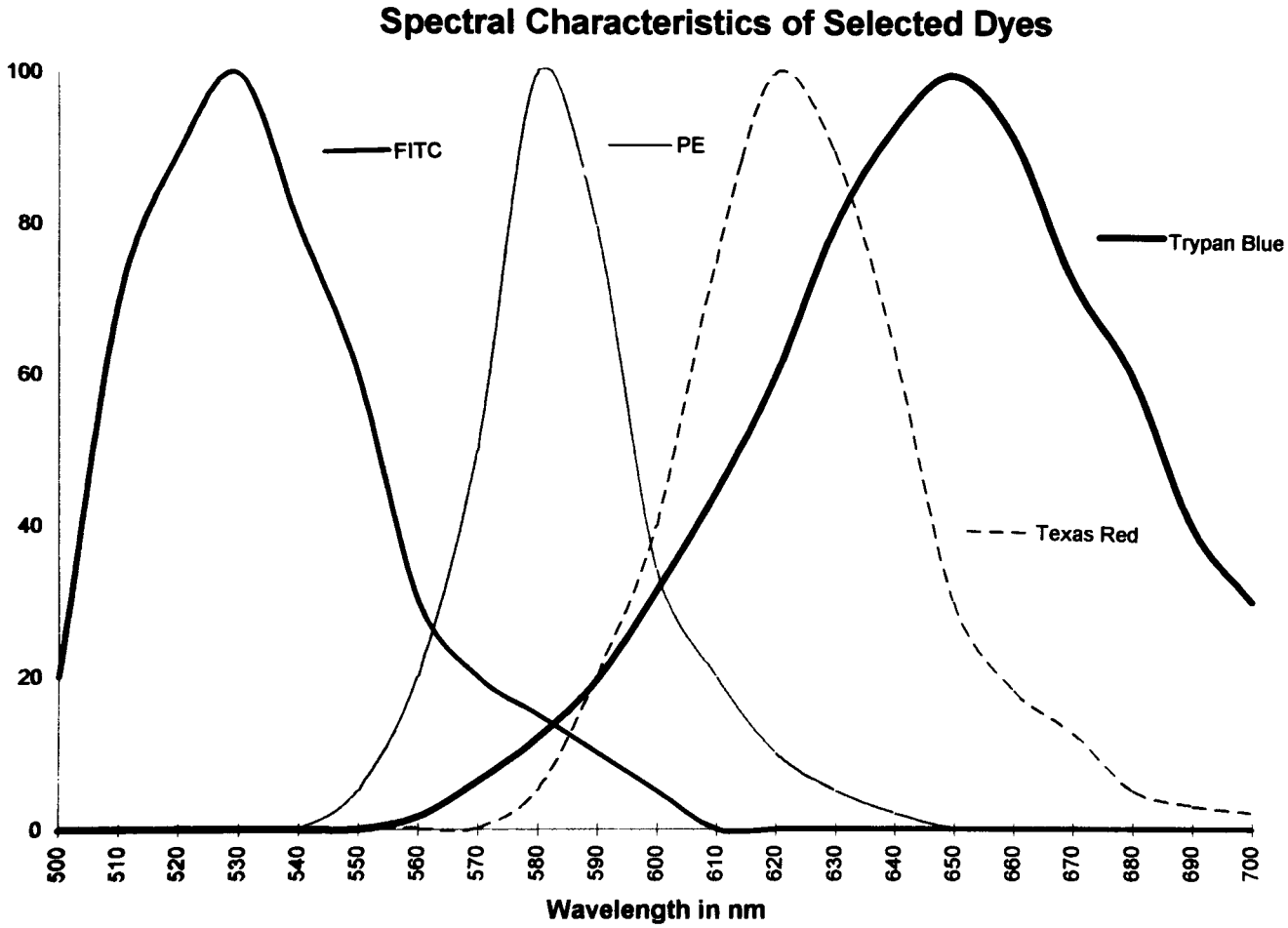

FIG. 1. Distributions of fluorescence intensity vs. wavelength for dyes typically used in three-color immunofluorescence and for trypan blue excited at 488 nm demonstrating the approximate overlap of the emission spectra. FITC, PE, and trypan blue are compensatable; however, dyes emitting at >610 nm are not recommended for use with this procedure. Peak heights were adjusted and do not represent actual fluorescent intensities.

vidual cells as they pass in front of the laser beam. After fixing and permeabilizing the cells, trypan blue is allowed to freely diffuse into the cell and uniformly distribute throughout the cytoplasm and nucleus. The low molecular weight (960.83 g/mole) of TB (9) facilitates its rapid diffusion through the cellular and nuclear membranes. At appropriate concentrations, this random distribution of dye molecules brings them within proper distance and orientation to autofluorescent, or nonspecifically bound fluorescent molecules, to allow quenching to occur (17).

MATERIALS AND METHODS

PBMC Separation

Lymphocytes were separated from peripheral blood by ficoll-hypaque density gradient (1077). Buffy coat was removed, cells were washed once in 1 × PBS, counted, and prepared as below.

Antibody Staining

For each sample, 1×10^6 cells were stained with 10 µl of antibody for 20 min at 4°C. Stained cells were washed once in 1× PBS prior to fixation. Antibodies used included Cytostat Coulter Clone B4-RD1/J5 FITC, Cytostat Coulter Clone KC56 FITC, and Cytostat Coulter Clone Isotype FITC (Coulter Corporation, Miami, FL).

Fixation

All fixations were done in 1× Ortho Permeafix (Ortho Diagnostics Systems, Inc., Raritan, NJ), 50 µl/10^6 cells, at room temperature for 1–18 h, followed by 1 wash in 1× PBS.

Hybridization

Cells were fixed as above and hybridized as described below. Briefly, cells were washed once in 2× SSC (1–2 ml) and then resuspended in hybridization cocktail with mRNA probe. Probes used were a ''negative'' probe directed toward HPV mRNA, and a ''positive'' probe directed towards 28S ribosomal mRNA (Aprogenex, Inc., Houston, TX). Probe cocktail mix was prepared in bulk for each batch of samples to ensure even distribution of the probe

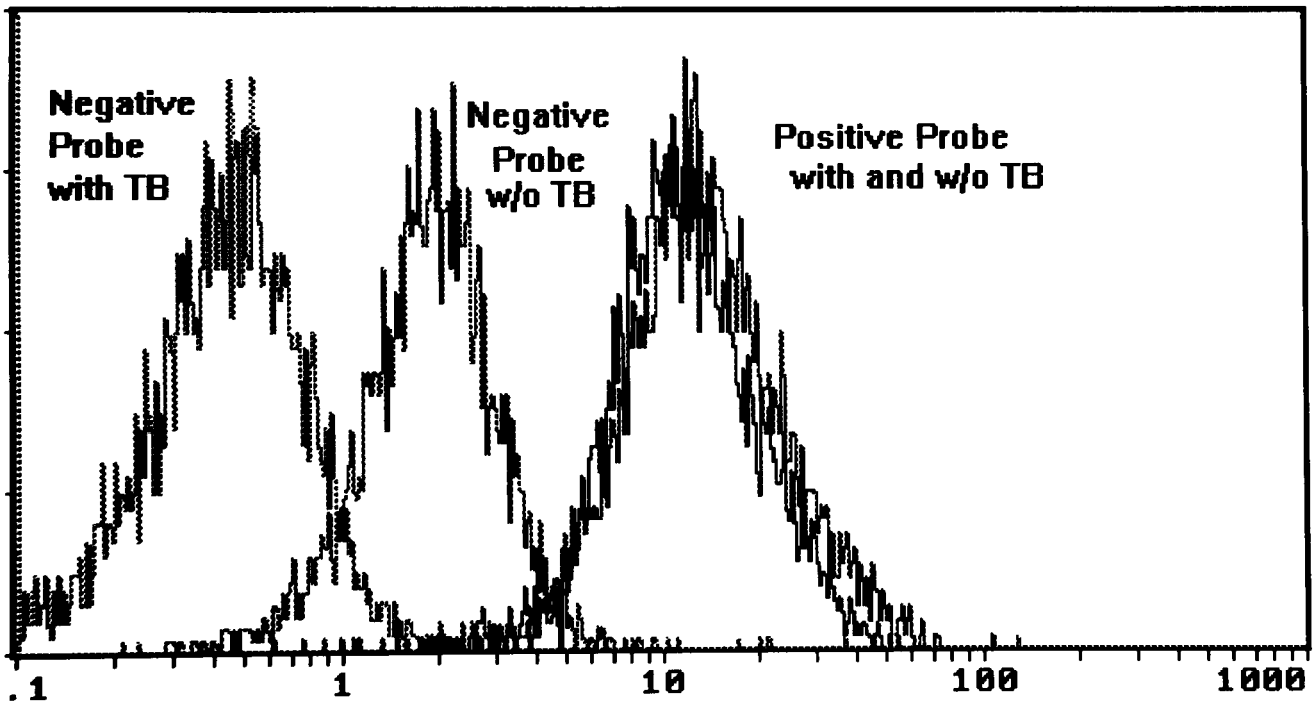

FIG. 2. Histogram showing fluorescence intensity of FITC-labeled mRNA control probes (negative and positive), demonstrating shift in background fluorescence intensity with and without (as indicated) trypan blue counterstaining.

into each sample tube; 1 µl of reconstituted probe into 50 µl of hybridization cocktail per sample (Aprogenex Inc., Houston, TX). Probe cocktail was incubated with cells in 42°C water bath for 30 min. Cells were washed in 2 ml of Wash A (Aprogenex, Inc., Houston, TX) that had been heated to 42°C. After centrifugation, cells were resuspended in Wash B (Aprogenex, Inc., Houston, TX), vortexed, and left in a 42°C water bath for 30 min. After incubation cells were centrifuged as above and resuspended in 500 µl (1 µg/ml solution) ice-cold trypan blue counterstain solution (Aprogenex, Inc., Houston, TX). Cells were incubated with counterstain for 10 min at 4°C. Samples were centrifuged once again and resuspended in 500 µl of 1× PBS for flow cytometric evaluation.

Flow Cytometry

All samples were analyzed on a Coulter EPICS XL flow cytometer (Coulter Corporation, Miami, FL). The instrument was equipped with a standard four color filter set-up (FITC; 550DCLP, 525BP. PE; 600DCLP, 575BP. TB; 645DCLP, 675BP). Calculations of S/N and fold changes were based on linear data values supplied by the instrumentation.

RESULTS

To explore the feasibility of using TB as a quenching dye in flow cytometric applications, it was necessary to characterize the dyes response to laser excitation. Trypan blue has intrinsic fluorescence in the far-red region of the spectra (600–720 nm). It also excites and absorbs over a broad range extending from the blue to the orange regions. We performed scanning spectrometry to characterize the emission spectra of TB after 488 nm excitation. The results shown in Figure 1 demonstrate the emission spectra of trypan blue as compared to several other commonly used fluorochromes. As shown, there is minimal overlap with FITC and PE emission regions, but considerable overlap with dyes typically used in single laser three or four color immunofluorescence applications. Thus, TB is not recommended for use in applications requiring PE-molecular conjugate dyes (such as PE-Cy5 or PE-Texas Red) or PerCP (Becton Dickinson, Mountain View, CA), which also emit in the far red (>610 nm) region of the spectra.

The next aspect characterized was determination of this technique's utilization in resolving dim internal probes after hybridization. As shown in figure 2, improved signal to noise was achieved by reducing the mean channel fluorescence of the internal negative probe with the addition of TB. The mean channel fluorescence of the 28S mRNA probe remained unchanged with the addition of TB, supporting the fact that TB blue quenches comparatively little specifically bound fluorescence.

We obtained the appropriate working concentration of TB by observing the changes in S/N ratio of the aforementioned mRNA probes. The resulting concentration curve for these experiments, graphically represented in Figure 3, was determined by the following method. After individually hybridizing the positive and negative probe pairs, they were combined at a 1:1 ratio and treated with differing concentrations of trypan blue. The mode of the positive

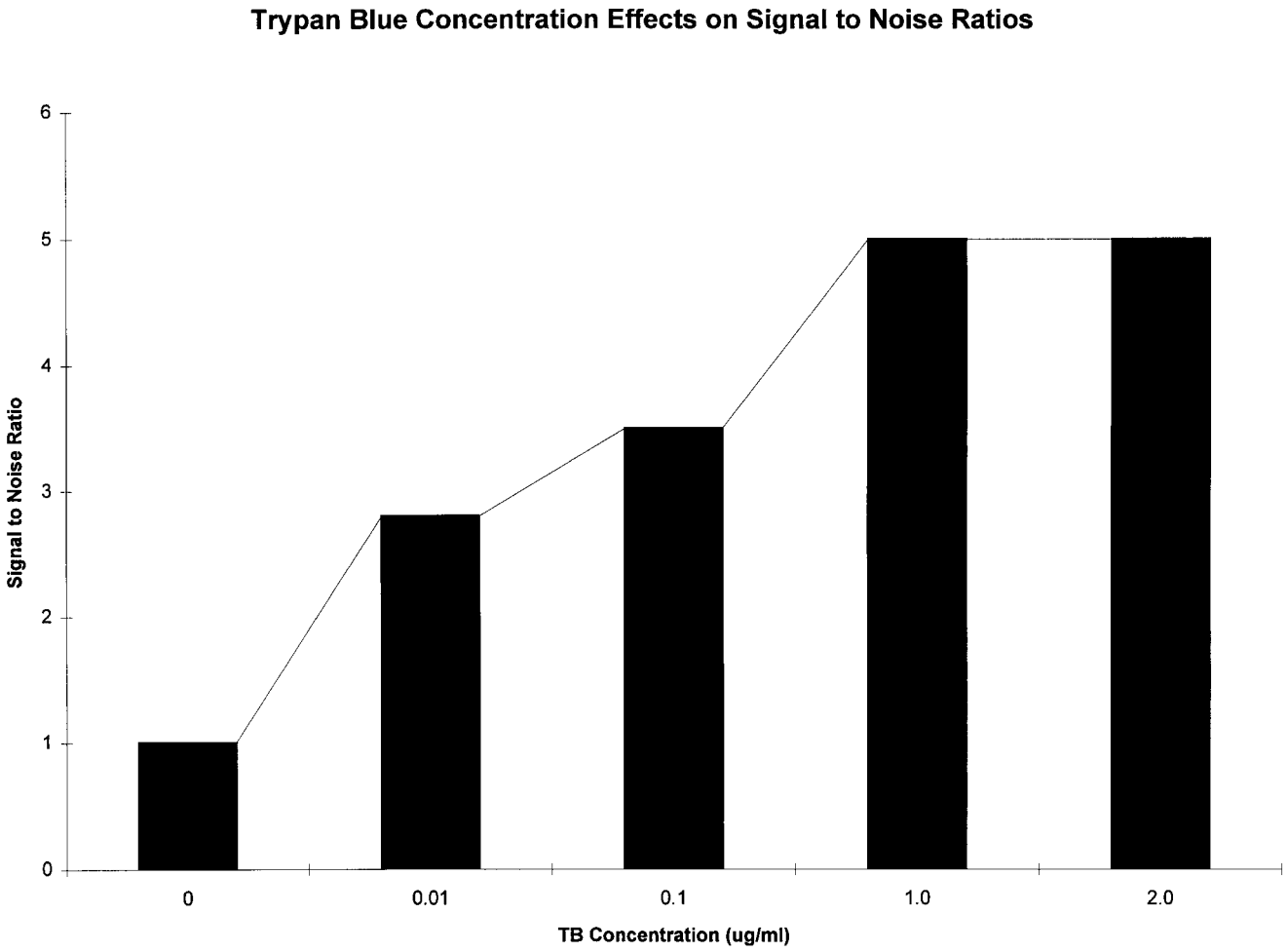

FIG. 3. Signal to noise ratio versus trypan blue concentration. This shows signal to noise as calculated by dividing the modal channel of the fluorescence distribution for two mRNA probes in hybridized cells. Cells were hybridized with either a positive probe directed against 28S mRNA, or a negative probe directed against HPV mRNA. After testing a range of trypan blue concentrations, maximal separation between the positive and negative probe signals occurred at a TB concentration of approximately 2.0 μg/ml. This concentration was enough to quench cellular autofluorescence without significantly reducing the fluorescence intensity of the positive probe signal that may occur at significantly higher dye concentrations (>20 μg/ml).

probe sample was divided by the mode of the negative probe sample to get the S/N value. A maximal increase in the S/N ratio of approximately 5-fold was achieved at 0.002% w/v TB. This is consistent with concentrations previously used with similar azo compounds in immunofluorescent microscopy, which report a range between 0.0002% and 0.10% w/v (4,7). There was little improvement with higher concentrations of TB.

The final characterization to prove this methods' broad-based utility was to apply it to a clinical specimen. With the inclusion of TB, we were able to resolve a dim CD10 positive population by improving the signal to noise. We compared the marker antibody staining to their respective isotypes and internal negative controls (cells within the positive signal sample that did not express that marker). As ascertained in Figure 4, the isotypes' downward shift along the fluorescence axis shows a decrease in mean channel fluorescence, while the mean of the positive antibody remains relatively unchanged.

Although this procedure is technically simple, one must proceed with care when establishing use in differing applications. One pitfall is the potential to quench specifically bound fluorescence if the trypan blue concentration is too high. While the concentrations in this study have worked well in a variety of applications, optimum concentrations may vary under other conditions. Properties influencing TB concentration include several factors: 1) the intensity and distribution of specific fluorescent marker, 2) type of fluorochrome used, 3) initial levels and characteristics of intrinsic cellular autofluorescence of target cells, and 4) effects of treatments such as thermocycling and hybridization.

After establishing appropriate concentrations, this technique is robust and removes potential operator bias in instrument set-up. For example, subtraction techniques based on measurement of autofluorescence in the red wavelength spectrum (1), while easily implemented, are dependent on instrument compensation settings which

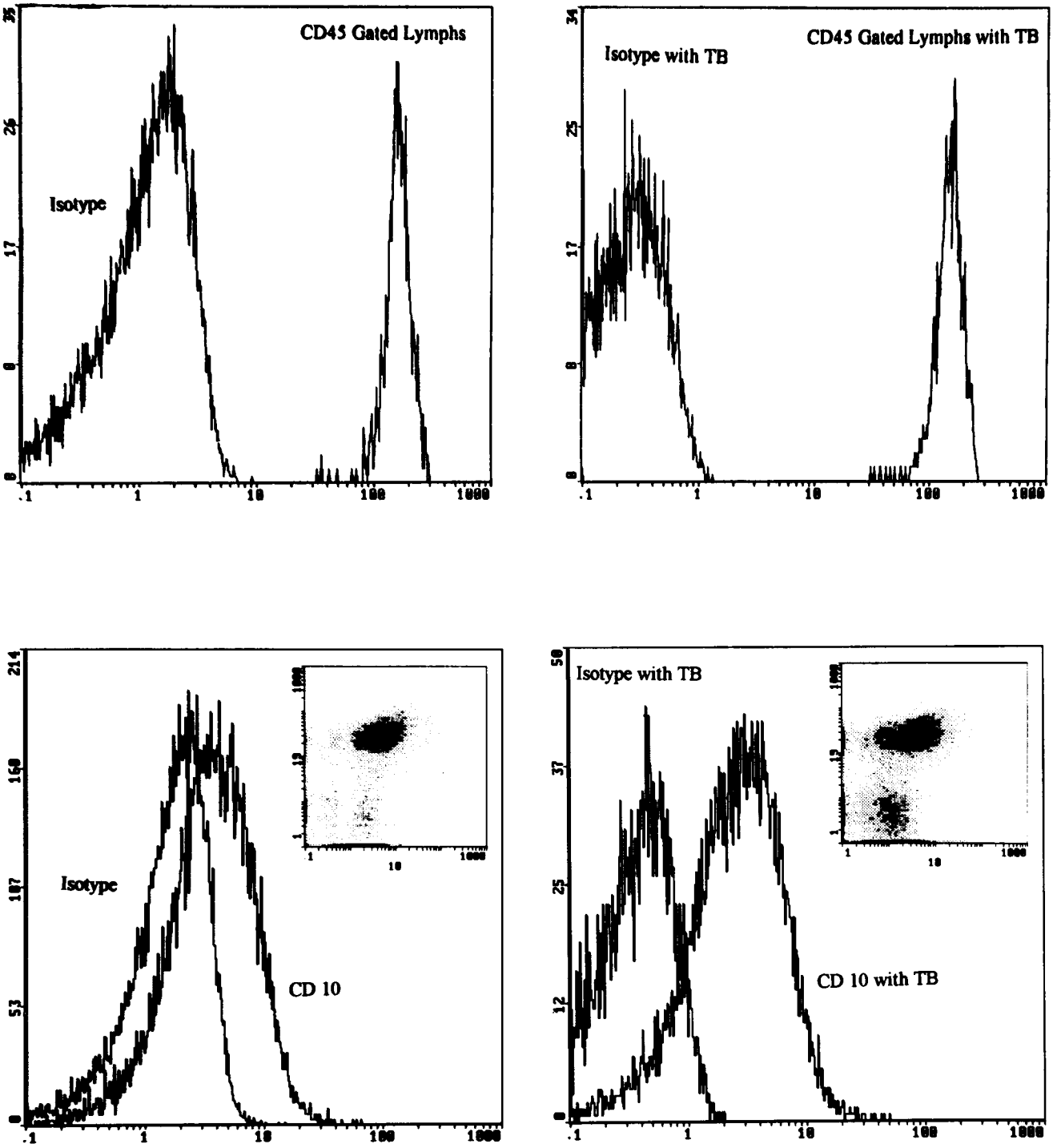

FIG. 4. Practical demonstration of trypan blue use with single (FITC) and dual (FITC/PE) labeled surface antibodies. **Top** two histograms display CD45 staining of scatter gated lymphocytes vs. isotype control, both with and without trypan blue. The **bottom** two histograms show use of dual color staining to resolve a CD10+/CD19+ population in a follicular lymphoma patient peripheral blood sample. Single histograms represent lymphocytes gated on CD19 PE positive cells. Insets show dual color (FITC/PE) dot plots gated on lymphocytes only.

are operator determined. Additionally, this alternative technique explicitly depends on the assumption that the red autofluorescence emission following 488 nm excitation is directly proportional to the autofluorescence in the green region of the spectra. Although this may be true in homogeneous populations, it needs to be verified for heterogeneous populations. Cell populations that have been thermocycled and/or hybridized would also need

evaluation, as alterations in the spectral characteristics of the cellular autofluorescence may occur (unpublished data).

DISCUSSION

Cellular autofluorescence is a common obstacle in flow cytometry, interfering with detection of low level fluorescence. Even though technologies exist to facilitate single copy gene (10,11) and sparse or diminished antigen detection (1,12,15), problematic background interference resulting from internal and non-specific fluorescent signals still limit these practices. Background fluorescence also causes significant problems in related techniques such as in situ probe detection in slide preparations and tissue specimens by FISH.

One solution to enhance current detection schemes is to amplify fluorescence on the probes and antibodies, and/or target material for these probes. Generally, these methods that introduce bulkier and/or highly modified probes can reduce specificity and potentially increase background signal (16). The technique described above is a simple yet elegant method that requires no special instrumentation or significant changes in protocol. The only limitation set is by the emission spectra overlap of antibodies and probes used with the intrinsic fluorescence characteristics of TB. However, one should note that subtle changes in the magnitude of the quenching effect might occur in differing cell populations. This is mainly due to differences in cell permeability (dye loading) and variance of intracellular distribution of autofluorescence molecules within the cell.

As demonstrated, use of this technique is appropriate with single and dual fluorescent surface markers, internal probes, and any combination thereof in intact cells and tissue specimens. This technique is rapid and adaptable. With the continued expansion of available markers and probes this methods' utility will prove very helpful in resolving dim positivity in a variety of sample preparations.

LITERATURE CITED

1. Alberti S, Parks DR, Herzenberg LA: A single laser method for subtraction of cell autofluorescence in flow cytometry. Cytometry 8:114–119, 1987.
2. Aubin, JE: Autofluorescence of viable cultured mammalian cells. J Histochem Cytochem 27:36–43, 1979.
3. Benson RC, Meyer RA, Zaruba ME, McKhann GM: Cellular auto fluorescence: Is it due to flavins? J Histochem Cytochem 27:44–48, 1979.
4. Cowen T, Haven AJ, Burnstock G: Pontamine sky blue: A counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry. Histochemistry 82:205–208, 1985.
5. Cubbage ML, Ju SC, Prashad N, Weber WD, Bressler J: Background-reducing compounds for probe-mediated fluorometric flow cytometric assays. United States Patent number 5582982, 1996.
6. Lehnert BE, Valdez YE, Stewart CC: Translocation of particles to the tracheobronchial lymph nodes after lung deposition: Kinetics and particle-cell relationships. Exp Lung Res 10:245–66, 1986.
7. Lundemose AG, Lundemose JB, Birkelund S, Christiansen G: Detection of Chlymidia in postmortal formalin-fixed tissue. APMIS 97:68–74, 1989.
8. Lynch JA, Derbyshire JB: Application of a modified indirect fluorescent antibody test for the detection of antibodies to bovine respiratory syncytial virus in Ontario cattle. Can J Vet Res 50:384–389, 1986.
9. The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals. Merck and Co., Inc., New Jersey, 1983. Monograph number 9592, p 1398.
10. Patterson BK, Goolsby C, Hodara V, Lohman KL, Wolinsky SM: Detection of CD4+ T cells harboring human immunodeficiency virus type 1 DNA by flow cytometry using simultaneous immunophenotyping and PCR-driven in situ hybridization: evidence of epitope masking of the CD4 cell surface molecule in vivo. J Virol 69:4316–4322, 1995.
11. Patterson BK, Till M, Otto P, Goolsby C, Furtado MR, McBride LJ, Wolinsky SM: Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science 260:976–979, 1993.
12. Roederer M, Murphy RF: Cell-by-cell autofluorescence correction for low signal-to-noise systems: Application to epidermal growth factor endocytosis by 3T3 fibroblasts. Cytometry 7:558–565, 1986.
13. Shapiro HM: Practical Flow Cytometry. Alan R. Liss, Inc., New York, 1988.
14. Steinkamp JA, Crissman HA: Resolution of Fluorescence signals from cells labeled with fluorochromes having different lifetimes by phase-sensitive flow cytometry. Cytometry 14:210–216, 1993.
15. Steinkamp JA, Stewart CC: Dual-laser, differential fluorescence correction method for reducing cellular background autofluorescence. Cytometry 7:566–574, 1986.
16. Wallner G, Amann R, Beisker W: Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14:136–143, 1993.
17. Wu P, Brand L: Orientation factor in steady-state and time resolved resonance energy transfer measurements. Biochemistry 31:7939–7947, 1992.