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Novel Flow Cytometry Compensation Standards: Internally Stained Fluorescent Microspheres With Matched Emission Spectra and Long-Term Stability

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In flow cytometry, the emission spectral overlap of fluorescein and R-phycoerythrin is usually corrected by electronic color compensation using microspheres surface labeled with the same fluorochromes. However, the inherent chemical instability of these fluorochromes may cause inaccurate compensation. To overcome these problems, CompenFlow beads, a new type of compensation standards were developed. The CompenFlow beads are a set of 6.0-μm-diameter polystyrene microspheres that are internally stained with selected BODIPY dye combinations. When excited by the 488-nm argon laser line, these beads show a nearly perfect emission spectral match to fluorescein-stained, R-phycoerythrin–stained and unstained lymphocytes, respectively. Moreover, since the dye molecules are oil soluble, they are contained inside the microsphere matrix instead of merely on the surface; thus, the molecules are shielded from environmental factors that could affect an exposed fluorochrome. Our results show a stable fluorescence spectral profile and constant intensity for at least 2 years stored either refrigerated or at room temperature. Cytometry 33:244–248, 1998. © 1998 Wiley-Liss, Inc.

Key terms: fluorescent probe; clinical cytometry; lymphocyte immunophenotyping

Using multicolor analysis in a modern flow cytometer, subsets of immunofluorescently stained cells can be resolved. However, the emission spectra of the fluorochromes commonly used for cell labeling, e.g. fluorescein and R-phycoerythrin (R-PE), are broader than the light collection windows generated by a flow cytometer's filters. Thus, the fluorescence signal of a dye may be received in more than one fluorescence detector. The emission overlap can be corrected by color compensation. In two-color flow cytometry, the green and red circuits of the flow cytometer can be adjusted to eliminate effects of spectral overlap and to set quadrant boundaries appropriate for the unstained, fluorescein- and R-PE–stained cells. Currently, the most popular way to conduct color compensation is by using fluorescent microspheres as compensation standards. The two important issues with this method are to have matched emission spectra and long-term stability of these standards. Polystyrene (or other polymers, such as poly[methyl methacrylate]) microspheres labeled with fluorescein and R-PE through reactive groups on the beads' surface are widely used for this purpose. These beads have an almost perfectly matched emission to the real cell labeled with fluorescent probes or fluorescent antibody conjugates. However, because of the inherent chemical instability of these fluorochromes (1), beads directly labeled with them cannot provide the same long-term stability as beads labeled with certain surrogate fluorochromes internally incorporated into the polymer matrix.

In this report, we introduce a new approach that addresses both of the concerns of spectral matching and long-term stability of flow cytometry standards. We have developed a set of uniform fluorescent microspheres stained with mixtures of novel 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) dyes (Molecular Probes, Inc., Eugene, OR) (2). The dye molecules are internally incorporated, such that the fluorophores occupy the space within the polymer matrix and are not present on the surface of the microsphere. In this way, the fluorophores are shielded by the microsphere matrix from many environmental factors that cause degradation, fluorescence quenching, or photobleaching. The ratio of the different dye molecules has been designed to give the microspheres spectral

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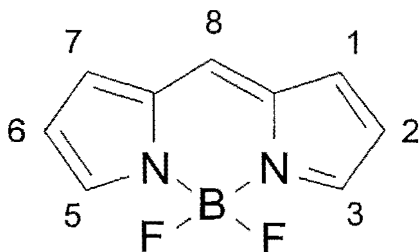

FIG. 1. The molecular structure of the BODIPY parent compound.

characteristics of intensity and wavelength distribution, that, when excited by an argon-ion laser, can be made virtually identical to the spectral characteristics of lymphocytes labeled with fluorescein or R-PE or even to the autofluorescence of unstained lymphocytes. These internally stained microsphere standards demonstrate long-term stability for at least 2 years stored either refrigerated or at room temperature. We have successfully used this set of three CompenFlow spectral matching beads (fluorescein-like beads, R-PE–like beads, and control beads) as compensation standards.

MATERIALS AND METHODS

Microspheres and Dyes

Premium grade 6.0-μm-diameter polystyrene microspheres (with a standard deviation of 0.042 μm) crosslinked with 2% divinylbenzene were purchased from Bangs Laboratory (Fisher, IN).

Derivatives of BODIPY (Fig. 1) were specially developed for use in preparing fluorescent microspheres (3). The fluorescence emission spectra of these BODIPY derivatives are adjusted by modifying the attached side groups. The pH-dependence of fluorescence intensity (FL) of BODIPY FL (emission maximum at 515 nm) and fluorescein were compared using a PTI spectrofluorometer (Photon Technology International, Princeton, NJ).

Staining Microspheres

Multiple BODIPY dyes were combined in specific ratios for the preparation of the fluorescein-like, R-PE–like, and control beads. The microspheres were gently swelled in a mixture of organic solvents containing the BODIPY dyes during constant stirring to ensure a homogeneous suspension. The organic solvents were then evaporated, returning the microspheres to their original size. The stained microspheres were then washed with distilled water containing 0.05% Tween 20 (J.T. Baker, Phillipsburg, NJ) a detergent, until the supernatant was free of dye molecules, and then sonicated in a bath sonicator. Visual analysis of a dilute aqueous suspension of the prepared microspheres by fluorescence microscopy using standard fluorescein or R-PE filter sets showed uniformly dyed green or orange fluorescent particles that were highly monodisperse. All qualified batches were shown by flow cytometric analysis to have narrow diameter and fluorescence intensity distributions.

Fluorescent Antibody Staining of Blood Cells

Whole blood from a donor was stained with a combination of anti-CD4–PE and/or anti-CD8–fluorescein isothiocyanate (FITC) (Coulter Corp., Miami, FL) for 30 min at 4°C followed by lysis with Q-prep reagent (Coulter) following the manufacturer's recommended procedure. The cells were washed with phosphate-buffered saline (PBS) containing 0.1% sodium azide and resuspended in PBS containing 0.5% formaldehyde (Polysciences, Inc., Warrington, PA).

Spectral Determination

The fluorescence spectral characteristics of the BODIPY dye-stained microspheres and fluorescent antibody-stained cells were determined using a PTI spectrofluorometer. Scattered light from the microspheres was corrected by subtracting the spectra obtained using unstained microspheres.

Flow Cytometry Measurement

All the cell and microsphere samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, [BDIS], San Jose, CA), equipped with a 15-mW 488-nm argon-ion laser, following an established procedure for a typical clinical cytometry laboratory (4). Forward- and side-scatter detectors were set for linear amplification, whereas the detectors for the fluorescence of FITC and R-PE (FL1 and FL2, respectively) were set for logarithmic amplification. A drop of each color of CompenFlow beads was added to 2 ml of sheath fluid (S/P blood bank saline; Baxter, McGraw Park, IL) and stored at 4°C until the experiments were completed. The prepared sample was then analyzed daily over 6 days using the same PMT (photomultiplier) and compensation settings over the analysis period. CaliBRITE beads (BDIS) were also analyzed in parallel for comparison and as an independent control.

Fluorescent Microsphere Stability Tests

The fluorescein-like beads were stored at room temperature, protected from light in sealed dropper bottles. The microsphere samples were tested at designated time intervals on a FACScan flow cytometer for several basic stability-related parameters, i.e., the fluorescence intensity (mean value of FL1), fluorescence uniformity (coefficient of variation [CV] on the mean value of FL1) and the CVs of forward and side scatter. Parallel samples were run on a spectrofluorometer (PTI) after being washed twice with 5 ml of sheath fluid (HAEMA-LINE 2; Baker Instruments Corp., Allentown, PA).

RESULTS AND DISCUSSION

Spectral Matching of the Fluorescein-like and R-PE–like Beads

The CompenFlow beads show a nearly perfect spectral match to labeled cells. When excited at 488 nm by an argon-ion laser, the fluorescein-like beads emit yellow-green fluorescence that matches the spectrum of fluores-

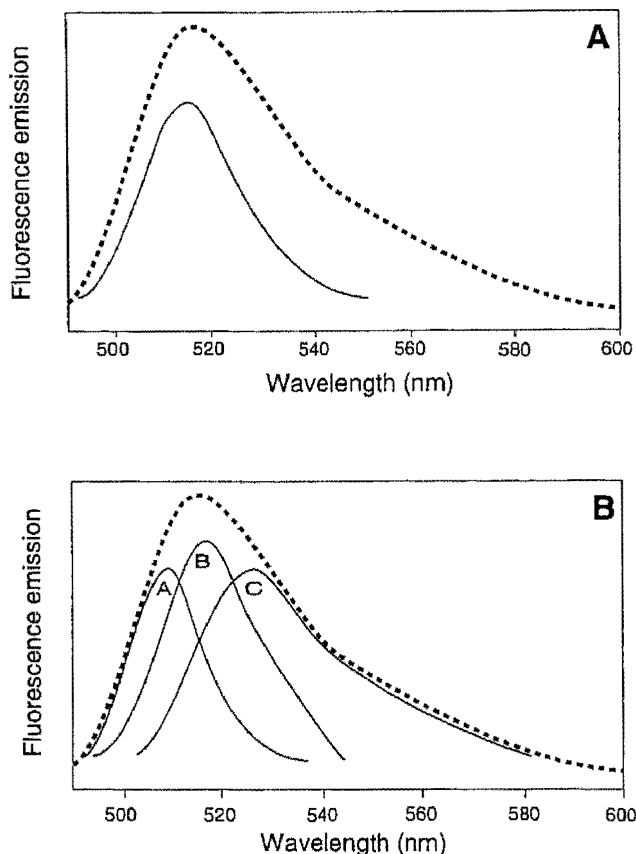

FIG. 2. Emission spectral matching. A: FITC-stained cells show a broader emission spectrum (dashed line) compared to that of single BODIPY® dye-stained microspheres (solid line). B: To obtain a near-complete spectral match, we used a mixture of BODIPY derivatives A, B and C. All of the samples were excited at 488 nm.

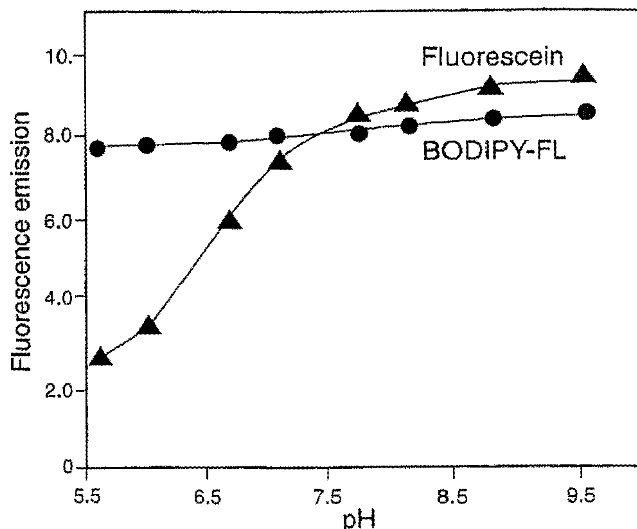

FIG. 3. Comparison of pH-dependent fluorescence of fluorescein (▲) and BODIPY-FL (●). Fluorescence intensity was measured for an equal concentration of the two dyes in 50 mM phosphate buffer, using an excitation/emission maximum of 480/515 nm.

cein-labeled cells (emission maximum approximately 515 nm) and the R-PE-like beads emit red-orange light that matches the spectrum of R-PE-labeled cells (emission maximum approximately 575 nm) (Fig. 2). Control beads, stained with a low level of the dye combinations used for both the fluorescein- and the R-PE-like microspheres, mimic the intensity of the natural autofluorescence of unstained human lymphocytes. The simplified spectral characteristics of the control beads (compared with human lymphocytes) facilitates the compensation process.

To achieve the near perfect spectral matching, the following characteristics of the BODIPY dyes are crucial: (a) they are pH-insensitive (Fig. 3); (b) there is a substantially linear relationship between dye concentration in the beads and FL where the concentrations are below the dye-dye interaction distance when fluorescence quenching occurs; (c) the narrow spectra of the dyes facilitate more precise spectral tuning with the dye mixtures; and (d) the dyes are sufficiently fluorescent to give a useful signal at the low concentrations needed to avoid most dye-dye interactions (2,4).

In determining the proportion of each of the dyes to be used to match the emission spectra of the labeled cells, the intensity values for the spectral profiles of each of the individual dye-stained beads were determined at the target excitation wavelength—the 488 nm line of the argon-ion laser, the excitation source most commonly used in flow cytometers. The intensity values for each spectral profile were added together to get a composite spectral emission profile, and the relative proportions of each of the dyes were adjusted by calculation and further optimized in practice to give a composite spectral emission substantially similar to that of the target material. Figure 4 shows how to use several BODIPY dyes that have relatively narrow emission spectra to match the broader emission spectrum of fluorescein. The final concentration of each dye incorporated into the microspheres depends on a number of factors, including size, tertiary structure, and hydrophobicity of the dye molecule and the degree of swelling of the microspheres based on solvent composition.

Internal Labeling and Long-Term Stability of Microspheres

One of the major factors contributing to the stability of the CompenFlow beads is that the dipyrrometheneboron difluoride compounds that make up BODIPY dyes are oil soluble (5). By being freely soluble in organic solvents and very sparingly soluble in water, BODIPY dyes can easily be introduced by organic solvent-based addition to previously manufactured polymer microspheres. This allows loading of the fluorophores into the matrix of each microsphere, which shields the dye molecules from many environmental factors that cause chemical instability, photobleaching, or fluorescence quenching. Furthermore, the dyes used in the microspheres cannot be significantly removed from the microspheres by water-based solvents that are commonly used as the suspension medium for the micro-

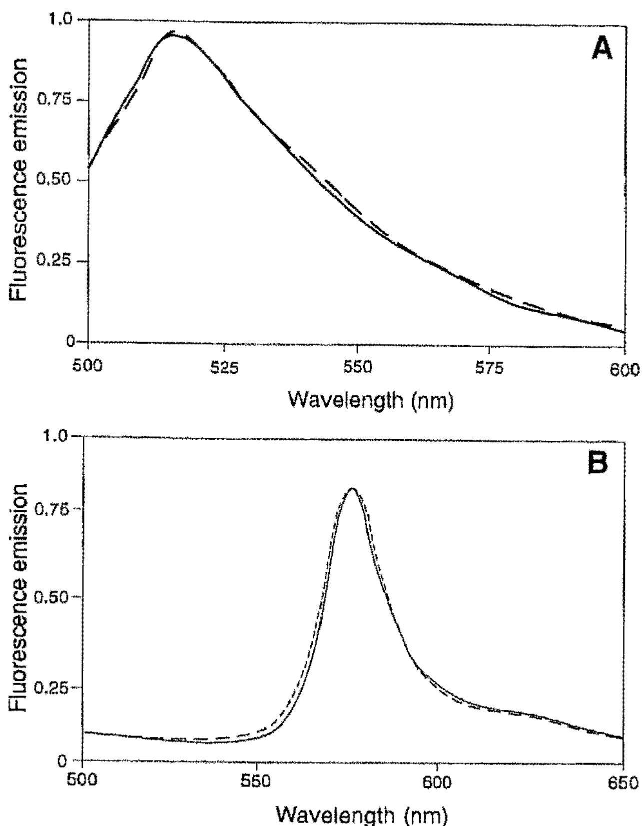

FIG. 4. **A:** With our special approach (see Materials and Methods), fluorescein-like beads (dashed line) internally stained with a mixture of BODIPY dyes show a near-perfect emission spectral match to FITC-labeled cells (solid line). **B:** A near-perfect emission spectral match is seen between R-PE-stained cells (solid line) and the R-PE-like beads (dashed line).

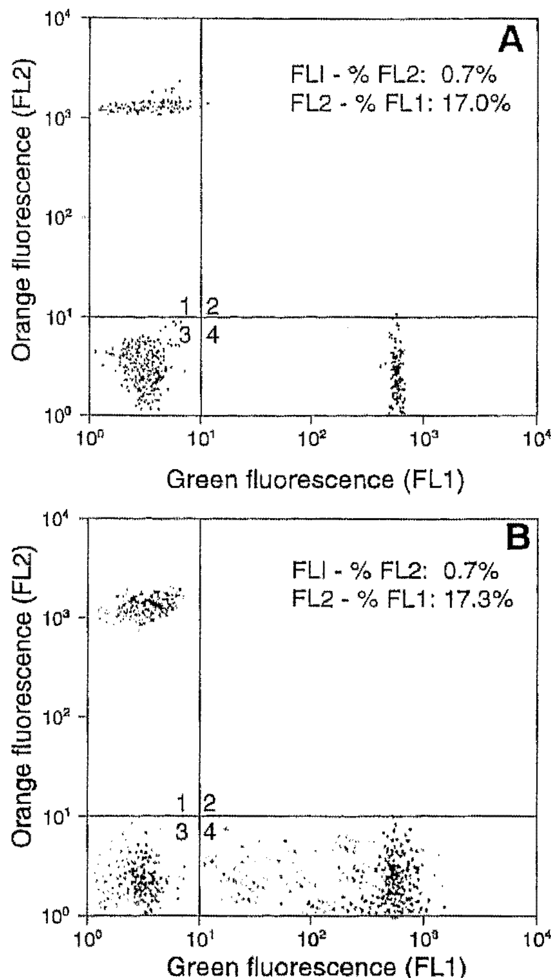

FIG. 5. Proper color compensation setting with a set of CompenFlow beads (A) in a FACScan flow cytometer. The difference of the mean intensity value of FL1 of the control beads (quadrant 3) and the R-PE-like beads (quadrant 1) was less than 4% (8 channels), whereas the difference of the mean intensity value on FL2 of the control beads (quadrant 3) and the fluorescein-like beads was less than 5% (12 channels) (averaged over 12 tests). **B:** A similar display can be achieved without significant adjustment of the settings for a set of human lymphocytes samples that is analyzed for expression of CD4 and CD8 antigens (labeled with anti-CD8-FITC and anti-CD4-PE antibodies).

sphere standards. Consequently, both the fluorescence intensity and the spectral profile of the fluorescein-like, R-PE-like, and control beads remained constant after 2 years of storage, protected from light, either at room temperature or refrigerated. We have determined that even after dilution into sheath fluid, the bead suspensions are still stable for at least 1 week. The relative hydrophilicity of FITC and R-PE requires these dyes to remain solely on the microsphere's surface, where they are exposed to many environmental factors that reduce fluorescence intensity.

Clinical Applications

Baseline settings were established for the FACScan on the first day by running the FACScomp program using CaliBRITE beads. The same settings were used each day, and freshly mixed CompenFlow beads were analyzed. Our data indicated that the x and y means for each group of beads (i.e., fluorescein-like, R-PE-like, and control beads) did not change significantly over the 6 days of testing when freshly mixed. However, if the mixture is stored at room temperature, there is a slight shift in the means after 3 days. Therefore, if the beads are to be stored at room temperature, they should be stored in separate bottles.

Next, compensation was set on the FACScan using CompenFlow beads as shown in Figure 5A. Human lymphocytes were then analyzed for expression of CD4 and CD8 antigens (labeled with anti-CD4-PE and anti-CD8-FITC antibodies) at the similar settings. Typical results are shown in Figure 5B. These results are similar to those obtained with other compensation beads, and the lymphocytes are compensated at a level acceptable for clinical testing. An acceptable level was defined such that both the difference of the mean intensity value of FL1 of the lymphocytes autofluorescence and the anti-CD4-PE antibody-labeled cells and the difference of the mean intensity value of FL2 of the lymphocytes autofluorescence and the anti-CD8-FITC antibody-labeled cells are less than 5%. The data indicate that this CompenFlow kit has met all of the specifications to serve as a compensation standard for flow cytometry. However, as with all bead

compensation standards, the final compensation settings should be established with the cells and antibodies that will be analyzed in the laboratory.

Flow cytometry assays are widely used in clinical laboratories. To limit the possibility of any artifact introduced in the assays, the methodology and/or instrumentation is critical (6). Sophisticated hardware and software for flow cytometric analysis have been redesigned and optimized frequently in response to customer requests. For calibration standards, most efforts have been focused on protocol-related issues, not on quality issues of the standards themselves. In contrast with the rapid advance in instrumentation, most of the fluorescence standards available today have remained old-fashioned. As interest in quantitative fluorescence cytometry grows, the continuous improvement of calibration standards becomes increasingly important (7,8). The development of internally stained fluorescent microspheres with both long-term stability and emission spectra matching those of commonly used fluorochromes represents a promising new tool for both basic calibration studies and practical implementation in the biomedical laboratory.

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