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Fluorescent Erythrocyte Ghosts as Standards for Quantitative Flow Cytometry

S.K. Doberstein, G. Wiegand, L.M. Machesky, and T.D. Pollard

Departments of Cell Biology and Anatomy (S.K.D., L.M.M., T.D.P.) and Medicine (G.W.), Johns Hopkins University School of Medicine, Baltimore, Maryland

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We report here a quick and inexpensive method for preparing standards of known fluorochrome content for calibration and quantitation of flow cytometry fluorescence signals. Erythrocyte ghosts prepared by hypotonic lysis are filled with solutions containing fluorescently labeled dextran. Standards prepared by this technique have a narrow range of fluorescence and a linear response of fluorescence to fluorochrome content up to 2×10^6 fluorochrome molecules/cell. The volume of ghost standard particles is roughly 70 femtoliters (fl)/cell. The fluorescence of ghost standards is nearly identical to that of commercially available microbead standards of similar fluorochrome content. Ghost standards have stable fluorescence for at least 3 weeks at 4°C. These standards can be made with any fluorochrome or combination of fluorochromes over a wide concentration range. © 1995 Wiley-Liss, Inc.

Key terms: FACS, fluorescence, erythrocyte ghosts, quantitative cytometry, flow cytometry standards, dextran, fluorescein isothiocyanate, rhodamine

Quantitation of fluorescence measured by flow cytometry requires calibration of the fluorescence channel number with standards of known fluorochrome content (1,5). The standards define the relationship between the observed fluorescence level of the cell and the number of bound fluorochrome molecules per cell. Quantitation of the fluorescence signal is used in many applications, including determination of the number of fluorescent antibody molecules bound to a cell surface (6) and measurement of kinetic rate constants for binding of fluorescent ligands to cell surface receptors on living cells (4).

We describe here the use of erythrocyte ghosts filled with solutions of fluorochrome-labeled dextran as quantitative standards for flow cytometry. These preparations are easy and inexpensive to produce, and yield standard curves comparable to commercially available microbead standards.

MATERIALS AND METHODS

Production of Fluorescent Erythrocyte Ghosts

We produced erythrocyte ghosts by a modification of the method of Steck and Kant (11). We centrifuged 5 ml of freshly drawn human blood for 5 min at 800g in a clinical centrifuge at 4°C. After decanting the supernatant, we resuspended the pellet in 40 ml of phosphate buffered saline (PBS)-EDTA (150 mM NaCl, 7.5 mM $NaPO_4$, pH 7.5, 1 mM EDTA) and centrifuged the suspension for 5 min as described above. We carefully removed the supernatant and buffy coat and repeated this washing procedure 2 more times. We then lysed the purified erythrocytes by rapid dilution and mixing of the pellet in ice cold 5P75 (5 mM $NaPO_4$, pH 7.5). We stored this suspension on ice for 10 min and then pelleted the erythrocyte membranes by centrifugation for 15 min at 22,000g in a Beckman JA-20 rotor at 4°C. We washed the ghosts 3 times in cold 5P75 followed by centrifugation as described above. After each spin we separated the loose ghost pellet from the small underlying pellet of unlysed cells. After the final pelleting we resuspended the purified ghosts in 2 ml of 5P75 and stored them on ice until use, generally the same day. For samples used in storage life studies, we added NaN_3 to 0.02% and stored the samples at 4°C.

We prepared 100 mg/ml stock solutions of either rhodamine- or fluorescein-labeled dextran, molecular weight 70,000, in 5P75 (Sigma Chemical Co., St. Louis, MO, or Molecular Probes, Eugene, OR). The standard solutions were made by dilution of a stock solution of fluorochrome-dextran, which was made from the dry reagent and buffer. We therefore know the concentrations by cal-

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S.K. Doberstein is now at the Department of Molecular and Cell Biology, Division of Neuroscience, 519 LSA, University of California, Berkeley, Berkeley, CA 94720. Address reprint requests there.

L.M. Machesky is now at the Medical Research Council Laboratory of Molecular Biology, Cambridge, England.

culation. The appropriate dilutions were made into 5P75 to a volume of 900 μl, and 100 μl of the purified ghosts was added. We incubated the ghost/fluorochrome suspension on ice for 30 min. We used the manufacturer's specification for the ratio of moles fluorochrome/mole dextran in our calculations.

The ghosts were resealed by addition of 110 μl of 10 × PBS-EDTA (1.5 M NaCl, 75 mM $NaPO_4$, 10 mM EDTA, pH 8.0) and incubation at 37°C for 45 min. We centrifuged the sealed fluorescent ghosts for 5 min at 10,000g in an Eppendorf Model 5415 microfuge at room temperature. After aspirating off the supernatant, we resuspended the ghosts in 1 ml PBS-EDTA and centrifuged as above. We repeated this wash step 3 times. After the last wash, we suspended the fluorescent ghosts in 3 ml PBS-EDTA with 0.02% NaN_3. We stored the fluorescent ghosts at 4°C until use. We calculated the number of molecules of fluorochrome per erythrocyte ghost by assuming a volume of 70 femtoliters (fl) for an average erythrocyte ghost as determined by calibration of ghost standards against stock solutions of fluorochrome-labeled dextran (see below).

Flow Cytometry

We measured the fluorescence of ghost standards using a FACStar+ flow cytometer (Becton Dickinson Immunocytometry Division, San Jose, CA). The light source for excitation was an Innova 70 argon ion laser (Coherent, Palo Alto, CA) regulated to 50 mW in the TEM00 mode monitored by the flow cytometer. We periodically checked the laser power output and stability with an external thermocouple power meter (Coherent). The transit time for a 7 μm diameter particle through the 20 μm laser beam was adjusted to about 27 μs to assure consistent excitation energy with minimal quenching. The emission flash was collected and focused on a Hamamatsu R1477 side window photomultiplier tube by a high numerical aperture (NA) objective lens. We excluded scattered light from the fluorescence channel with a multilayered bandpass filter in the emission light path. For fluorescein measurements, we used 488 nm excitation with a 530DF30 multilayered bandwidth filter. For rhodamine measurements, we used 514 nm excitation with a 600LFA long pass filter.

To test the accuracy of the log amplified signal, microbead standards were allowed to form doublets and their fluorescence was measured on the flow cytometer. The log amplified signals of the 1 × and 2 × pairs are consistent. The data suggest that we have a constant transfer function throughout the entire range of the amplifier. Pulse heights from the amplifier were digitized and displayed as a histogram. The population fluorescence means were calculated using the LYSYS 2 cytometry data collection and analysis software (Becton Dickinson Immunocytometry Division). For shelf life testing, the flow cytometer was set up using the same calibration settings each time. The calibration was checked using commercial microbead standards.

Verification of Fluorochrome Content and Volume Determination

We verified the fluorochrome content of ghost standards by comparing the fluorescence of standard suspensions with the fluorescence of several fluorochrome-dextran solutions of known concentrations, and adjusting for the number of ghosts per unit volume. We performed fluorescence measurements in a Perkin-Elmer model 650–10S fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) using the same cuvette for all measurements made with this instrument.

Since this calibration requires knowledge of the concentration of ghosts in the suspension, we counted the number of ghosts per milliliter in the ghost suspensions with a Coulter counter model ZF (Coulter Electronics, Hialeah, FL). We performed Coulter counter measurements on lysed ghosts that were not resealed as a control. Ruptured ghosts scatter light differently from filled ghosts and can be discriminated (and eliminated from consideration) on that basis, both in Coulter counter measurements and in flow cytometry experiments.

Comparison to Microbead Standards

We compared the fluorescence of ghost standards to that of quantitative fluorescent microbeads (Quantum Series, Flow Cytometry Standards Corporation, Research Triangle Park, NC) as measured by flow cytometry (see above).

RESULTS

Erythrocyte ghost standards yield narrow, repeatable fluorescence profiles by flow cytometry (Fig. 1A). The standard deviation of the fluorescence in our experiments ranges from 22% to 30% of the mean fluorescence. The median fluorescence and mean fluorescence of a given standard preparation typically differ by less than 3%.

Ghost standards have a linear response of fluorescence to molecules of fluorochrome over a range of 100,000 to 2 million fluorescein isothiocyanate (FITC) molecules per cell (Fig. 2A) or 100,000 to 2 million rhodamine molecules/cell (Fig. 2C). Above 2 million fluorochrome molecules/cell the fluorochromes undergo intermolecular quenching which renders the response nonlinear although still usable (Fig. 2B,D). We did not measure ghosts containing less than 100,000 molecules per cell.

We directly verified the amount of fluorochrome per ghost by measuring the fluorescence of a suspension of known fluorescein-ghost concentration and comparing it to the fluorescence of standard solutions of fluorescein-labeled dextran. By this method we determined the volume of fluorochrome solution in each ghost to be 65 fl.

The fluorescein ghost standards have nearly equivalent fluorescence to commercially available standards of similar fluorescein content (Fig. 3). Fluorescein ghost standards and microbead standards of similar fluorescein content have very similar fluorescence distributions (Fig. 1).

Storage at 4°C in PBS with 0.02% *sodium azide has no*

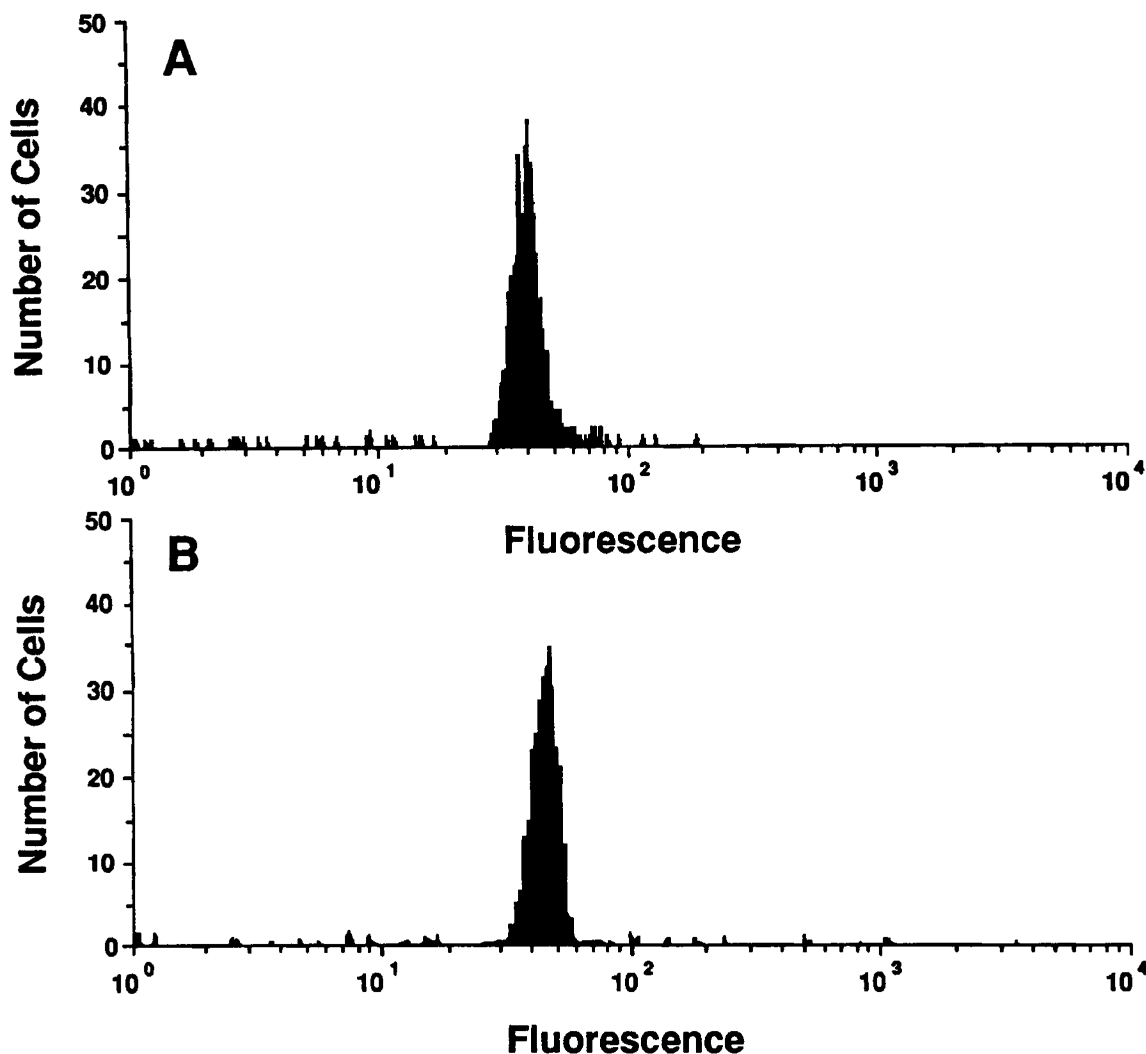

FIG. 1. Histograms of fluorescence level of standards as measured by flow cytometry. A: Ghost standards filled with fluorescein-labeled dextran. B: Commercial quantitative microbead standards (FCSC Quantum™ microbeads, Flow Cytometry Standards Corporation) of similar equivalent fluorochrome content.

effect on the fluorescence of erythrocyte ghosts containing up to 10^6 fluorescein molecules/cell for at least 3 weeks (Fig. 4). Fixation with 3% paraformaldehyde eliminates ghost fluorescence completely within 24 h (data not shown). Some high concentration standards ($>10^7$ fluorescein molecules/cell) increase in fluorescence by as much as 2-fold over 2–3 weeks.

DISCUSSION

Plastic microbeads were first used as standards for quantitative flow cytometry by Sklar et al. (10) and have now received wide acceptance (1,5). Most commercially available quantitative standards consist of acrylic microbeads with fluorochrome molecules dispersed throughout the plastic or attached to the surface. Beads with large numbers of fluorochrome molecules are larger in size, implying that the matrix has some defined capacity for attachment or inclusion of fluorochrome. The microbeads are typically calibrated to standard solutions of fluorochrome by the manufacturer to yield the number of equivalent soluble fluorescent molecules. Plastic microbeads were also used as standards by Oonishi and Uyesaka (8). These workers covalently linked FITC to the surface of plastic microspheres. The amount of FITC bound to the microspheres was quantitated by solubilizing the spheres, measuring the fluorescence of the resulting solution in a fluorimeter, and comparing that fluorescence to standard fluorochrome solutions. Quantitative microbead standards typically deviate from nominal fluorescence by as much as 15–22% (4).

Two other particulate standards have been described. Fixed calf thymocyte nuclei labeled with fluorescein (2) were used for quantitation of flow cytometry fluorescence and gave excellent results in an international study

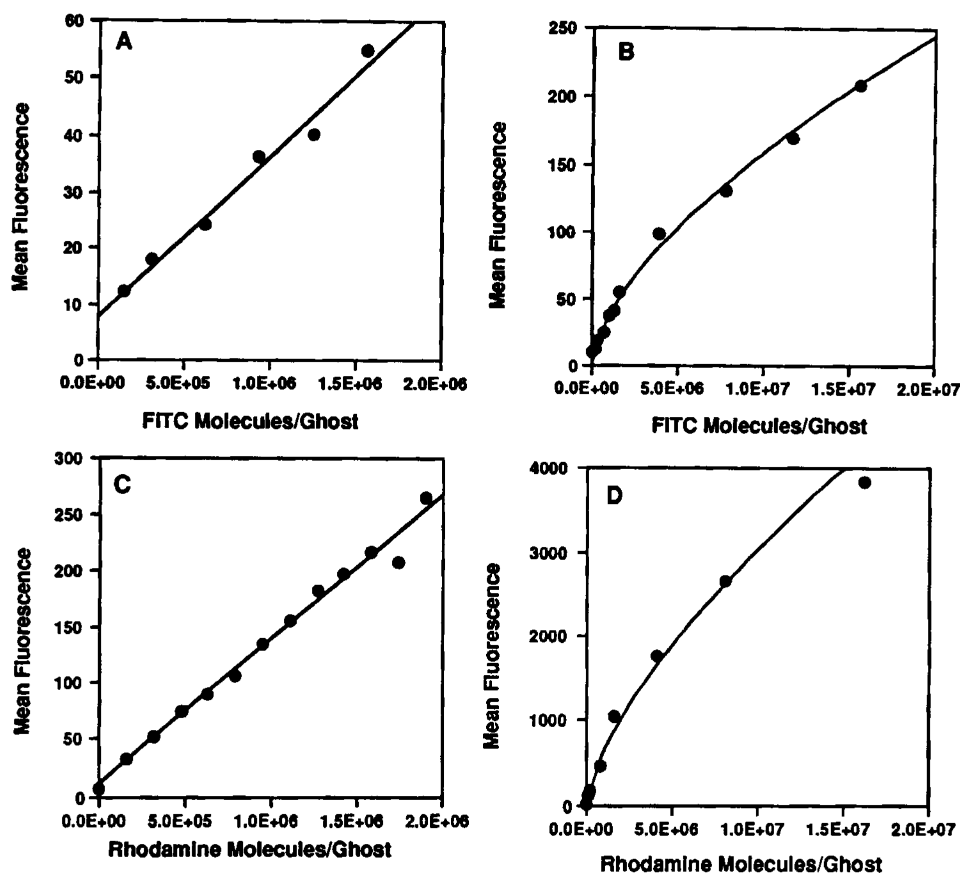

FIG. 2. Plots of mean fluorescence vs. number of fluorochrome molecules for ghost standards filled with fluorochrome-labeled dextran as measured by flow cytometry. **A**: FITC-labeled dextran, linear range. **B**: FITC-labeled dextran, total range. **C**: Rhodamine-labeled dextran, linear range. **D**: Rhodamine-labeled dextran, total range.

(12). The use of glutaraldehyde-fixed chicken erythrocytes as standards was described by Kávai et al. (6). The glutaraldehyde in these intact cells becomes fluorescent after 48 h fixation. Kávai et al. (6) calibrated this preparation by comparison to fluorescent dye solutions using steady-state fluorimeter methods. This technique is inexpensive but inaccurate since only a single standard point is used for calibration.

Ledbetter et al. (7) used fluorescein standard solutions to calibrate flow cytometers. Since almost all commercially available flow cytometers require a pulsed signal from a particle passing though the laser beam to trigger data collection, the use of (pulseless) solution standards is inconvenient.

Erythrocyte ghosts loaded with fluorescent dextran have several advantages over currently available microbead standards. First, the cost of production of ghost standards is very low. Laboratory quantities cost less than $50.00 per gram to produce, and the manufacturing technique is simple and rapid. Second, the size of the standards is the same throughout the range of fluorescence. Third, the method is applicable to any soluble fluorochrome or combination of soluble fluorochromes that can be conjugated to a macromolecule, including proteins. Fourth, the number of fluorochrome molecules is explicitly known and is not determined by comparison to other standards. Fifth, for quantitation of antibody binding, the ghosts may be filled with the labeled antibody and used directly for self-standardization. This avoids the problem of controlling for quenching of the fluorochrome fluorescence by the antibody to which it is conjugated. Sixth, the fluorochrome is contained in a volume and in an ionic environment similar to that of the cells being tested by flow cytometry.

The distribution of fluorescence for a preparation of ghost standards is narrow, with a standard deviation of fluorescence of less than 30% of the mean fluorescence (Fig. 1). By comparison, in our hands commercial microbead standards typically have a standard deviation of about 25% of the mean fluorescence.

Erythrocyte ghost standards have a linear response of fluorescence to fluorochrome content up to 2×10^6 molecules/ghost (Fig. 2). Above this limit, quenching of the fluorochrome leads to deviations from linearity. The

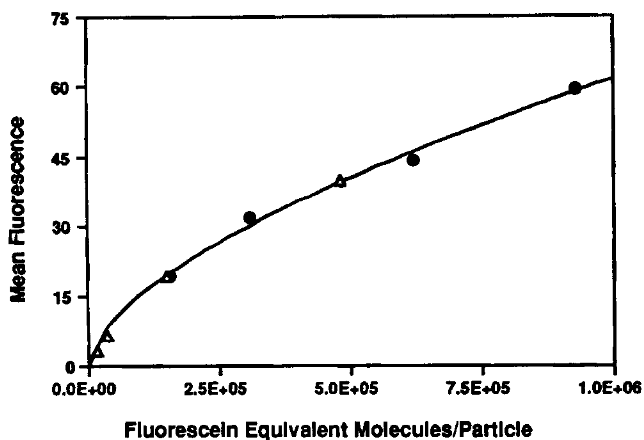

FIG. 3. Plot of mean fluorescence vs. number of fluorescein molecules per particle for ghost standards and quantitative microbead standards as measured by flow cytometry. (△) Ghost standards; (●) FCSC Quantum™ microbeads.

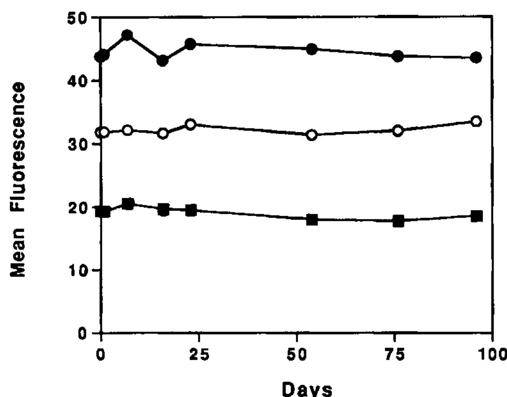

FIG. 4. Plot of mean fluorescence of ghost standards vs. time in storage at 4°C as measured by flow cytometry. (●) 6.2 × 10^5 FITC molecules/ghost; (○) 3.1 × 10^5 FITC molecules/ghost; (■) 1.55 × 10^5 FITC molecules/ghost.

magnitude of these deviations is consistent with measurements of fluorescence quenching of similar fluorochrome solutions in small vesicles (3,9).

We indirectly measured the volume of erythrocyte ghost standards by measuring the steady-state fluorescence of a suspension of ghost standards and comparing this to stock fluorochrome-dextran solutions. We find that the volume measured by this technique is 65 fl. It is possible, however, that the fluorochrome molecules inside the ghosts are quenched, which would lead to an underestimate of the volume (3).

Ghost standards containing up to 10^6 FITC molecules/ghost are stable for at least 3 months when stored at 4°C in PBS containing sodium azide (Fig. 4). Some preparations containing highly concentrated fluorescein-dextran solutions (>10^7 molecules/cell) appear to increase in fluorescence after 2–3 weeks of storage. The increase is as much as 2-fold, with no apparent change in the light scattering profile of the sample. Standard preparations with FITC content in the range of that normally used in flow cytometry (i.e., <10^6 molecules/cell) are quite stable, and higher concentrations are accurate if used within 1 week of manufacture.

LITERATURE CITED

1. Agrawal YP, Mahlamäki EK, Pentitilä IM: Use of quality control standards in clinical flow cytometry. Ann Med 23:127–133, 1991.
2. Brown MC, Hoffman RA, Kirchanski SJ: Controls for flow cytometers in hematology and cellular immunology. Ann NY Acad Sci 468:93–103, 1984.
3. Chen RF, Knutson JR: Mechanism of fluorescence concentration quenching of carboxyfluorescein in liposomes: Energy transfer to nonfluorescent dimers. Anal Biochem 172:61–77, 1988.
4. Fay SP, Posner RG, Swann WN, Sklar LA: Real-time analysis of the assembly of ligand, receptor, and G protein by quantitative fluorescence flow cytometry. Biochemistry 30:5066–5075, 1991.
5. Horan PK, Muirhead KA, Slezak SE: Standards and controls in flow cytometry. In: Flow Cytometry and Sorting. New York, Wiley-Liss, 1990.
6. Kávai M, Bodolay E, Szöllősi J: Characterization of human monocyte subpopulations by flow cytometry. Immunology 47:255–262, 1982.
7. Ledbetter JA, Rouse RV, Micklen HS, Herzenberg LA: J Exp Med 152:280, 1980.
8. Oonishi T, Uyesaka N: A new standard fluorescence microsphere for quantitative flow cytometry. J Immunol Methods 84:143–154, 1985.
9. Plant AL: Mechanism of concentration quenching of a xanthine dye encapsulated in phospholipid vesicles. Photochem Photobiol 44: 453–459, 1986.
10. Sklar LA, Finney DA, Oades ZG, Jesaitis AJ, Painter RG, Cochrane CG: The dynamics of ligand-receptor interactions. J Biol Chem 259: 5661–5669, 1984.
11. Steck TL, Kant JA: Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol 31: 172–180, 1974.
12. Vogt RF Jr, Cross GD, Phillips DL, Henderson LO, Hannon WH: Interlaboratory study of cellular fluorescence intensity measurements with fluorescein-labeled microbead standards. Cytometry 12: 525–536, 1991.