

Classification and Properties of 64 Multiplexed Microsphere Sets

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Received 8 June 1998; Accepted 10 June 1998

We describe a practical method for the analysis of multiple analytes in a single sample. The vehicle for each separate measurement consists of a set of microspheres identifiable by characteristic fluorophores embedded in the particles. The use of robust, bench-top flow cytometers (flow microfluorimeters) for the analysis of the multiple sets of microspheres is facilitated by hardware and software, which acquire the data from the cytometer, classify the microspheres according to sets, and collate measurement information from each microsphere set in real time. This measurement system can analyze up to 64 analytes in a single sample. The advantages of multiplexed assays using flow cytometry include robust

measurements, because each microsphere set is measured repeatedly. The advantage of the assay's is consistent with simultaneous measurement of many parameters as well as the speed with which the flow microfluorimeter (cytometer) makes measurements (many hundreds per second). Here, we describe the properties of the microspheres, the calibration of the cytometer, and the influence of the properties of the microspheres on the sensitivity of measurements. Cytometry 33:234-243, 1998. © 1998 Wiley-Liss, Inc.

Key terms: flow microfluorimetry; microspheres; multiplexing; molecular analysis; measurement space; calibration

Although a patent was issued in 1976 to Fulwyler (1), Horan and Wheelless (2) made the first broadly published suggestion for the use of flow cytometers for the determination of analytes in serum or other fluids by reactions at the surface of microspheres in 1977. A more complete description of such an assay (3) and many additional demonstrations of the use of microspheres for analysis of serum proteins followed this first demonstration (4–25). Recent reviews (26–27) have covered the many uses of such assays and the advantages they afford for rapid, sensitive, and multiplexed analysis of antibodies, antigens, and other soluble molecules, including nucleic acids, drugs, and enzymes.

Taking into consideration favorable features of the microspheres, a multiplexed system was designed that, including the hardware and software necessary for the facile analysis of the microspheres, uses widely available bench-top flow cytometers (flow microfluorimeters) such as FACScan and FACSCalibur.

The hardware and software were designed to identify, in real time, sets of microspheres based on their fluorescence. The basic strategy of the multiplexed system was to use three parameters, (side scatter, orange fluorescence [Fl 2], and red fluorescence [Fl 3]) to classify sets of FlowMetrix microspheres. A fourth parameter, green fluorescence [Fl 1], was reserved to measure the chemical or

ligand-ligate reaction occurring on the surface of each microsphere set (28).

In this report, we describe the measurement of the microspheres and their classification by the FlowMetrix System. The standardization and calibration of the flow cytometer is a critical feature in the development of an accurate flow cytometric measuring system. In addition, we discuss features of the multiplexed system that outline the range of the assay parameters.

MATERIALS AND METHODS

Microspheres

Microspheres were the generous gift of the Luminex Corporation (Austin, TX).

Hardware

A GPIO cable extends from the FACScan or FACSCalibur to a switch box and cables are connected to the original computer furnished with the FACScan (Hewlett Packard

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or Macintosh) and a PC bearing an ISA card with GPIO connector. The PC requires an Intel Pentium processor of 133 MHz or higher, 32 MB of RAM, and available I/O ports from hexadecimal 0240 through 0245M. It also must be running FlowMetrix software (Luminex Corporation). The ISA board uses a digital signal processor.

Software

The FlowMetrix software runs under Windows 95. The software controls the FACScan and allows the user to set compensation parameters, amplifier values, and PMT voltages. The system provides additional information to the user, including flow rates and progress of the data collection.

The classification procedure simplifies the data analysis from a highly multiplexed system. In general, the board and software function to control the cytometer; capture data from the FACScan; process the data; and classify microspheres into sets on the basis of light scatter, orange, and red fluorescence information. Only information that passes through a side-scatter gate is evaluated, so only uniform single particles are classified.

Standardization and Calibration

The FlowMetrix software currently uses two calibration microspheres (provided by Luminex Corporation), and each instrument is calibrated with this standard. An automatic calibration feature of the software adjusts the PMT voltages so that microspheres of each calibration type fall within the classification parameters allocated for them. When calibration is achieved, the 64 different microspheres fall within the allocated gate characteristics for side scatter, Fl 2, and Fl 3. The voltage of the PMT for Fl 1 is also set based on the "spillover" fluorescence. In this fashion, each instrument performs at the same level for classification.

Data Collection

A FACScan and a FACStar Plus were used to collect the data presented in this report. Data from the calibration of a FACSCalibur were also used. The FACScan was used with both Lysis II and the FlowMetrix system; FACStar Plus data were collected with Lysis II. FlowMetrix, WinMidi 2.6, and Lysis II software were used to process the data. For the calculations of statistical parameters, the FCS files were converted to ASCII text as channel numbers. The channel numbers were converted into fluorescence intensities, and then mean fluorescence intensity (MFI), standard deviation (S.D.), and coefficient of variation (CV) values of the channel numbers were calculated using SYSTAT. WinMidi, SYSTAT, or SigmaPlot was used to create the graphs.

RESULTS

Design and Construction of FlowMetrix Microspheres

The design of microspheres weighs different properties to achieve a useful material; the *Latex Course Book* (27)

discusses some of the issues. The FlowMetrix microspheres were cross-linked during polymerization for greater physical and thermal stability. The polymerization was modified so that many carboxylate groups were available at the surface, to provide stability in high-ionic-strength media and to serve as a functional group for bond formation in the coupling of molecules to the microspheres. The microspheres were of sufficient size (5.6 μm) and uniformity ($<1\%$ CV for diameter) that the surface would couple enough molecules ($1\text{--}2 \times 10^6$) to each microsphere to ensure high signals and a satisfactory range when the reporter molecules associate with a microsphere set. For convenience, we called the molecules attached to the microsphere surface "target" molecules and the labeled molecules that bind to the microsphere "reporter" molecules.

Although the structures of the proprietary dyes are not described here, the fluorescence properties of the dyes within the microspheres provide levels of orange (575 nm) and red (>620 nm) fluorescence. To ensure the uniformity of chemical coupling technology, the microspheres used were the same size, prepared from a single large lot. Because the surface features were common to all sets of microspheres, optimization with one set of microspheres would suffice for all other microspheres sets.

Scatter of Laser Illumination by Microspheres in Flow Cytometers

Laser illumination of the microspheres during analysis in a flow cytometer produces forward- and side-scatter values. As viewed with electron microscopy, the diameter of the microsphere population is highly uniform, with a 1% CV. As viewed with forward-angle light scatter in the FACScan and the FACStar Plus, the population also appears to be highly uniform. Data of the unstained microspheres obtained from the FACStar Plus with linear amplification show a Gaussian peak, with a CV of 0.0251% (Fig. 1A). With the use of log amplification and a geometric estimation of mean value, we observed a CV of 0.0096%. Similar values were obtained with a FACScan. Microspheres were suspended in water and run with water as the sheath fluid. When side scatter was analyzed, a spread of values was obtained. A sizable fraction of the population has a higher level of side scatter. In contrast, the forward-angle light scatter showed high uniformity. This pattern of forward and side light scatter was shown by Doornbos et al. (29) to be a consequence of the Lorenz-Mie scatter by refractive, uniform polystyrene spheres. The side-scatter values have a wide range because very small differences in size within the population of microspheres give large differences in side scatter.

Correlation of Light Scatter and Fluorescence in Microsphere Populations

The variation in particle size by side scatter suggests that different microspheres may contain different amounts of

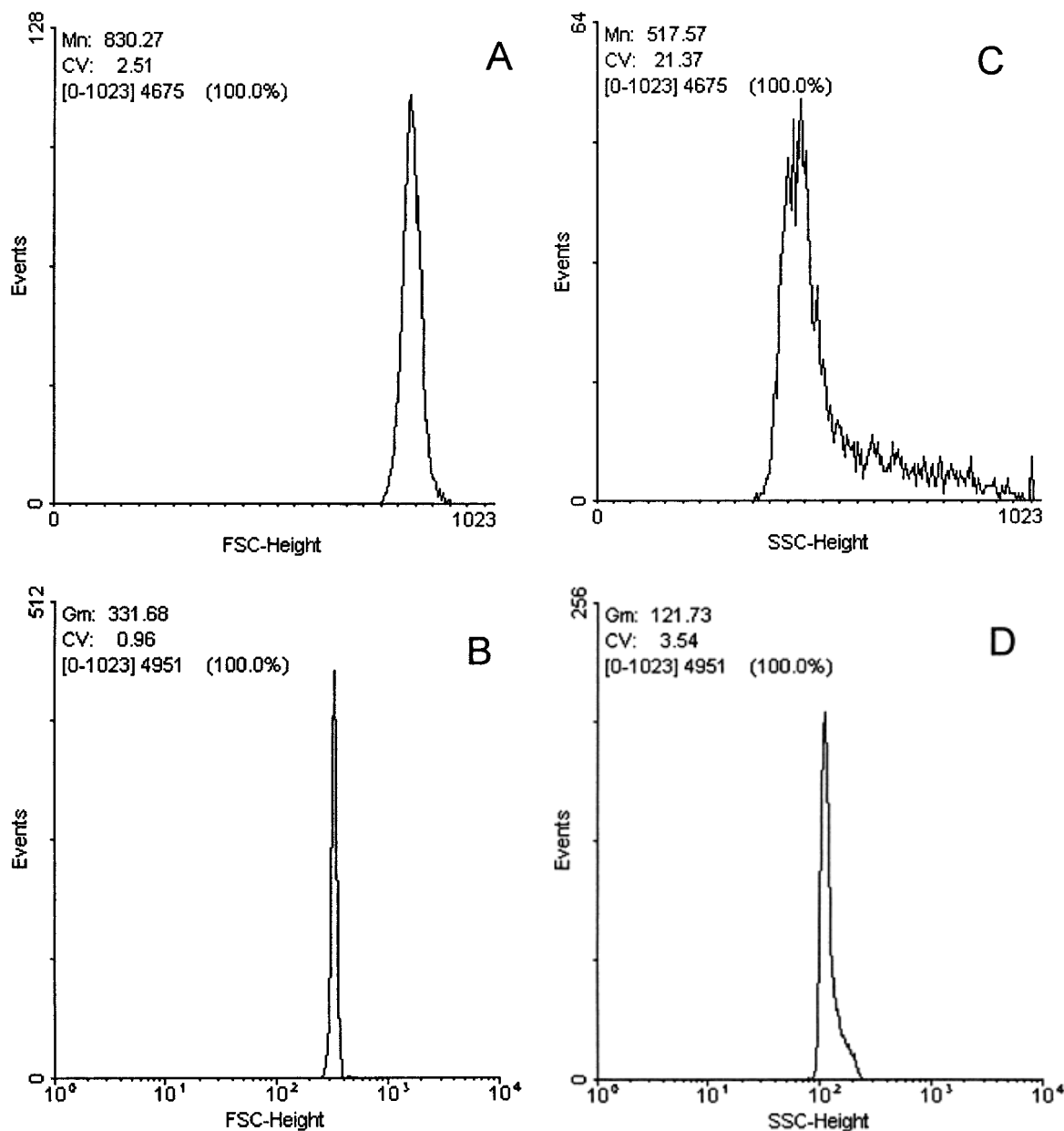


FIG. 1. Light scatter by FlowMetrix microspheres. **A:** Forward-angle light scatter of unstained microspheres used for the construction of microsphere sets. Data were collected with a FACStar Plus using linear amplification. **B:** Forward-angle light scatter of the same microspheres,

with logarithmic amplification. **C:** Side scatter of the same microspheres, with linear amplification. **D:** Side scatter of the same microspheres, with logarithmic amplification.

dye, which leads to variation in the level of fluorescence within a microsphere population. There is a strong correlation between side scatter and red fluorescence (Fl 3) in a stained microsphere set (Fig. 2, middle). The same microsphere set shows a slight negative correlation between Fl 3 and forward light scatter.

Sorting experiments (data not shown) show that the microspheres with high side scatter and high red emission are distinct and can be sorted from the other microspheres. Thus, this variation is not due to orientation

artifact and the higher levels of light side scatter (and forward scatter) are true measures of differences in particle size.

Calibration of the Data Space With Software and Standard Particles

To reproducibly and reliably classify 64 distinct microsphere sets, the PMTs for each flow cytometer were adjusted so that the appropriate microsphere sets fell within the gate limits for the classification of each micro-

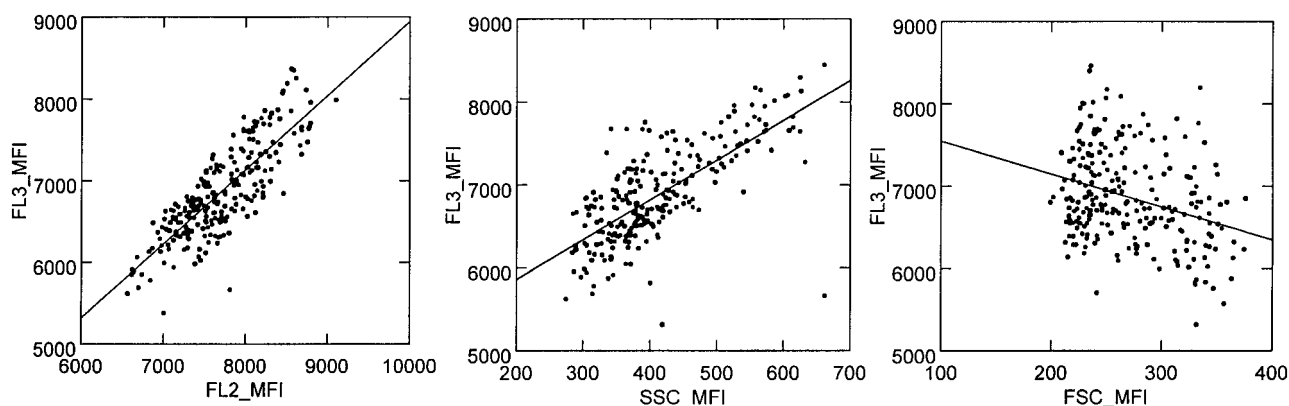


FIG. 2. Correlation within a microsphere population of light scatter and fluorescence. **Left:** Correlation of FL 2 and FL 3 within the microsphere set with the largest fluorescence values. Slope is 0.731 ± 0.033 S.E., and r^2 is 0.668. **Middle:** Correlation of FL 3 with the side scatter. Slope is $0.107 \pm$

0.0066 S.E., and r^2 is 0.520. **Right:** Correlation of forward scatter and FL 3. Slope is -0.024 ± 0.0048 S.E., and r^2 is 0.0944. The sample size was 247 microspheres.

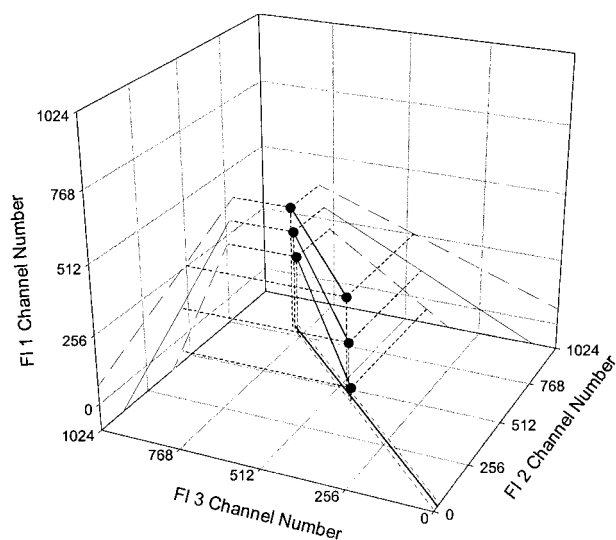


FIG. 3. Properties of microspheres used to calibrate flow cytometers for multiplexed microsphere classification. The middle line joining the middle points is the regression line of the two populations used for classification for FL 1, FL 2, and FL 3; top and bottom points and lines indicate ± 3 S.D. for the three values. Regression lines are projected on the three two-dimensional planes, and dashed lines are the ± 3 S.D. limits.

sphere set. To standardize the PMT voltages, a software routine was constructed to adjust the PMTs with the aid of two specific microsphere sets: one with low and the other with high levels of both the FL 2- and FL 3-emitting dyes. The software adjusts the FL 2- and FL 3-PMT voltages until the mean measure of FL 2 and FL 3 falls within a predetermined channel value.

The characteristics of the two microsphere sets used for this calibration procedures are shown as a three-dimensional plot in Figure 3. Six points are joined by three lines. The middle line (z-axis) joins the mean channel numbers of each population, and the two other lines represent ± 3 S.D. On the three planes (FL 2 $\times$ FL 3, FL 1 $\times$ FL 2, and FL 1 $\times$ FL 3), the mean ± 3 S.D. of the two parameters are

Table 1
PMT Values Found Upon Calibration of Eight Bench-top Flow Cytometers

Instrument	Date of calibration	PMT setting			
		SSC	FL 1	FL 2	FL 3
A	3/18/98	239	668	573	658
B	3/4/98	320	619	663	660
C	3/17/98	307	661	574	681
D	3/19/98	251	549	566	560
E	4/9/98	269	586	621	521
F	5/18/98	291	538	548	572
G	5/22/98	309	639	553	670
H	5/20/98	245	676	652	631
Mean		279	617	594	619
Range		239–320	538–676	548–663	521–681

projected. The slope of the regression line for FL 2 $\times$ FL 3 is 1.0358, with a correlation coefficient (r^2) of 0.9968. These data suggest that over four decades of amplification (1,024 channels), the amplified value is within 1% of the expected value.

In contrast, the slope of the regression lines for FL 1 $\times$ FL 2 and FL 1 $\times$ FL 3 are more than one (1.437 [$r^2 = 0.859$] and 1.490 [$r^2 = 0.857$], respectively), which reflects a complex origin of the green fluorescence within each population. The variation of green fluorescence within each population is also greater than expected based on anticipated photon sampling errors.

With the use of the same set of calibration microspheres, eight bench-top flow cytometers (FACScans and FACSCaliburs) were calibrated, and the results of the most recent calibrations for each instrument are shown in the Table 1. There is considerable variation in the PMT voltage necessary to place the calibration microspheres at the desired mean channel number. In Table 1, the mean and range for the eight instruments are indicated; there seems to be little pattern in the values observed. Variation in the values obtained could be attributed to the use of different filter sets among the instruments, variation in the age and

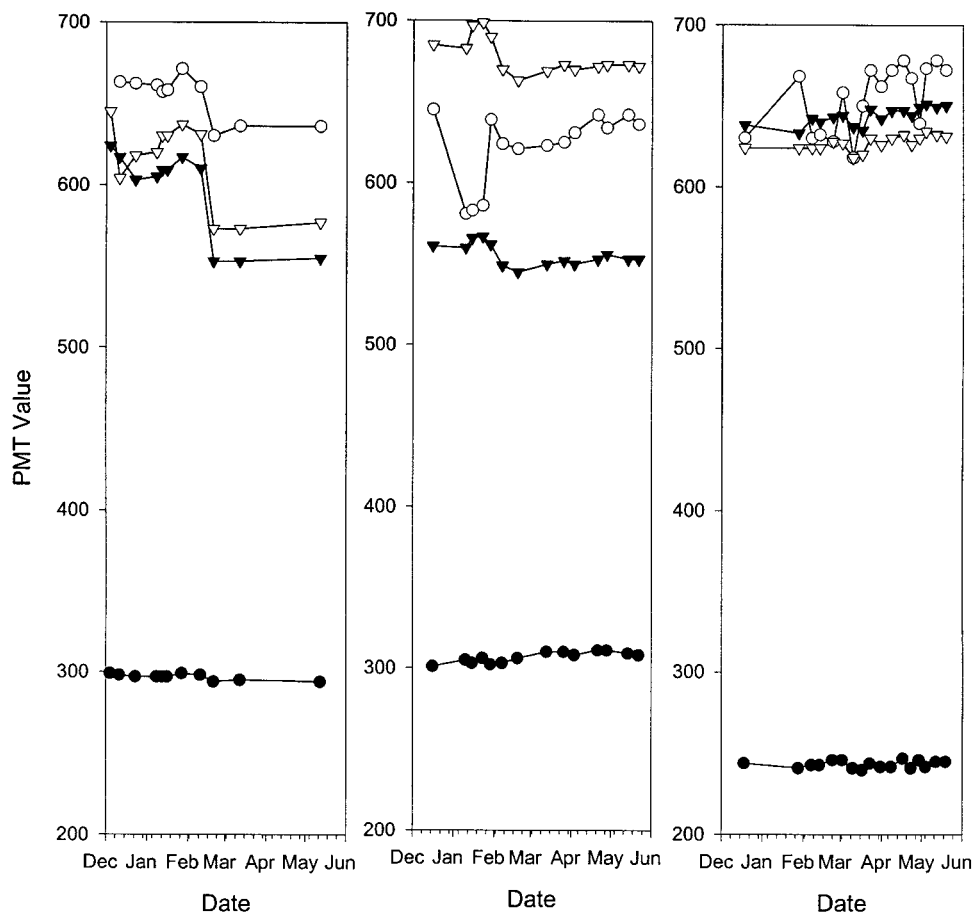


FIG. 4. Stability of flow cytometer performance on calibration. Three different bench-top flow cytometers (two FACScans and a FACSCalibur) were enabled with FlowMetrix software and hardware and calibrated at several intervals with the use of the routine furnished by the Luminex Corporation. The panels show the values of the PMT found after the automated calibration routine on the three different systems. PMT values are for side scatter (●), Fl 1 (○), Fl 2 (▼), and Fl 3 (▽).

efficiency of the PMTs, calibration of the log amps of each instrument, or the linearity of the log amps.

Although variation between instruments is high, the same instrument on repeated calibration over a period of several months shows similar calibration values (Fig. 4). The values for four parameters over a period of six months are indicated for three instruments: two FACScans and one FACSCalibur. As shown in the Table 1, there is considerable variation in PMT settings between instruments; there is less variation on repeated calibrations. The setting for side light scatter is particularly constant. Shifts in the calibration values were usually gradual but on two occasions precipitous. The two precipitous variations were due to the recalibration of the log amps on those instruments to "factory standards."

Standardizing the Cytometer: Characterizing Instrument Parameters

With the use of standard microspheres from Flow Cytometry Standards Corporation, the Fl 1 scale was standardized. Our goal was to understand the relationship between MFI (channel number) and the number of molecules of fluorescein that could be detected on the microsphere surface. The base value for unmodified microspheres (not stained with dyes) was about 200 molecular equivalents of soluble fluoroscein (MESF) and had an MFI of about 1. A difference in the MFI of 1.5 could be

determined statistically by analyzing 130 microspheres each of the control (unmodified microspheres) and experimental type, using Student's *t*-test and a null hypothesis test (data not shown). The limit of sensitivity was at least equivalent to binding of 200 molecules of ligand per microsphere. For microsphere populations whose background Fl 1 values were >3 , the number of microspheres required to statistically differentiate a MFI difference of 1.5 MFI increased significantly (as determined using Student's *t*-test).

Sets of Microspheres for Multiplexed Analysis

The Luminex Corporation has prepared 64 microsphere sets that can be discriminated when analyzed as a mixture by the FlowMetrix system. A three-dimensional display of microspheres is shown on the cover of this issue. Figure 5 displays a dot plot in Fl 2 $\times$ Fl 3 bivariate space. An average of 300 single particles of each type displayed. Of more than 19,000 particles in the display, 0.44% do not fall clearly into one of the 64 populations displayed.

Classification of Microspheres by FlowMetrix Hardware and Software: Measurement Space and Use of Ratios

The microsphere sets are constructed and undergo quality control analysis so that 80% of the particles have

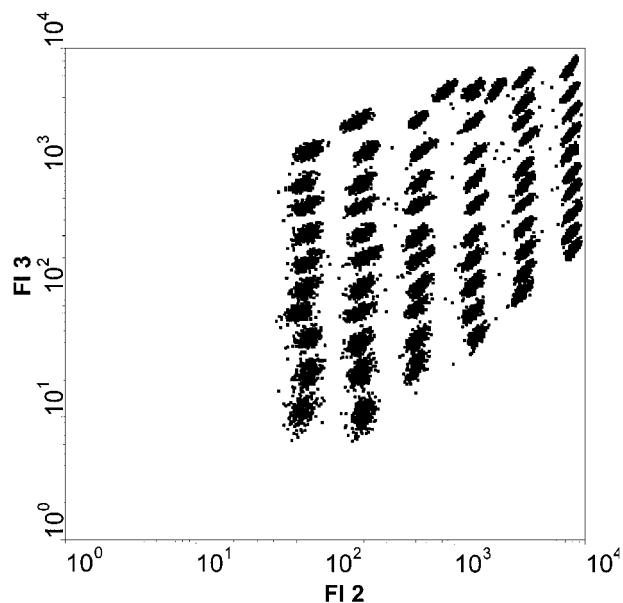


FIG. 5. Dot plot shows the FI 2 and FI 3 values (log amplification) for a mixture of 64 different microsphere sets. An average of 300 members of each set are displayed. Only single microspheres were analyzed. About 0.44% of the values does not fall within limits of one of the microsphere sets.

FI 2 and FI 3 values within preset gate areas. In addition, <0.3% of any microsphere set falls within a neighboring gate area. Figure 6A illustrates a statistical evaluation of the measurement space used by the 64 microsphere sets. The center of each of the 64 crosses is the mean for FI 2 and FI 3 for each microsphere set. Each bar extends ± 2 S.D. from the mean, representing a circular area in which 98% of the microspheres are expected. This display suggests that there is overlap in the measurement space that may potentially result in misclassifications of some microspheres from adjacent sets. The actual classification gates are not circular but include information from the correlation of values within the set of microspheres.

Figure 6B is a better representation of the classification algorithm. In this measurement space, the mean value of FI 3 is plotted against the mean value of the ratio of the FI 2/FI 3 value for each particle in the population. Again, the length of the bars is ± 2 S.D. from the mean, for a total of 4 S.D. In the regions of apparent overlap in Figure 6A, there now appears to be no overlap. In regions of apparent overlap in Figure 6B, there is no overlap in Figure 6A. In areas where there is significant correlation between FI 2 and FI 3, the classification gate regions reflect that correlation. Where there is no correlation, classification gate areas are circular as appropriate for populations in which the parameters are not correlated.

Limits for the Discrimination of Microsphere Sets: Analysis of Error, Especially Photon Sampling Error

In the parameters used for designing microspheres to be used for multiplexing, uniformity (low CV) is an important issue. The more uniform the population, the more sets that

can be blended and used in the same mixture. Several criteria have been established for the current set of microspheres. The median MFI of the population (FI 2 and FI 3) should be placed as close to the center of the classification criteria as possible. Microspheres from one set do not fall into the classification criteria for a neighboring set of microspheres. This feature is related to the uniformity of the fluorescence among the members of each microsphere set, that is, the CV.

Three major features play a role in the definition of the CV. The first is the absolute number of photons collected by the optical system (photon CV); the greater the efficiency, the lower the CV. If the number of photons is small, then photon sampling error becomes the major factor in defining the photon CV. The second major feature is the actual variation in the molarity of dye in each set (dye CV). The third major contributor to the CV is noise from both the source of illumination and the from noise of the electronics system (noise CV). The total CV is the square root of the sum of the squares of the CV of each identifiable and measurable component (28).

To understand the role of each of these factors in designing a multiplexed system in which microspheres with a wide range of MFIs are to be classified successfully, the MFI of FI 2 and FI 3 for each microsphere set was plotted the against the respective standard deviation for the same set (Fig. 7). The slopes of the regression lines for FI 2 and FI 3 are close to 1 (0.892 [$r^2 = 0.996$] and 0.915 [$r^2 = 0.995$], respectively). This relationship is expected when the principal source of variance is photon sampling error. For FI 1, however, the slope is linear but lower than expected when photon sampling error is the principle source of variance (0.603 , $r^2 = 0.976$).

Additional analyses were performed to confirm photon sampling error as the major source of variance. Sets of microspheres with low or high levels of fluorescence (FI 1, FI 2, or FI 3) were exposed to different intensities of 488-nm light with a FACStar Plus. As the light intensity was increased, the CV decreased as expected (data not shown). This observation shows that the variability in amounts of dye for each microsphere is not limiting for the levels of illumination used in the FACScan.

The illumination of the FACScan (488 nm) is not optimal for the excitation of the two dyes used to emit FI 2 and FI 3. With the use of a FACStar Plus, several microsphere sets were excited by several intensities of 488-nm or 514-nm radiation, and the MFIs and CVs were compared. Excitation by the same level of 514-nm light resulted in larger MFIs and lower CVs (data not shown). These observations confirm the notion that the variation in the amount of dye per microsphere is not a source of variability and that the principal source of variance is photon sampling error of emitted radiation.

In another experiment, neutral density filters were placed in front of the PMTs for FI 2 and FI 3, and the MFIs and CVs were determined for several sets of microspheres. As the neutral density filters diminished the MFI, the CV increased as expected until instrument "noise" of the FACStar Plus became the limiting feature of the signal.

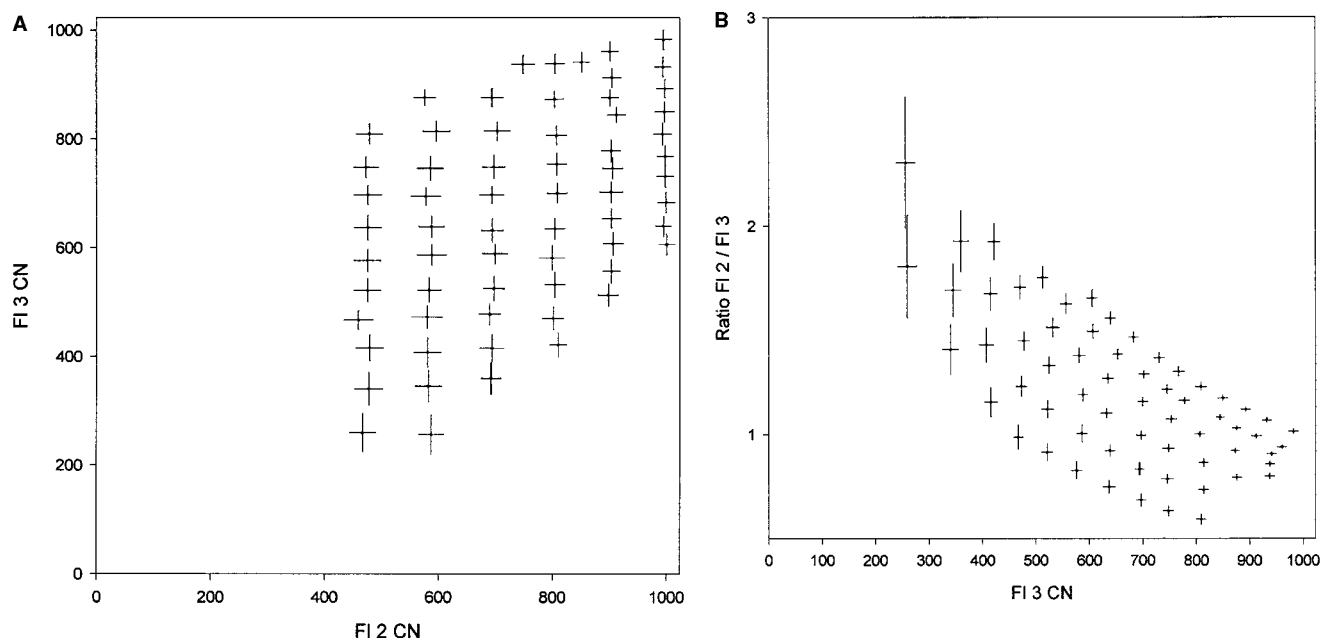


FIG. 6. Maps of the measurement space occupied by 64 sets of microspheres. **A:** FI 2 and FI 3 measurement space. The center of each cross is the mean of FI 2 and FI 3. The length of the vertical and horizontal bars is a total of 4 S.D. (± 2 S.D.). The amplification is log, and the scale is

channel number (CN). **B:** Measurement space using the means of FI 3 values and the means of the ratio of values of FI 2 and FI 3. The center of each cross is the mean of FI 3 or ratio of FI 2/FI 3. The error bars are ± 2 S.D.

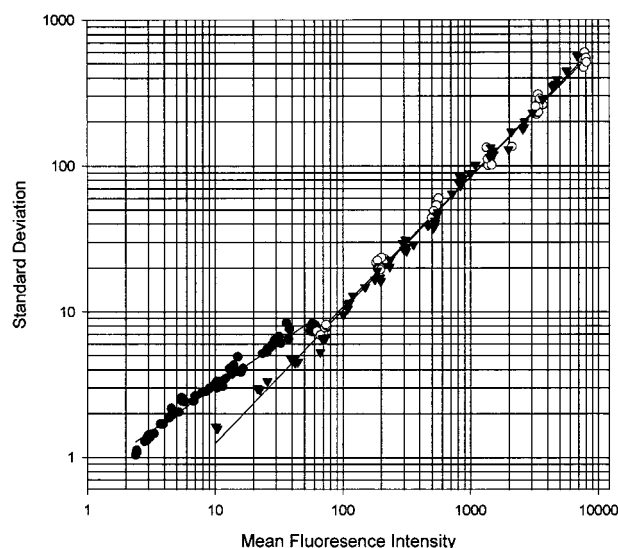


FIG. 7. The log of the mean fluorescence intensity (MFI) of FI 1, FI 2, and FI 3 for 64 sets of multiplexed microspheres is plotted against the standard deviation for that measurement of each population. $\bullet$: FI 1 values; $\circ$: FI 2 values; $\blacktriangledown$: FI 3 values. The slopes of the respective regression lines are as follows: FI 1 slope 0.603, $r^2 = 0.976$; FI 2 slope 0.892, $r^2 = 0.996$; and FI 3 slope, 0.915, $r^2 = 0.995$.

All of the above observations contribute to the idea that the principal source of variance in the FI 2 and FI 3 values for the microspheres is the photon sampling error, that is, a feature of the level of excitation, the quantum efficiency of the dye, the frequency of the radiation, the amount of dye, and the efficiency of the measuring instrument.

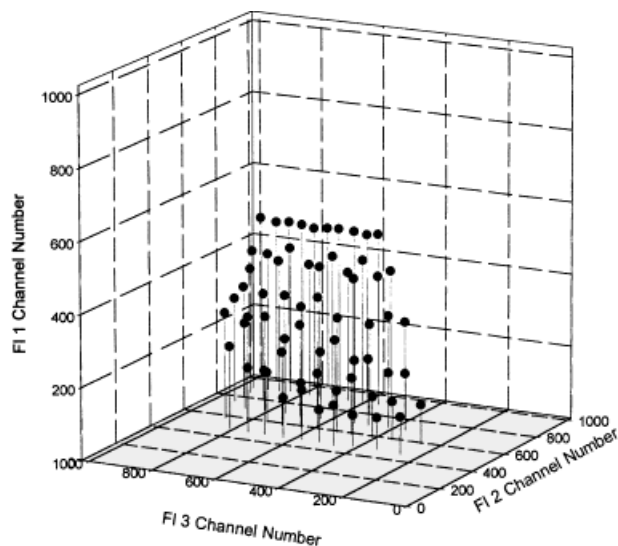


FIG. 8. The relationship among FI 1, FI 2, and FI 3 values for 63 microsphere sets. The three-dimensional plot shows the MFI of FI 1 (z-axis), FI 2 (x-axis), and FI-3 (y-axis). Data are given as channel number, and all values were for log amplification.

Origin of FI 1 "Background" Fluorescence

We anticipated that the level of FI 1 would be a function of the amounts of FI 2- and FI 3-emitting dye used in the dyeing process. The level of FI 1 fluorescence is indicated in Figure 8 for 63 sets of microspheres. The level of FI 1 fluorescence was independent of the level of FI 3 and constant when a high level of FI 2-emitting fluorophore was present (Fig. 8, back row of values). This is expected if

only the level of Fl 2-emitting fluorophore contributed the Fl 1 fluorescence. The front row of values in Figure 8 illustrates that when there is a low level of Fl 2-emitting dye, the level of Fl 1 is dependent on the level of Fl 3-emitting dye. Therefore, the origin of the Fl 1 background fluorescence does not directly correlate with the amounts of Fl 2- and Fl 3-emitting dyes but instead results from a complex concentration-dependent interaction between the two dyes.

DISCUSSION

Design of FlowMetrix Microspheres

Several observations have been made regarding the use of dyes dissolved in the microspheres. Because the dyes are inside the microspheres, solvent conditions will not affect the dye characteristics. Image analysis (data not shown) indicates that the dyes are spread throughout the microsphere matrix. Although the microsphere population is reasonably uniform, small differences in size and or composition alter the relative dyeing efficiency. Additionally, as the dye content is increased, the spectrum of the combination of two dyes changes.

Because the efficiencies of each dye are very different when stimulated with 488-nm light, to achieve useable signal levels, different concentrations of each dye must be used. The molarity of the orange-emitting dye is lower than that of the red-emitting dye. This difference has practical consequences when applied to fluorescence resonance energy transfer (FRET). Each fluorophore must have overlapping emission and absorption spectra and must be within a certain distance for FRET to be effective (<60 angstroms). FRET can occur in microspheres (commercial products depend on this feature for their microsphere absorption and emission characteristics). By keeping at least one of the dyes at a lower molarity than the other, FRET can be kept to a minimum.

Influence of the Reporter Molecule on the Classification of Multiplexed Microspheres

The green fluorophore (Fl 1 reporter) used to determine a molecular interaction at the surface of the microspheres can affect the classification of the microspheres in several ways. The green fluorescence can influence the level of orange fluorescence collected for each microsphere set because of spectral overlap of the green fluorophore. High levels of perturbation of the orange fluorescence can cause the microsphere to fail to satisfy the classification criteria and result in a serious loss of data. By using the fluorescence compensation feature of the FACScan or within the FlowMetrix software, Fl 2 can be corrected by the use of subtraction of a part of the Fl 1 signal.

The spectrum of each of the reporter fluorophores may not be the same. If very high levels of fluorophore are used for modification of the reporter, there may be interaction between the attached fluorophores, which results in shifts of the emission (or absorption) spectrum (28). For example, at high density, BODIPY (Molecular Probes, Inc., Eugene, OR) undergoes a spectral shift to red emission (Fl

3). The FlowMetrix software has been written to allow compensation between Fl 1 and Fl 3 signals, a feature not present in the FACScan system.

Because the dyes we used for classification are inside the microsphere and the green fluorophore is restricted to the surface, we expect no significant FRET. However, if the level of green fluorescence gets high enough, inner filter effects can become significant and cause misclassifications of the microspheres because of absorption of green emission of the reporters by the orange-emitting fluorophore inside the microsphere. This effect has been encountered only at very high levels of green reporter molecules.

Determining the Surface Properties of Each Microsphere Set

One of the considerations in constructing the microsphere sets was the use of a single type or even a single lot of microspheres so that all members of all the sets would have the same surface properties, thus ensuring uniform coupling chemistry. Such has proven to be the case. When a mixture of 64 microsphere sets was coupled with a target molecule and analyzed with a fluorescent reporter that binds with the protein, all the microsphere sets in the mixture had the same MFI for Fl 1 (MFI of 1,300 with an inter-set CV of <5%). These results demonstrated that each set was modified to the same extent (data not shown). The specification is still under development and will take into account different lots of microspheres.

Light Scatter of Multiplexed Microspheres

As others have pointed out (29), the light scatter of uniform microspheres of a high refractive index is complex and extremely sensitive to the size of the microsphere and the angle at which the light scatter is measured. Those who use different-sized microspheres for multiplexing have noted the difficulty of using side scatter as a classification parameter (23,24). We have noted (data not shown) that the forward-angle light scatter is extremely sensitive to the core flow rate, diameter, and composition. In contrast, the side scatter was insensitive to alterations in the flow rates, the composition of the core material, and composition of the sheath fluid. Therefore, use of side scatter is the most robust way to identify single microspheres. During the analysis of light scatter and fluorescence of the microsphere sets, both positive and negative correlation was noted. These variations are entirely consistent with the calculations of others on the expected patterns of light scatter with particle size (29). At this point, it has not been determined whether the variation in total fluorescence within a microsphere set is dependent on variations in microsphere size, permeability, refractive index, or some combination of these parameters.

Reliable Calibration of the Bench-Top Flow Cytometer

Reliable classification of the microsphere sets depends on each set producing its characteristic level of fluorescence and the instrument amplifying those signals to the same extent. Logarithmic amplification is used during the

classification of the multiplexed microspheres, leaving only the PMT voltage as a means of regulating the amplification to yield the same level of signal from instrument to instrument. To calibrate the system, two sets of calibration microspheres were used to set the voltages of Fl 2 and Fl 3 PMTs, so the calibrators produced the desired level of signal. Fl 1 value settings were established by using the "spillover" from the Fl 2- and Fl 3-emitting dyes. It should be mentioned that there are two critical requirements to achieving reliable calibration. First, the instrument must be stable, which requires operation for about 2 h. Second, the log amps optimally should be at "factory conditions"; that is, there should be four orders of amplification from channels 0 to 1023, and the amplification should be log uniform. If these conditions are not observed, then calibration will lead to useable but not optimal classification of the microspheres.

Considerations of Statistical Analysis in Determining the Sensitivity of FlowMetrix Assays

The sensitivity of the assay systems using multiplexed microspheres depends on the background or "noise" level of each microsphere set. Because many measurements can be made of the same microsphere set rapidly, the collection of many repetitions of the data is a major advantage of a flow cytometer-based analysis. The background level of the microspheres consists of the noise level of the cytometers as well as the nonspecific binding of the reagents to the microsphere surfaces. In the flow cytometers used so far, the noise level is about 200 MESF, which gives a signal of about 1 MFI in a calibrated instrument. The MESF per MFI under those conditions is also in the range of 200. The amount of nonspecific binding that occurs is highly variable for different reagents. If a population of microspheres increases from 1 to 2 MFI, then each microsphere will have gained an average of 200 MESF of reporter.

Distinguishing two populations that differ by 1 MFI depends on the variance of each population and the number of replicate values in each population. For example, it has been observed that two populations differing by 1 MFI can be distinguished (Student's *t*-test, test of the null hypothesis) if each population contains at least 30 replicates. A limiting feature will be the variances of the Fl 1 signal of the control population of microspheres, which has an unexpectedly high variance (Fig. 7).

We have reported the design and characteristics of 64 microsphere sets that can be distinguished in a calibrated bench-top flow cytometer. The system has been used to develop multiplexed methods for genetic analysis as well as immunoassays (30).

ACKNOWLEDGMENTS

The authors thank the Luminex Corporation (Austin, TX) for the FlowMetrix microspheres. We especially thank the editors of this special issue of *Cytometry* for the opportunity to report our findings on calibration and standardization of multiplexed flow analysis systems.

LITERATURE CITED

1. Fulwyler MJ: Method for detecting and separating antigens and antibodies in blood or other samples. UK Patent #1561042, 1976.
2. Horan PK, Wheelless LL: Quantitative single cell analysis and sorting. *Science* 198:149-157, 1977.
3. Horan PK, Schencj EA, Abraham GN, Kloszewski MD: Fluid phase particle fluorescence analysis: Rheumatoid factor specificity evaluated by laser flow cytophotometry. In: *Immunoassays in the Clinical Laboratory*, Nakamura RM, Dito WR, Tucker ES (eds). Alan R Liss: New York, 1979, p 185.
4. Lisi PJ, Huang CW, Hoffman RA, Teipel JW: A fluorescence immunoassay for soluble antigens employing flow cytometric detection. *Clin Chim Acta* 120:171-179, 1982.
5. Saunders GC, Jett JH, Martin JJ: Amplified flow-cytometric separation-free fluorescence immunoassays. *Clin Chem* 31:2020-2023, 1985.
6. McHugh TM, Stites DP, Busch MP, Krowka JF, Stricker RB, Hollander H: Relation of circulation levels of human immunodeficiency virus (HIV) antigen, antibody to p24, and HIV-containing immune complexes in HIV-infected patients. *J Infect Dis* 159:1088-1091, 1988.
7. McHugh TM, Miner RC, Logan LL, Stites DP: Simultaneous detection of antibodies to CMV and HSV by using flow cytometry and a microsphere-based fluorescence immunoassay. *J Clin Microbiol* 26:1957-1961, 1988.
8. Wilson MR, Wotherspoon JS: A new microsphere-based immunofluorescence assay using flow cytometry. *J Immunol Methods* 107:225-230, 1988.
9. Wilson MR, Mulligan SP, Raison RL: A new microsphere-based immunofluorescence assay for antibodies to membrane-associated antigens. *J Immunol Methods* 107:231-237, 1988.
10. Presani G, Perticarari S, Mangiarotti MA: Flow cytometric detection of anti-glucan antibodies. *J Immunol Methods* 119:197-202, 1989.
11. Scillian JJ, McHugh TM, Busch MP, Tam M, Fulwyler MJ, Chien DY, Vyas GN: Early detection of antibodies against rDNA-produced HIV proteins with a flow cytometric assay. *Blood* 73:2041-2048, 1989.
12. McHugh TM, Wang YJ, Chong HO, Blackwood LL, Stites DP: Development of a microsphere-based fluorescent immunoassay and its comparison to an enzyme immunoassay for the detection of antibodies to three antigen preparations from *Candida albicans*. *J Immunol Methods* 116:213-219, 1989.
13. Lim VL, Gumbert M, Garovoy MR: A flow cytometric method for the detection of the development of antibody to Orthoclone OKT3. *J Immunol Methods* 121:197-201, 1989.
14. Lindmo T, Bormer O, Ugelstad J, Nustad K: Immunometric assay by flow cytometry using mixtures of two particle types of different affinity. *J Immunol Methods* 126:183-189, 1990.
15. Saunders GC, Martin JC, Jett JH, Perkins A: Flow cytometric competitive binding assay for determination of actinomycin-D concentrations. *Cytometry* 11:311-313, 1990.
16. Kim K, Han DY, Yoon DY, Cho B, Choi MJ, Choe IS, Chung T-W: Solid-phase immunoassay using a flow cytometer: quantitative and qualitative determination of protein antigens and a hapten. *Immunol Lett* 31:267-272, 1992.
17. Elkhaila MY, Kiechle FL, Gordon SC, Poulik MD: A flow cytometric method to detect anti-pyruvate dehydrogenase antibody in primary biliary cirrhosis. *Am J Clin Pathol* 97:202-220, 1992.
18. Best LM, Veldhuyzen van Zanten SJ, Bezanson GS, Haldane DJ, Malatjalian DA: Serological detection of *Helicobacter pylori* by a flow microsphere immunofluorescence assay. *J Clin Microbiol* 30:2311-2317, 1992.
19. Vlieger AM, Medenblik AMJC, van Gijlswijk RPM, Tanke HJ, van der Ploeg M, Gratama JW, Raap AK: Quantitation of polymerase chain reaction products by hybridization-based assays with fluorescent, colorimetric, or chemiluminescent detection. *Anal Biochem* 205:1-7, 1992.
20. Labus JM, Petersen BH: Quantitation of human anti-mouse antibody in serum by flow cytometry. *Cytometry* 13:275-281, 1992.
21. Frengen J, Schmid R, Kierulf B, Nustad K, Paus E, Berge A, Lindmo T: Homogenous immunofluorometric assays of alpha-fetoprotein with macroporous, monosized particles and flow cytometry. *Clin Chem* 39:2174-2181, 1993.
22. Renner ED: Development and clinical evaluation of an amplified flow cytometric fluoroimmunoassay for *Clostridium difficile* toxin A. *Cytometry* 18:103-108, 1994.
23. McHugh TM, Fulwyler MJ: Microsphere-based fluorescence immunoassays using flow cytometry instrumentation. In: *Clinical Flow Cytom-*

- etry, Bauer KD, Duque RE, Shankey TV (eds). Williams and Wilkins: Baltimore, MD, 1993, pp 535–544.
24. McHugh TM: Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. In: *Methods in Cell Biology*, Darzynkiewicz Z, Robinson JP, Crissman HA (eds). Academic Press, San Diego, CA, 1994, vol 42, pp 575–595.
25. McDade RL, Fulton RJ: True multiplexed analysis by computer-enhanced flow cytometry. Medical Device and Diagnostic Industry, April 1997.
26. The FlowMetrix[®] System from Luminex Corporation: Users Manual, 1st ed. Luminex Corporation, Austin TX, January 1997.
27. Bangs L, El-Aasser M, Seaman G, Gorman E, Price C, Sackrison J, Brinkley JM, Knox R, Kettman J, Meza M, Hemmes P: *Diagnostic Applications of Latex Technology*. Bangs Laboratories, Inc., Fishers, IN, 1997.
28. Shapiro HM: *Practical Flow Cytometry*, 3rd ed. Wiley-Liss, New York, 1995.
29. Doornbos RMP, Hoekstra AG, Duerloo KEI, De Grooth BG, Sloot PMA, Greve J: Lissajous-like patterns in scatter plots of calibration beads. *Cytometry* 16:236–242, 1994.
30. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR: Advanced multiplexed analysis with the FlowMetrix[®] system. *Clin Chem* 43:1749–1756.