

Is the Central Dogma of Flow Cytometry True: That Fluorescence Intensity Is Proportional to Cellular Dye Content?¹

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Received for publication January 8, 1982; accepted April 1, 1982

Measurements and theoretical calculations of fluorescent emission from four samples of polystyrene microspheres (diameter 0.92, 1.63, 1.90 and 4.18 μm) containing the same fluorescent dye show a general dependence upon particle size, emission angle, and polarization conditions. However, for the excitation and detection conditions used in flow cytometry, the relative fluorescent intensities measured for the four particle sizes are proportional to the dye content to +10% accuracy, independent of particle size. Accordingly, the central dogma of flow cytometry

that fluorescence is proportional to cellular dye content is valid to this accuracy for these solid, highly refractive polymer particles. Most mammalian cells are much less refractive, therefore, should conform more closely to the central dogma.

KEY TERMS: Flow cytometry, cellular dye content, fluorescent signal intensity, fluorescence polarization, fluorescence emission theory, fluorescent microspheres, particle size, fluorescence angular distribution

The central dogma of flow cytometry is that the fluorescence from cells stained with fluorescent dyes is proportional to cellular dye content (13). For example, the G₁ cells of a proliferating population stained fluorescently for DNA content are expected to give equal fluorescent signals despite significant variability in cell volume and morphology. Similarly, the G₂ and M cells are expected to give equal fluorescence signals despite the large differences in the geometric distribution of chromatin between interphase and mitotic cells. There are exceptions to the central dogma such as highly refractile flattened mammalian sperm (2, 7, 14) or situations involving energy transfer (10), but applications to most bio-

logical and medical problems tacitly assume the validity of the central dogma. Yet, when small particles containing fluorescent molecules are suspended in a medium of different index of refraction, there are intrinsic optical phenomena primarily due to the index mismatch which affect the angular distribution of both the intensity and polarization of the emitted fluorescence (1, 4-6, 16). This holds true when the particles are uniform microspheres, and when they are biological cells the additional factors of cellular morphology, internal dye distribution, and orientation of the cell with respect to the exciting illumination can also affect the fluorescence emission. In this context morphology means cellular size, shape, and internal distribution of refractive index. The questions which arise in flow cytometry are whether equal fluorescent intensities necessarily imply equal amounts of dye and also whether unequal intensities necessarily imply unequal amounts of dye.

The intensity and polarization of the illuminating radiation at the site of a fluorescent molecule determines its rate of excitation. In addition, the orientation of the molecules must be considered. Fluorescent molecules whose absorption dipoles are parallel to the electric vector of the exciting light at the molecular site will be preferentially excited. The illumination intensity and polarization at sites inside small particles

¹ The work at Clarkson College of Technology was supported by National Science Foundation Grant CHE-801144 and ARO Grant DAAG-29-79-6-005. Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48.

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may differ from that in a uniform bulk medium because the radiation which reaches a particular site in a particle may have been strongly affected by reflection, refraction, absorption and scattering enroute to that site. Similarly, longer wavelength fluorescence undergoes comparable processes enroute from the emitting molecule through the particle to the detector. Accordingly, to be considered are: effects of particle morphology, particle orientation, and dye molecule distribution and orientation upon transport of the radiation at the exciting wavelength to the molecule; also transport of the radiation at the emitting wavelength from the molecule. Fluorescence polarization generated by photo selection as described above will be reduced if the excited molecules can rotate before emitting their fluorescence. Each of these factors may affect the measured fluorescence intensities in flow cytometers.

Theoretical calculations of fluorescent emission from uniform microspheres of radius comparable to or less than the wavelength of the exciting light (1, 4-6, 16) have raised questions about the central dogma. These calculations, as well as a number of associated experiments (8, 11, 12), indicated that fluorescence emitted from uniform solid polymer microspheres of equal dye content was a strong function of microsphere radius for values of radius between 0.08 and 0.40 μm and exciting light of wavelength about 500 nm. Fluorescence emission also depends on the angle at which the fluorescence is viewed and upon the polarization of the exciting radiation.

These challenges motivated us to test the central dogma simply and realistically. Fluorescent emission intensities from four sizes of uniform fluorescent microspheres ranging in diameter from 0.93 μm to 4.18 μm were measured in a photometer with microspheres in suspension in a cuvette, and in 2 different flow cytometers with orthogonal geometry in two different laboratories. These experimental results were compared with the results of theoretical calculations to determine the extent to which particle size influences flow cytometric measurements when particle size approaches cellular dimensions. Measurements and calculations were carried out cooperatively at three laboratories: Clarkson College of Technology (CCT) Potsdam, NY Coulter Electronics Incorporated (CEI) Hialeah, FL; and Lawrence Livermore National Laboratory (LLNL) Livermore CA.

These experiments show that fluorescence emission is not isotropic and is a function of particle size which is in general agreement with theory. However, for commonly used flow systems with either orthogonal geometry or epiillumination, the results show that dependence of fluorescence emission on microsphere radius is small and should be even smaller for biological cells because they usually have an index of refraction much closer to that of the suspending medium (water) than do polymer microspheres. These results are consistent with the small coefficients of variation (cv) reported for flow cytometric measurements on cells of equal DNA content but variable morphology.

Materials and Methods

The overall experimental design was to measure fluorescence intensity per unit dye content by both flow cytometric and conventional photometric methods from several sizes of uniform polymer micro-

spheres (approximating the diameter of mammalian cells) containing fluorescent dye and to compare these results with theoretical predictions. Several measurements were made on each microsphere sample, including: a, particle size; b, particle relative dye content; c, angular distribution of fluorescence intensity in a light scattering photometer; d, relative fluorescence intensity by flow cytometry. The theoretical angular distribution of fluorescence intensity was also calculated.

Four samples of fluorescent polystyrene microspheres with smooth surfaces and narrowly dispersed diameters from Polysciences Inc. (Warrington, PA) were used. The surfaces of three of these as indicated in Table 1 were carboxylated. Particle sizes as determined at two laboratories (CCT and CEI) and nominal sizes supplied by the manufacturer are listed in Table 1. Sucrose gradient density centrifugation showed the density of each sample to be $1.049 \pm 0.002 \text{ gm/ml}$ thereby confirming the manufacturer's declaration that the materials are the same. The fluorescent dye used by the manufacturer is 7-diethylamino-3-(2-benzothiazolo) coumarine which has a yellow-green fluorescence and spectral properties approximating fluorescein. Excitation maximum is 478 nm and fluorescence maximum is 480 nm.

Particle size: The average particle size was determined by light scattering performed at CCT using an instrument which has been described in detail elsewhere (15). The geometry was identical for both light scattering and subsequent fluorescence measurements as shown in Figure 1. The sample, contained in a cylindrical glass cell with a plane entrance window, is illuminated by a plane parallel beam from a high pressure mercury arc source. The linearly polarized

Table 1
Diameters of Fluorescent Polystyrene Microspheres as Measured by Coulter Volume Analysis (CEI)^a and Light Scattering^b

Polysciences Catalog No.	Nominal Diameter (μm)	Coulter Diameter (μm)	cv %	Light Scattering Diameter (μm)
7776	0.88	0.93	2.4	0.91
9719	1.52	1.64	1.7	1.62
9847 ^c	1.83	1.90	1.6	1.89
9853	4.5	4.18	1.0	4.7

^a Work performed at Coulter Electronics Inc.

^b Work performed at Clarkson College of Technology.

^c Surface of samples other than this were carboxylated.

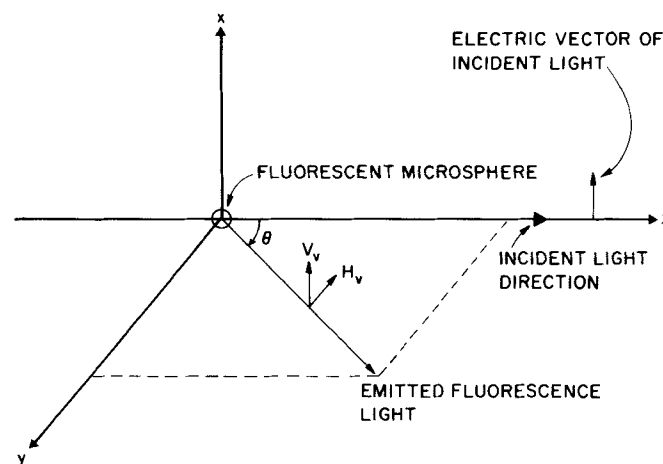


FIG. 1. In the optical geometry for both light scattering and fluorescence measurements, incident light propagates along z axis with its electric vector perpendicular to observation plane (yz). Scattered and fluorescent light from particle at origin is observed at angle θ , and has components whose electric vectors are perpendicular (V_v) and parallel (H_v) to the yz plane. For incident radiation polarized parallel to the yz plane, the scattered and fluorescent radiation each have corresponding components V_h and H_h .

incident illumination has its electric vector vibrating either vertically or horizontally; the scattered light may have components with both polarizations. With vertically polarized incident light, the vertically and horizontally polarized components of the scattered light are designated V_v and H_v , respectively; the subscript refers to incident light polarization. Similarly, with horizontally polarized light, the scatter components are V_h and H_h . For isotropic homogeneous spherical particles such as used here, V_h and H_v are zero. V_v and H_h were measured over the range of scattering angle $\theta = 10^\circ$ to 80° with incident light at 436 and 546 nm. The incident light was obtained by passing the light from a mercury arc source through an Oriel monochromator, model 7242 (Oriel Corp., Stanford CT). The average particle size was determined by comparison of the location of maxima and minima in the angular distribution of V_v and H_h with theoretical calculations based on Lorenz-Mie theory as elaborated on pp. 344-349 of reference (3). The refractive index of polystyrene relative to water was taken as $n = 1.21$. To eliminate clumping of the microspheres, samples were diluted with a solution of sodium dodecyl sulfate (SDS [special pure grade, BDS Chemical Ltd., England]) consisting of 3.0 mg/ml of doubly distilled water. Table 1 shows the measured values of microsphere diameter.

The Coulter Channelyzer Model C-1000 (Coulter Electronic Instruments, Hialeah, FL) with pulse editing and a 30 μm diameter aperture was used at CEI to measure the volume of the three smallest sizes. Particles not passing through the center of the aperture were electronically rejected in order to attain highest accuracy and precision. The sample consisting of the largest particles was sized on a Coulter Channelyzer Model H4 using a 50 μm diameter aperture, also with pulse editing. Measurements were calibrated against 5.108 μm diameter particles whose size had been determined by the National Bureau of Standards using the array method (9). In this method, microspheres are deposited on a microscope slide at high density so that close packed areas resembling a two dimensional crystal are formed. This allows measurement of linear sequences of touching microspheres, yielding a more accurate average diameter than is possible from measurements of isolated microspheres. The measured particle diameters and their cv obtained by the Coulter method are also listed in Table 1.

The particle sizes estimated by the two independent methods of light scatter and Coulter volume analysis agree well for the three

smallest sizes. The light scattering method becomes rather inaccurate for the larger size whereas the Coulter method becomes more accurate in this size range (see "Discussion" section). Accordingly, we believe the latter (diameter of 4.18 μm) more nearly represents the true size and we have utilized this value in subsequent calculations.

Analysis of relative dye content: Fluorescence emission spectra measured independently at CCT and LLNL on all four samples showed no variation among samples thereby confirming that each contained the same fluorescent dye. At CCT, solutions for spectral measurements were obtained by drying samples in a vacuum oven and then dissolving them in benzene whereas at LLNL a suspension of microspheres was measured in a spectrofluorometer. Each sample gave identical excitation and emission spectra. Figure 2 is typical of each of the four samples and shows the fluorescence emission spectra for various excitation wavelengths from 400 to 470 nm and an excitation spectrum for 480 nm. The band width of the excitation beam was about 10 nm which accounted for the slight emission at the shorter wavelengths for the 470 nm excitation.

Determination of the relative dye content was carried out in two independent ways. At CCT, samples first were dispersed ultrasonically and diluted with surfactant solution (0.003 g ml/SDS) to minimize clumping and attachment of the particles to the glass surfaces. Then a portion was added to a tared weighing bottle, dried under vacuum at room temperature for 30 hr, baked for 4 hr in a vacuum oven at 80° to 90°C , and weighed. The final weight included a correction for the amount of SDS which was small enough not to interfere with subsequent absorption and fluorescence measurements.

Solutions of known polystyrene concentration were prepared by adding benzene directly to the dried samples in weighing bottles. Complete dissolution took 2-3 hr during which some solvent evaporation occurred. A final weighing yielded the weight of benzene plus polystyrene allowing calculation of the polymer concentration. The relative dye content of each sample was determined by measurement of the absorbance at 440 nm using a Zeiss MQ3 photometer (Carl Zeiss, West Germany). This was the wavelength at which absorption was a maximum. The specific absorbance (measured absorbance divided by the polymer concentration) is listed for each sample in column 2 of Table 2; it is different for the 4 different sizes, and is assumed to be proportional to the dye concentration in the microspheres. Multiplication of the dye concentration in the microspheres

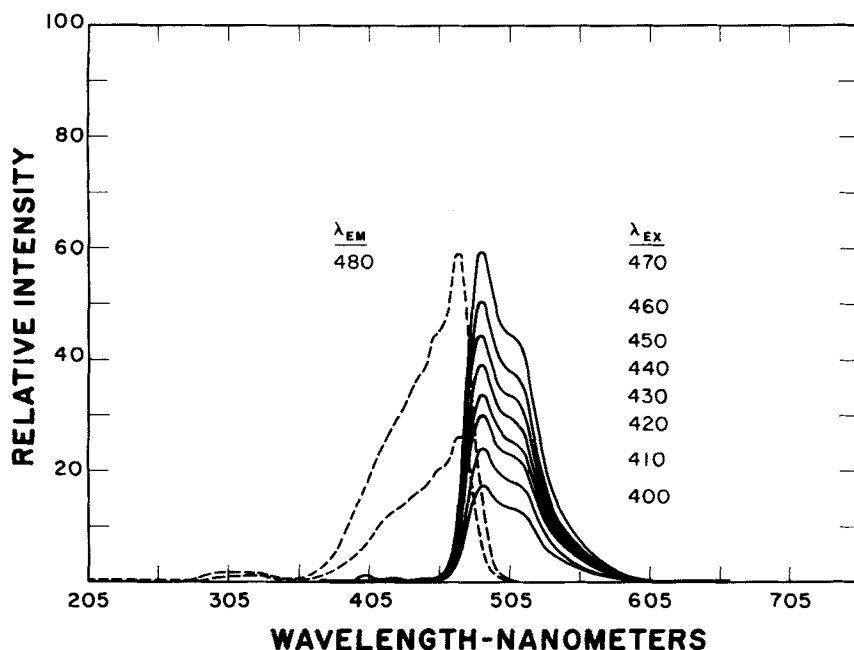


Fig. 2. Emission spectra of dye from 1.90 μm diameter polystyrene microspheres for 8 excitation wavelength from 400 to 470 nm. Polymer concentration in benzene is 2.54 mg/ml. Excitation bandwidth is 10 nm. Excitation spectrum is shown by dashed line.

by the cube of the microsphere diameter gives the relative dye content of each sample of microspheres. This quantity, normalized to 100 for the 1.64 μm diameter sample, is listed in column 3 of Table 2 for each of the microsphere samples.

A second method for measuring relative microsphere dye content utilized an image analyzing fluorescence microscope at LLNL. This system is a Zeiss epiillumination fluorescence microscope coupled to a (silicon intensified target) vidicon TV camera. Microspheres were mounted on a microscope slide in an oil (R. P. Cargille Labs, Cedar Grove, NJ, $n = 1.572$) with an index of refraction close to that of the microspheres. The excitation light was the 436 nm line from a mercury lamp reflected into the objective using a 460 nm long-wavelength pass dichroic mirror. Fluorescence emission was measured after passage through a 475 nm long-wavelength pass filter. This enabled measurements of relative fluorescence emission per microsphere with no optical effects due to index mismatch and hence the measured fluorescence intensities are proportional to the relative microsphere dye contents. These values, normalized to 100 for the 1.64 μm samples are shown in column 4 of Table 2.

Angular distribution of microsphere fluorescence: The instrument used for the measurement of the angular distribution of light scatter at CCT was also used to measure the angular distribution of the fluorescence from microsphere suspensions using vertically and horizontally polarized exciting light. In order to obtain high angular resolution the radiation is collected by means of optical stops, over a very narrow solid angle. With vertically polarized illumination, the vertically polarized emission component (V_v) was measured. With horizontally polarized illumination the horizontally polarized emission component (H_h) was measured. These fluorescent measurements were made on aqueous suspensions of microspheres (diluted to avoid multiple scattering and loss of fluorescence by self-absorption) in the same configuration as for the light scattering measurements. The 436 nm line of the mercury arc used for fluorescence excitation was obtained using the same Oriel monochromator. The fluorescent emission was measured after passing through a Baird-Atomic D-530 optical cut-off filter. This filter combined with the S-11 photosurface and the emission spectrum as shown in Figure 2 restricts fluorescence detection to a band which is effectively centered at about 530 nm allowing comparison with the theoretical calculations which assume monochromatic 530 nm fluorescence emission (in "Theoretical calculation" below). Unlike elastic light scattering from spheres for which $H_v = V_h = 0$, there are small but measurable values of H_v and V_h in the case of fluorescence; H_v is discussed below in section d.

A small amount of dye which dissolved in aqueous phase also contributed to the fluorescence. This was determined by fluorescence measurements on the supernatant obtained after ultracentrifugation for 2 hr at $43,000 \times g$. The contribution of the free dye was about 5% of the total signal and this was subtracted from that signal in each case. The resulting microsphere fluorescence values were corrected by multiplication by $\sin \theta$ for the change of viewing volume at different emission angles, θ . These measured angular intensities for each sample were then divided by the concentration of each sample and by the specific absorbance listed in Table 2, except for the 4.18 μm diameter sample. In that case the relative dye content obtained at CCT, which differed significantly from the LLNL value, was believed to be anomalous and so its value was corrected to correspond to the LLNL value.

The resulting angular distributions were then normalized for comparison with the theoretical results by setting the values of V_v at 90° for the 1.64 μm diameter sample equal to unity and by scaling all other values by the same multiplicative factor. These final results which are given in Table 3 represent the angular distribution of fluorescence emission from the microsphere suspension, in arbitrary units, per unit amount of fluorescent dye.

Microsphere fluorescence by flow cytometry: The fluorescence intensities of these same four samples were also measured by flow cytometry instrumentation at CEI and LLNL using the Coulter EPICS IV and a flow cytometer built at LLNL, respectively. Both of these instruments are based on the orthogonal geometry shown in Figure 1. These instruments measured both polarization components of the fluorescence emission of individual particles in flow excited by an argon ion laser beam vertically polarized. This signal is proportional to $V_v + H_v$ integrated over the angular range subtended by the light collection optics. Measurements at CCT showed that H_v is small and proportional to V_v for these samples, so that the relative values of $V_v + H_v$ and V_v are approximately the same. The illuminating wavelength used at CEI and LLNL was 476 nm; the fluorescent light traversed long-wavelength pass filters which cut on at 515 nm. The LLNL flow cytometer uses f1.0 light collection optics and internal flow chamber sensing through a flat window. The Coulter EPICS IV used f0.8 optics with jet-in-air sensing. Table 4 compares the flow cytometric and spectroscopic measurements of relative microsphere fluorescence per unit dye content for the 4 samples.

Theoretical calculations: Theoretical calculations were carried out using a model in which each fluorescent molecule is assumed to be a randomly oriented electric dipole fixed in space such that the excitation and emission are in the same direction, i.e., the dipole does not rotate (12). Excitation was at 436 nm and emission was at 530 nm

Table 3
Fluorescent Intensities per Unit Amount of Fluorescent Dye Measured in CCT Photometer for Four Sizes of Microspheres at Emission Angles, θ , between 45° and 138° ; and Normalized to $V_v = \text{unity for } \theta = 90^\circ$, and $d = 1.64 \mu\text{m}$

Angle θ	$d = 0.93 \mu\text{m}$		$d = 1.64 \mu\text{m}$		$d = 1.90 \mu\text{m}$		$d = 4.18 \mu\text{m}$	
	H_h	V_v	H_h	V_v	H_h	V_v	H_h	V_v
45	0.75	0.96	0.79	0.94	0.77	0.91	0.86	1.06
60	0.71	0.99	0.73	0.98	0.70	0.90	0.83	1.08
70	0.65	1.00	0.69	1.00	0.63	0.97	0.75	1.13
80	0.56	1.02	0.68	1.00	0.63	0.97	0.70	1.13
90	0.51	1.00	0.68	1.00	0.63	1.01	0.67	1.09
100	0.56	0.98	0.69	1.00	0.62	1.00	0.66	1.06
105	0.54	1.03	0.70	1.08	0.64	1.02	0.72	1.08
110	0.60	1.08	0.73	1.08	0.65	0.98	0.75	1.12
115	0.65	1.07	0.75	1.10	0.72	1.02	0.78	1.09
120	0.71	1.09	0.79	1.09	0.76	1.04	0.82	1.12
125	0.73	1.09	0.83	1.13	0.80	1.07	0.77	1.12
130	0.76	1.10	0.87	1.07	0.88	1.13	0.98	1.13
135	0.80	1.10	0.92	1.16	0.96	1.13	1.04	1.15
138	0.84	1.10	0.95	1.17	1.01	1.14	1.07	1.16

Table 2
Average Relative Dye Content per Microsphere as Measured by Two Independent Methods (CCT^a and LLNL^b)

Microsphere Diameter (μm) (CEI)	Specific Absorbance ($\text{cm}^{-1} \text{gm}^{-1} \text{cm}^3$) at 440 nm (CCT)	Relative Microsphere Dye Content		Average Relative Microsphere Dye Content ^d
		(CCT) ^c	(LLNL)	
0.93	65.2	4.41	3.60	4.01
1.64	269.1	100	100	100
1.90	259.1	150	162	155
4.18	130.2	800	498	498

^a Clarkson College of Technology

^b Lawrence Livermore National Laboratory.

^c Product of (column 1)³ and column 2 normalized to the 1.64 μm size.

^d Anomalous value of 800 in column 3 omitted.

in accordance with the CCT experiments. The particles were presumed to be monodisperse with diameters as measured by light scattering except for the largest size, in which case the value $4.0\ \mu\text{m}$ was used (other calculations showed the results in this range to be rather insensitive to small changes in size). The refractive index of the microspheres relative to water was taken to be 1.21 at both the excitation and emission wavelengths. The calculations were normalized with the value of V_v at 90° for the $1.64\ \mu\text{m}$ diameter sample equal to unity for comparison with the experiments in Table 3. The computed angular distributions V_v and H_h are plotted in Figure 3. Table 4 (final column) shows the computed values of V_v at 90° for comparison with the experimental measurements of the same quantity.

Discussion

The measurements of microsphere diameters made by Coulter volume analysis and light scattering agree to an accuracy of 1–2% for three sizes. For the fourth, and largest size, there is a discrepancy of 12%. The Coulter procedure is expected to be most accurate for the largest size since measurement difficulty decreases with increasing size, whereas the light scattering value is expected to be least accurate for the largest size due to the small separation of maxima and minima in the angular scattering pattern and clumping problems. Therefore in this paper, we use the Coulter measurements as the best estimate of true diameter of the largest particle.

Table 2 shows the results of the optical absorption measurements on weighed samples of microspheres dissolved in organic solvent. The specific absorbance values, proportional to microsphere dye concentration, are of the same order of magnitude, but by no means equal. This is not unexpected since in the polymerization process used to manufacture them the final dye concentration cannot be tightly controlled from batch to batch. Also shown in Table 2 are the relative dye contents per microsphere calculated from the product of the cube of the diameter and the relative dye concentration (i.e., specific absorbance) measured at CCT, and also as measured in the LLNL image scanning fluorescence microscope. Agreement is good except for the largest size, where it appears the CCT measurement of specific absorbance is anomalously high; we have no explanation for this anomalous value, but greater confidence must be assigned to the LLNL method which is simpler and more direct. This CCT value has been omitted in the final column (averages) of Table 2.

The measurements of microsphere fluorescence are summarized in Tables 3 and 4. Table 3 shows the results from the CCT photometer, in which the fluorescence intensities per unit dye content, V_v and H_h , were measured over a range of

emission angles from $\theta = 45^\circ$ to 138° . If these measured signals had been directly proportional only to the amount of dye per microsphere and independent of particle size, emission angle, and polarization, all values in Table 3 would be unity. That is obviously not the case. The fluorescence signals vary with polarization, emission angle, and particle size. The small depolarized component, H_v , was also measured (not shown) and is proportional to V_v . Thus V_v is directly comparable to the fluorescence signals measured in the orthogonal geometry flow cytometers at CEI and LLNL used in this work.

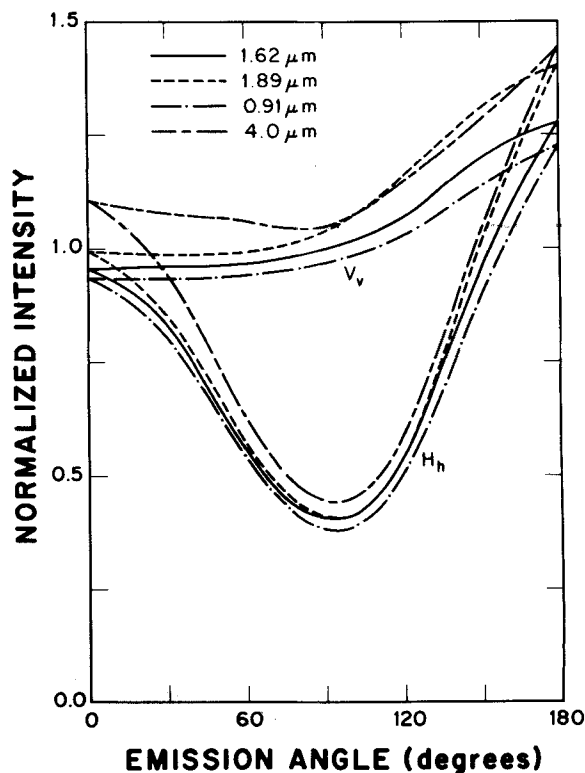


Fig. 3. Computed intensity of polarized fluorescence components V_v and H_h versus emission angle θ (Fig. 1) for microspheres with uniform distribution of dye and relative refractive index 1.21. Excitation and emission wavelengths are assumed to be 436 and 530 nm. All values of V_v were normalized to unity for the $1.64\ \mu\text{m}$ diameter microspheres at $\theta = 90^\circ$. Fluorescence intensity values are also adjusted to correspond to the same total amount of dye.

Table 4
Experimental and Theoretical Values of Fluorescence Intensity per Unit Dye Content

Microsphere Diameter (μm) (CEI)	Av. Relative Dye Content per Microsphere ^a	Fluorescence Signal per Microsphere in 2 Different FCM ^b			Av. FCM Signal per Unit Dye Content	Fluorescence Signal per Unit Dye Content (V_v at 90°) (CCT)	Theoretical Fluorescence Signal per Unit Dye Content (V_v at 90° in CCT computations)
		CEI	LLNL	Av			
0.93 ± 0.02	4.01	4.8	4.0	4.4	1.10	1.00	0.95
1.64 ± 0.03	100	100	100	100	1.00	1.00	1.00
1.90 ± 0.03	155	153	148	151	0.97	1.01	1.05
4.18 ± 0.04	498	457	461	459	0.92	1.05	1.05

^a Measured by two independent methods at CCT and LLNL. One anomalous value (800 obtained for the largest size at CCT) omitted. Normalized to $1.64\ \mu\text{m}$ diameter values.

^b Normalized to $1.64\ \mu\text{m}$ diameter values.

These experimentally measured angular distributions have been smoothed and compared graphically in Figure 4 with the theoretical expectation for a distribution of dipoles randomly oriented in a bulk medium. All results in Figure 4 have been normalized so that $V_v = 1$ at 90° both for the experimental results with the 1.64μ sample and the theoretical results for the bulk medium. In the bulk medium the theoretical expectation is that V_v will be independent of θ since vertically polarized illumination will preferentially excite dipoles with approximately vertical orientation. This assembly of excited dipoles is symmetrical about the vertical axis and will emit a V_v component independent of θ . In the bulk medium the angular dependence for $H_h = (1 + 2 \cos^2 \theta/3)$. This function has a minimum at $\theta = 90^\circ$, as would be expected from the fact that the assembly of excited dipoles now tends to be oriented along the observation direction (y axis in Fig. 1), and hence emits weakly in that direction (an oscillating dipole does not emit along its axis). At $\theta = 0^\circ$ and $\theta = 180^\circ$, the geometry of the illumination electric vector and excited dipoles is the same as in the V_v case and hence $H_h = V_v$ at these two angles. The

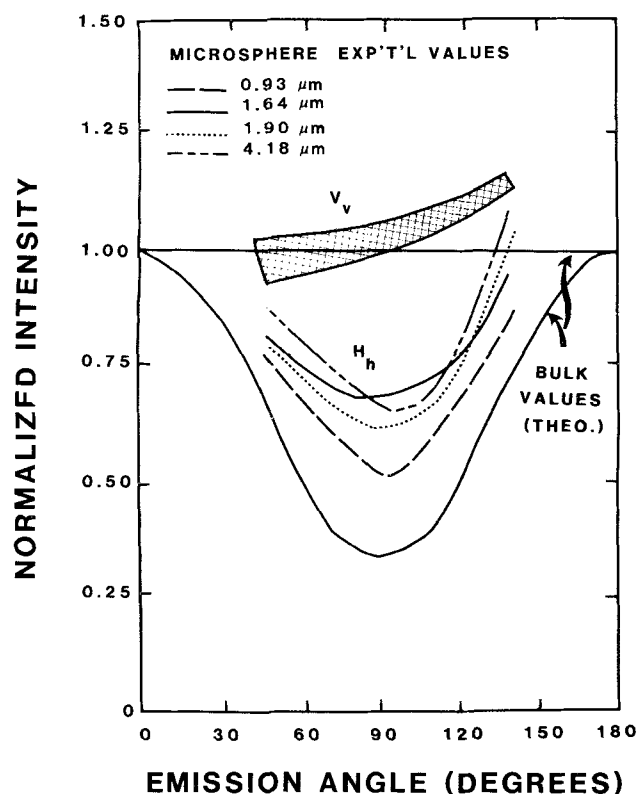


FIG. 4. Measured values of polarized fluorescence components V_v and H_h versus emission angle θ for fluorescent polystyrene microspheres of four sizes suspended in water. Excitation and emission wavelengths in water are centered at 436 and 530 nm, respectively. All values of V_v are normalized to unity for the 1.64μ diameter sample at $\theta = 90^\circ$. Values are also adjusted to correspond to the same total amount of dye. The H_h curves were obtained by smoothing the data in Table 3. The band, indicated by the cross hatching, contains all the V_v measurements. The horizontal line at unit normalized intensity (V_v) and the curve (H_h) labeled bulk values represent the theoretical expectation for the distribution of fluorescent dipoles randomly oriented in bulk medium.

functional form of H_h is the result of the average orientation of excited dipoles along the y axis and the increased emission as θ departs from 90° and approaches 0° and 180° .

The experimental values of V_v increase appreciably with angle of emission over the measured range, $\theta = 45^\circ$ to 138° as shown by the cross-hatched region. The width of the cross-hatched region indicates the difference in V_v among the 4 samples. However, the variability of the set of measurements for any one sample is as large as the sample-to-sample variability (2–5%). Thus, these measurements are not sufficiently accurate to detect any size dependence of V_v over this angular range. The experimentally observed increase of V_v with θ differs somewhat from the constant value in bulk medium expected from theory. This difference is due to optical effects at the microsphere surface, which acts as a lens of short focal length and creates a nonuniform excitation field inside the microsphere. The resulting fluorescence emission is affected by similar optical effects at the microsphere surface so that the geometric emission pattern is a function of θ . This emission pattern could be independent of θ only if the excitation field within the microsphere were either uniform or radially symmetrical, which it is not.

The results for H_h are strikingly different. The experimental values differ significantly from the bulk values and from each other and they show very strong dependence upon emission angle with a minimum value near, but not always precisely at, 90° (as in the bulk medium). The four H_h curves might be expected to nest; three do but the fourth (for the 1.64μ size) crosses over — not what physical intuition nor theory would suggest. However, the general shape of the four H_h curves is similar to although shallower than, the bulk value curve.

The theoretical results calculated on the basis of the microsphere model are shown in Figure 3. Compared with the experimental results in Figure 4, the general trends are the same. Theoretical values of V_v tend to increase with emission angle for the four sizes and overlap the experimental values. The theoretical values of V_v are significantly larger for the largest size microspheres for $\theta < 90^\circ$ than for the other three sizes which cluster rather tightly. The theoretical H_h curves are more closely clustered together than the experimental values. Over the middle range of emission angles which have been studied here, the theoretical values for the particles are only slightly larger than the bulk values and distinctly smaller than the experimental values.

The difference between the experimental and theoretical values for the microspheres may be explained in part by a reduction in the polarization of the fluorescence emission light due to effects such as rotational relaxation of the excited dye molecules before fluorescence emission occurs, lack of parallelism between absorption and emission dipoles, and energy transfer. The theoretical model does not take depolarization effects into account. Some of these depolarizing effects may vary from sample depending on the details of the manufacturing process; for example, the local environment of the dye molecules, and hence any rotational relaxation, may vary among samples. This may explain the larger spread among the four samples in the experimental data as compared to the theoretical calculations. H_h is theoretically more sensitive to depolarization effects than V_v , which is consistent with the fact

that there is a considerable discrepancy between the experimental and theoretical H_h curves but little or none between the V_v curves. Some of the discrepancy between the theoretical and experimental values of H_h may be due to other simplifications built into the model. For example the model assumes that the optical properties of the polystyrene are not changed by the dye, it neglects the band width of the emission spectrum and the spread of particle sizes about the mean, and it presumes that the excited molecules can be treated as classical oscillating electric dipoles. Furthermore, the model assumes that the dye is uniformly dispersed within the particle, that the absorption and emission dipoles are rigidly fixed in space and that they are randomly oriented.

It should be stressed that aside from anchoring of one of the theoretical points and one of the experimental points (1.64 μm diameter particles and $\theta = 90^\circ$) at $V_v = 1$, there are no adjustable parameters in this work. Each of the samples has a different experimentally determined dye content and particle size. Accordingly the agreement between the angular trends, the polarization effects, and the magnitudes of the experimental and theoretical results is gratifying.

We now consider the fluorescence from the four microsphere samples as measured in two different orthogonal geometry flow cytometers (CEI and LLNL), in the conventional photometer (CCT) and as calculated from the theoretical model. The CCT values in Table 4, both theoretical and experimental, correspond to V_v at 90° collected over a narrow angle whereas those from LLNL and CEI obtained by flow cytometry correspond to V_v subtending larger angles (approximately 50°) centered at 90° . However, because of the small angular dependence of V_v over this range, the CCT results are not changed significantly when averaged over the angular range and so these tabulated values can be compared directly.

The results, which are shown in Table 4, are in excellent agreement. Considering that the specific absorbance, which corresponds to the concentration of dye in the particle, varies by a factor of 4 over these samples, that the particle diameter varies by a factor of 4.5, and that particle volume varies by 100, the agreement of all of these signals and computations to within $\pm 10\%$ is impressive.

Conclusions

There are two conclusions, one relating to the general scientific question and the other to the specific question about flow cytometry raised at the introduction of this paper. In general, theoretical analysis suggests and experiment confirms that fluorescence emission from small polymer spheres suspended in water depends not only on dye content but also upon particle size, upon the polarization of the incident and emitted radiation, and upon the angle with respect to the incident beam at which the emitted radiation is viewed. These effects would be expected to be much less pronounced for biological cells for which the refractive index more nearly approximates that of water than the polystyrene investigated here. They would also be expected to be less pronounced if the cellular medium is sufficiently fluid so that random rotation by the dye molecules is comparable to or faster than the relaxation time of the fluorescence. In the latter case the appropriate model would be what has been referred to as

randomly oriented with uncorrelated emission (16) and the angular and polarization effects observed in Figures 3 and 4 would tend to wash out. The effects we have described would be expected to be more pronounced for highly nonspherical cells with a high refractive index and this does turn out to be the case for sperm cells (2, 7, 14).

However, dependence of fluorescence on size hardly occurs even with polystyrene microspheres for V_v over the measured angular range. These fluorescent intensities are independent of particle size to within $\pm 10\%$ and these results as measured independently at three laboratories using two quite different techniques and are consistent with the theoretical predictions. Since it is precisely V_v which is measured in orthogonal flow cytometers, these results support the central dogma of flow cytometry that fluorescence from stained cells is proportional to cellular dye content.

Although no measurements were made on epiillumination flow cytometers, the theoretical calculations shown in Figure 3 allow an estimate of the dependence of fluorescence signals on microsphere size. In epiillumination systems incoherent, unpolarized incident light is used with large numerical aperture optics, and θ centers on 180° . Hence, the size dependence of fluorescence signals for the four sizes of microspheres is expected to be slightly larger than for orthogonal geometry but the same conclusions hold.

Acknowledgments

We appreciate the assistance of Joseph Mazrimas (LLNL) in performing the sucrose density gradient centrifugation measurements. Drs. Marylou Ingram, Brandon Price and Dieter Grossegger at the Institute of Cell Analysis, Miami, Fla., were helpful in obtaining the CEI fluorescence results. We appreciate the helpful discussion and encouragement of Walter Hogg at Coulter Electronics, Inc.

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