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The Polarization of Fluorescence of DNA Stains Depends on the Incorporation Density of the Dye Molecules

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Background: The fluorescence induced by polarized light sources, such as the lasers that are used in flow cytometry, is often polarized and anisotropic. In addition, most optical detector systems are sensitive to the direction of polarization. These two factors influence the accuracy of fluorescence intensity measurements. The intensity of two light sources can be compared only if all details of the direction and degree of polarization are known. In a previous study, we observed that fluorescence polarization might be modified by dye–dye interactions. This report further investigates the role of dye density in fluorescence polarization anisotropy.
Methods: We measured the polarization distribution of samples stained with commonly used DNA dyes. To determine the role of fluorophore proximity, we compared the monomeric and a dimeric form of the DNA dyes ethidium bromide (EB), thiazole orange (TO), and oxazole yellow (YO).
Results: In all dyes sampled, fluorescence polarization is less at high dye concentrations than at low concentrations. The monomeric dyes exhibit a higher degree of polarization than the dimeric dyes of the same species.
Conclusions: The polarization of fluorescence from DNA dyes is related to the density of incorporation into the DNA helix. Energy transfer between molecules that are in close proximity loosens the linkage between the excitation and emission dipoles, thereby reducing the degree of polarization of the emission. © 2004 Wiley-Liss, Inc.

Key terms: fluorescence polarization; energy transfer; fluorescence depolarization; reabsorption; ethidium bromide (EB); thiazole orange (TO); oxazole yellow (YO); TOTO-1; YOYO-1; ethidium homodimer (EBEB)

The fluorescence emissions in flow cytometry are often polarized. The degree of polarization is related to the mobility of the fluorophores, and the orientation of the excitation dipole relative to the emission dipole (1). The emission dipole of stationary molecules tends to be parallel to their excitation axis, but if these molecules rotate between the absorption and the emission of a photon, the correlation between excitation and emission dipoles will be partially lost, causing the fluorescence to have less polarization anisotropy. The linkage between fluorescence polarization and dye mobility has led to special assays in which the polarization of a probe molecule is used to determine the microfluidity of different cell types (2).

In a recent study, we showed that fluorescence polarization is a widely spread phenomenon that is exhibited by many common flow cytometry dyes (3). The degree in polarization of different dye species is generally in line with the fluorophore mobility theory (1). DNA-bound dyes show high polarization values near the theoretical maximum of 0.5. More mobile molecules such as antibody-bound FITC and fluorescein diacetate (FDA) are less polarized, although significant polarization values were observed. Detailed observations revealed some paradoxical trends. When phycoerythrin (PE) and FITC are coupled to antibodies, the larger phycoerythrin shows no polarization while the smaller, assumingly more mobile, FITC exhibits a significant degree of polarization. The polarization of DNA dyes seems to be related to dye concentration. When dye is added to already stained cells,

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the polarization decreases. Because DNA dyes are held tightly in a stacked arrangement, a change in mobility seems unlikely. Dye molecules in plastic calibration beads, assumingly totally immobilized, do not show any polarization at one excitation wavelength, but exhibit some polarization at another. These observations indicate that dye mobility is not the only manner in which excitation and emission dipoles can be uncoupled. Dye–dye interactions also seem to play a role.

Mechanisms that influence fluorescence polarization have practical consequences in flow cytometry. It is a common practice to infer relative dye concentrations from relative fluorescence intensities. However, the intensity of a polarized fluorescent light source is not easily determined. One (trivial) reason is that all photodetectors, to a small degree, are polarization sensitive. Other elements on the detection path may have larger effects on the polarization of the measured light. Transmissive surfaces, such as a dichroic mirror or a beam pick-off at 45°, transmit p-polarized light more efficiently than s-polarized light. In a cytometer, fluorescent or scattered light passes several of these interfaces. By the time light reaches the detector, it will be biased toward one of the two polarization states. The measured signal from a polarized light source, therefore, depends not only on the light intensity, but also on the orientation of the detector assembly. Depending on the layout, the difference in detector sensitivity for the two main polarization components can be expected to be in the range of 10–40% (3). Another, more difficult problem is that polarized light sources are anisotropic. A cluster of polarized dye molecules emits different amounts of light in different directions. The emitted intensity is usually greatest in the plane orthogonal to the prevalent polarization axis, and minimal in a direction parallel to the polarization axis (3). Depending on the direction of observation, different amounts of light will be collected. Because of the combination of detector sensitivity and directionality of the fluorescence emission, the intensity of a point source of polarized light cannot be expressed unequivocally in a single value. To derive the concentration of a fluorescent dye, one must know the degree and direction of polarization, the sensitivity and orientation of the detector, and the direction of observation. If the same dye can exhibit different degrees of polarization, relative fluorescence intensities do not necessarily indicate relative concentration.

The mechanisms that cause fluorescence to be polarized are described in detail elsewhere (3,4). Briefly, an electromagnetic wave most easily excites molecules whose excitation dipoles are parallel to the wave's electric (E-) field vector. As a result, polarized illumination sources, such as the lasers used in flow cytometry, will selectively excite a subset of molecules whose excitation dipoles tend to be aligned with the incident waves. If these molecules do not change orientation between excitation and emission, the emission dipoles will be in the same plane as the excitation dipoles, causing the fluorescence to be directional with a preferred polarization.

Under certain conditions, dye molecules may have direct dipole–dipole interactions without the generation of intermediate photons. The excitation energy can transfer from molecule to molecule, providing that: 1) there is spectral overlap between the acceptor's absorption and the donor's emission spectrum; and 2) the molecules are in very close proximity. Because of the nature of the dipole–dipole interaction, the probability of energy transfer decreases with the sixth power of the distance between the donor and acceptor molecules. Beyond 10 nm or so, the likelihood of energy transfer is negligible, but its probability rises rapidly at shorter distances. Energy transfer is easily observed for dye pairs with different emission spectra. The dye pairs Hoechst 33342-chromomycin, and mithramycin-ethidium bromide show spectral shifts indicative of energy transfer (5,6). Because the donor and acceptor molecules do not necessarily have the same orientation, the E-field of the emitted photon may not align with the excitation dipole of the donor molecule, resulting in fluorescence depolarization. The characteristic distance for a given pair of molecules, Förster's radius, is in the order of a few nanometers for the dyes studied. While energy transfer is usually studied between two molecules of different species, fluorescent molecules with small Stoke's shifts will also engage in resonant energy transfer (RET) (1). A change in the fluorescence anisotropy is a telltale sign that resonant energy transfer is taking place. DNA dyes such as thiazole orange (TO), oxazole yellow (YO), and ethidium bromide (EB), all have significant overlaps between their emission and excitation spectra and incorporate into the DNA helix at close proximities. Energy transfer between the same molecules seems a possibility. If energy transfer between these molecules occurs, significant changes in fluorescence anisotropy can be expected. The depolarization of the fluorescence from DNA dyes at higher concentrations may be a sign of resonant energy transfer and can be used to study the incorporation densities and relative orientations of these molecules.

In order to investigate whether energy transfer between dye molecules affect fluorescence polarization, we tested several dyes as monomers and as dimers. The dimeric DNA dyes consist of two identical DNA-binding groups connected by a flexible bis-cationic amino linker. Because they are connected, the two fluorophores insert in close proximity in the DNA helix, generally 2-bp apart. If energy transfer is important, dimeric dyes should exhibit a lower degree of fluorescence polarization than their equivalent monomers. Observations with varying concentrations of monomeric and dimeric DNA dyes confirm that energy transfer significantly reduces signal polarization. This leads to the conclusion that fluorescence polarization—and therefore fluorescence detection efficiency—is not a fixed property of a particular dye species, but varies considerably with dye density. The capricious nature of fluorescence anisotropy must be considered in the development of robust DNA concentration measurements in flow cytometry.

MATERIALS AND METHODS

Instrumentation

All measurements were performed using a flow cytometer designed and developed in our laboratory. This instrument is derived from the MoFlo (DakoCytomation, Fort Collins, CO) prototype that was developed in our laboratory. A research instrument of a design similar to our current instrument is being manufactured under the name InFlux (Cytopeia, Seattle, WA). A more detailed description of the electronics and special detector for the polarization measurements can be found in our previous papers (7,8). Briefly, a polarizer (03PTA401; Melles Griot, Irvine, CA) was placed in the optical pathway, directly in front of a photomultiplier (H957 series; Hamamatsu, Bridgewater, NJ). The polarizer was allowed to rotate over an angle of 260°, while a potentiometer in a voltage-divider circuit produced a voltage signal that varied linearly with the angle of the polarizer. For each measurement of fluorescent intensity, the output of the voltage divider circuit was digitized as a measure of the polarization angle.

Sample Preparation

Fluorescent microspheres (Fluoresbrite YG, cat# 18860; Polysciences, Inc., Warrington, PA) were diluted 1:100 in distilled water prior to analysis. Mouse thymocytes were harvested from B10.PL(73NS)/Sn mice. Samples were placed into a 15-ml tube, pelleted by centrifugation at 1,000 rpm for 10 min, and resuspended at 1×10^6/ml in 1× Dulbecco's PBS (pH 7.1, cat# 14080-055; Gibco BRL, Gaithersburg, MD). Detergent (2.5% Triton-X) was added to make the cell membranes permeable for the DNA dyes. Boiled RNAse (10 mg/ml) was also added to destroy any RNA in the sample. The cells were placed in a 37°C water bath for 20–30 min. The samples were put into 6-ml sample tubes. Varying amounts of each dye were added from a stock solution of 2 mg/ml in deionized water. The dyes used in this study were EB, YO, TO, ethidium homodimer (EBEB), oxazole yellow dimer (YOYO), and thiazole orange dimer (TOTO). These dyes were chosen for their frequent use in flow cytometry. They were purchased from Molecular Probes, Eugene, OR (cat# E-3565, Y-3603, T-3602, E-1169, Y-3601, and T-3600). The stained samples were allowed to equilibrate at room temperature for at least 20 min prior to measuring. The sheath fluid of the flow cytometer contained 1× Dulbecco's PBS (pH 7.1).

Measurement Procedure

The measurement procedure and data analysis are described in more detail in our previous work (3). Both the angle of the polarizer and the light intensity collected through the polarizer were recorded in a list file. For each fluorescence anisotropy measurement, ≈40,000 particles were recorded per sample to insure a good distribution over the range of the polarizer. From this distribution, the data were binned and averaged to create a single curve consisting of ≈250 point pairs (angle x_n, intensity I_n) summarizing the dataset. A method for nonlinear least squares fitting was used to compute the best-fit curve for the dataset to the function

$$I_n = \alpha \sin^2(x_n) + \beta \cos^2(x_n) \,.$$

The P-value, representing the degree of polarization, was calculated from the best-fit parameters using $P = \frac{|\alpha - \beta|}{\alpha + \beta}$. P varies from 0 (no polarization) to 1.0 (light fully polarized). The maximum degree of polarization expected from a fluorescence source is 0.5 (3). The samples were analyzed sequentially, in order, from the most dilute to the most concentrated sample. For samples of the same dye, but different concentrations, the system was allowed to back-flush for several minutes. When changing the dye sample, the sample tubing was thoroughly back-flushed for 15 min. The samples were filtered thorough a 40-μm nylon filter before analysis to prevent clogging the nozzle.

RESULTS

We measured the relationship between dye concentration and polarization anisotropy for three DNA dyes: EB, TO, YO, and their homodimers (EBEB, TOTO, and YOYO). In the presence of detergent, mouse thymocytes were incubated with varying amounts of dye, after which the cells were analyzed in a flow cytometer with a polarization filter in front of the fluorescence detector. The polarization value (P-value) was calculated from a theoretical fit to measurements at different angles of the polarization filter, as described in the preceding section.

Figure 1 shows results obtained with the three dyes in both their monomeric and dimeric forms. All three dyes exhibit fluorescence anisotropy. The degree of polarization depends on the amount of dye that is added to the cell samples, ranging from the theoretical maximum of 0.5 to almost no polarization ($P < 0.1$). In all cases, the fluorescence polarization of the monomeric dyes is higher than that of the dimeric forms. The P-values for the different monomeric dyes are very similar, ranging from 0.478 ± 0.085 at the lowest dye concentrations, to a value of 0.090 ± 0.05 when the cells are incubated with approximately 5-μmol dye. In general, the polarization of the tandem dyes is much lower than that of the monomeric forms. In this case, there are qualitative differences between the different dye species. The polarization of the EBEB is fairly constant at a value of 0.272. The dimers TOTO and YOYO decrease in polarization with increased amounts. The lowest polarization is observed for YOYO. At an incubation concentration of 5 μmol, this dye shows virtually no polarization with a P-value less than 0.1 (0.090 ± 0.05).

DISCUSSION

The fluorescent properties of dye molecules that are bound to identical substrates are generally considered to be constant. When flow cytometrists compare the fluores-

cence intensity from two stained particles, it is inherently assumed that the spectral characteristics—fluorescence efficiency, absorption, and emission spectra—are the same. In this study of common DNA dyes, we observed that fluorescence polarization, a factor that greatly influences the apparent brightness of fluorescent objects, is not constant, but varies with the amount of dye that is bound to DNA. As the density of bound dye molecules increases, the emitted fluorescence becomes less polarized. Apparently, fluorescence polarization is not an intrinsic property of a stain, but is also determined by the staining conditions.

The importance of this observation is appreciated when we realize that polarized fluorescence is anisotropic. Polarized fluorescent light sources emit different amounts of light in different directions (3,4). A maximally polarized fluorescent light source (P = 0.5) emits twice as much light into a direction orthogonal to the polarization vector than in the direction along the polarization axis. In contrast, completely depolarized light (P = 0) is evenly distributed, with an intensity that is two-thirds of the maximum intensity seen with a perfectly polarized fluorescence source. Most flow cytometers collect light in a direction orthogonal to both the direction and the polarization of the excitation beam. Under these conditions, the collection efficiency for fully polarized light is 1.5 times greater than the efficiency for depolarized light. In typical flow instruments, the detection efficiencies for polarized and nonpolarized fluorescence can be very different.

We suspect that the different degrees of fluorescence polarization are related to the incorporation density of the dye molecules into the DNA helix. In our experiments, we added varying amounts of dye to 1-ml samples, each with 10^6 cells. The amounts of dye added (0.05–5.00 nmol/ml) fall within the typical range for DNA staining experiments. Many publications about DNA dyes incorrectly consider the amount of dye added and the free dye concentration as equivalent. Dye molecules with a high affinity for DNA partition themselves among the solution and the DNA, with most dye molecules bound to DNA. Of the three dyes, EB has the weakest DNA affinity, with a reported (9) binding constant of $K_b = 1.5 \times 10^5$/mol. Each cell contains 6×10^9 bp of DNA. Therefore, at 10^6 cells/ml, each sample presents 10 nmol of potential binding sites to the monomers and somewhere between 3 and 5 nmol of potential binding sites for the homodimers. The binding constant predicts that at 0.05 nmol of dye added per sample, the fraction of bound dye molecules is greater than 0.9 for all of the dyes except EB. For EB, the bound fraction is approximately 0.6 when less than 1 nmol of dye is added. With 0.5 and 5 nmol added, the calculated bound fractions of EB are 0.592 and 0.525, respectively. Even for the weakest binder, at the highest concentrations, the majority of the dye molecules is in the bound state. The bound fractions of the other monomers and dimers at concentrations less than 1 nmol/ml are higher, typically greater than 0.9. We calculated the bound fraction using reported binding affinities (9–13). These considerations indicate that in our experiments the incorpo-

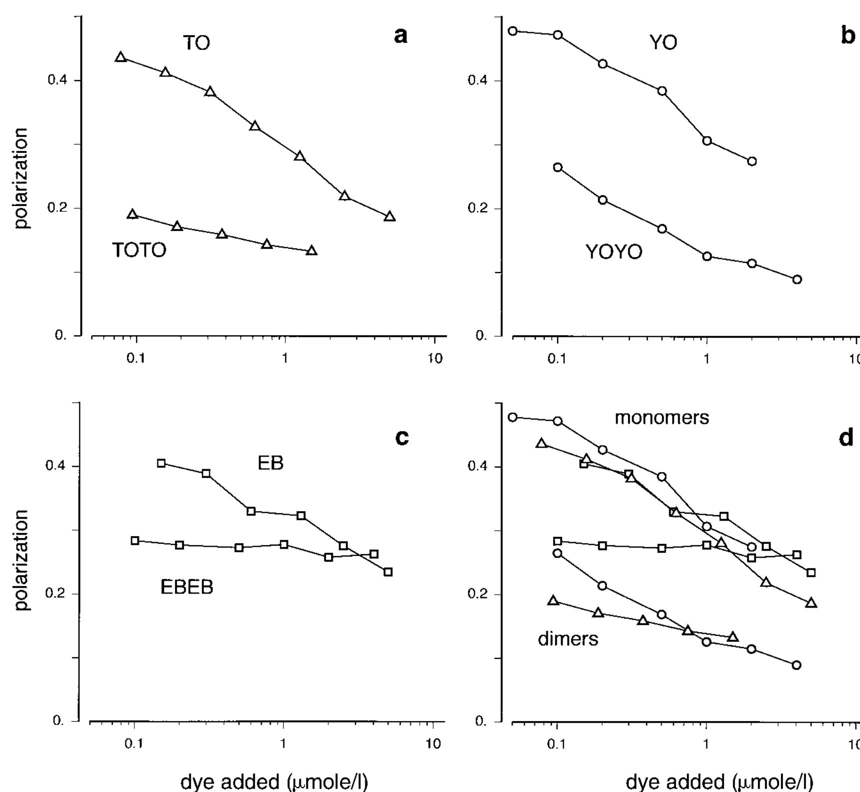

FIG. 1. Polarization for intercalating dyes in their monomeric and dimeric forms as a function of the concentration of dye added to the staining solution. **a:** Thiazole orange (TO) and its homodimer TOTO. **b:** Oxazole yellow (YO) and its dimer (YOYO). **c:** Ethidium bromide (EB) and its homodimer (EBEB). **d:** Results comparing all of the monomers to the dimeric forms. The dye concentration is μmol dye added per liter of solution.

ration ratio varied from one dye molecule for every 200 binding sites to a high range of nearly one dye molecule for every binding site.

The high degree fluorescence polarization that is observed at low dye incorporation ratios is easily understood. The rungs of the DNA ladder between which the dye molecules insert themselves hold the molecules firmly in place. The molecules have restricted mobility and therefore the emission dipoles will be well correlated with the excitation dipole. At the lower dye concentrations, in the order of 1 dye molecule per 100 base pairs, the polarization of the fluorescence of all monomeric dyes is near the theoretical maximum of 0.5 (Fig. 1).

The loss of fluorescence polarization at increasing dye concentrations is unexpected because the mobility of the newly bound molecules should not be greatly different from those that are already seated in the DNA helix. We therefore attribute this decrease in polarization to energy transfer between closely-spaced dye molecules.

One explanation for the fluorescence depolarization at high dye/DNA base pair ratios is resonant energy transfer (RET). This phenomena is described in detail elsewhere, and has been exploited as a spectroscopic ruler (1,14). The RET effect is characterized by the $\frac{1}{r^6}$ nature of the dipole interaction and by the requirement that the donor molecule's emission spectrum overlap with the acceptor molecule's excitation spectrum. At a characteristic distance, known as Förster's radius, one-half of all excited molecules transfer their energy to the donor molecules via this mechanism. There is no requirement that the molecules have to be different; RET can occur between similar molecules having small Stoke's shifts. If the excitation and emission spectra of a donor and acceptor dye molecule are known, then Förster's radius can be calculated (1) using $R_f^6 = 0.211\left[\frac{\kappa^2 Q}{n^4}J(\lambda)\right]$ in Å. In the preceding, κ^2 is a geometric factor that relates the relative orientation of the dipoles ($\kappa^2 = 2/3$ for randomly oriented dipoles), n is the index of refraction of the media (typically between 1.4 and 1.6 for cells), Q is the quantum efficiency of the *donor* molecule, and $J(\lambda)$ is the overlap integral defined as

$$J(\lambda) = \int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda\ .$$

In this equation, $F(\lambda)$ is the emission spectrum of the donor molecule normalized to unit area, $\varepsilon(\lambda)$ is the acceptor molecule's extinction coefficient in units $mol^{-1}cm^{-1}$, and for our purposes, λ is in nanometers (nm). The emission and extinction spectra for EB, YO, and TO are readily obtained (15), and one can calculate that the Förster's radii for these dyes are 19.97, 57.89, and 54.76 Å, respectively. The conditions for homologous energy transfer are met for the dye molecules that we have studied, particularly YO and TO, as evidenced by their relatively large Förster's radii. This is less so for EB, but the degree of overlap between its emission and excitation spectra allows for energy transfer at incorporation densities greater than 5 bp per dye molecule.

At these high incorporation densities, the dye molecule spacing falls within the appropriate range for energy transfer. The DNA helix makes one full turn for every 10 bp or 34 Å. At an incorporation density of 1:10, TO and YO will have on average two neighbors within energy transfer range in each direction. These molecules are held in a parallel orientation between the steps of the DNA ladder (16), further increasing the chance of dipole interactions. In contrast, at an incorporation density of 1:100, the average distance between dye molecules is 340 Å; well beyond the range of energy transfer for all dyes studied. The calculated spacing of intercalated molecules is consistent with the view that energy transfer is not a likely occurrence at low dye concentrations, but does become a significant phenomenon at the high end of the concentration range. Because the helix turns 36° for each base pair, the correlation between the emission dipoles is lost rapidly as energy is transferred between dye molecules.

Our observations with the dimeric dyes confirm that closely spaced dye molecules may interact (Fig. 1d). These dimers consist of two identical fluorophores linked by a short flexible chain (EBEB (≈1.54 nm), TOTO and YOYO (≈1.68 nm)). The linker allows both dye groups to intercalate simultaneously in close proximity, generally 2 bp apart (16). It is consistent with our theory that such molecules should interact causing a significant decrease in polarization.

In order to better understand the effects of energy transfer with dye incorporation density, we constructed a Monte Carlo model of the interaction using a pairwise interaction to account for the fluorescence depolarization. Dye molecules are randomly inserted into a linear DNA chain at a specific density. The fluorescence depolarization for each dye molecule with its neighbors is computed using the time averaged depolarization ratio (17)

$$R = \frac{\left(\frac{r}{R_f}\right)^6 + \frac{9}{4}\cos^4\phi + \frac{3}{4}\cos^2\phi}{\left(\frac{r}{R_f}\right)^6 + 3\cos^2\phi},$$

where ϕ is the in-plane angle between the fluorophores, r is the distance between the fluorophores and R_f is Förster's radius. The polarization is related to the depolarization ratio via

$$P = \frac{3RP_0}{3 - P_0 + RP_0}$$

where P_0 is the polarization extrapolated to zero binding density. For our purposes, we estimate P_0 from Figure 1 to be 0.45 for EB and TO and 0.48 for YO. This model assumes that the intercalators are inserted perpendicular

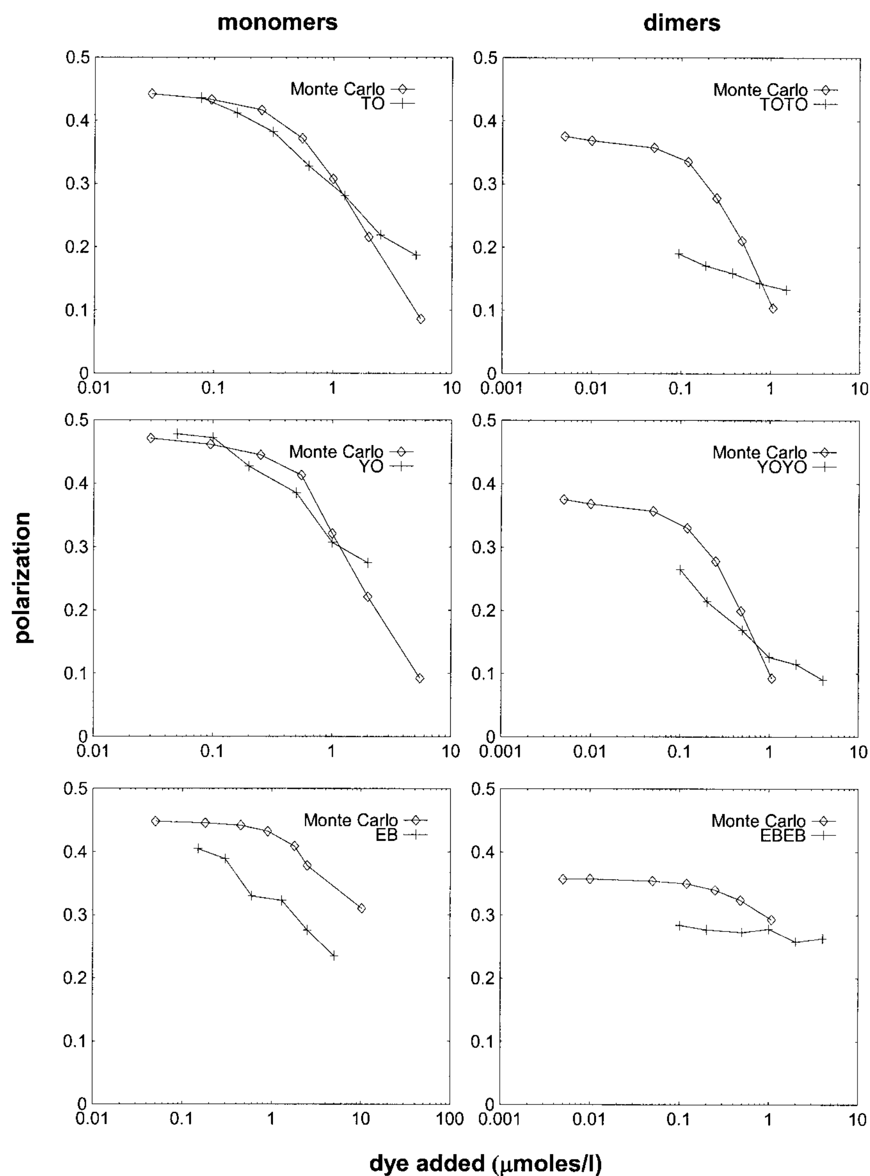

FIG. 2. Comparison of the experimental data to a Monte Carlo simulation that uses resonant energy transfer as the dominant mechanism for fluorescence depolarization. The results for the monomers are shown in the graphs on the left, and the results for the corresponding homodimers are shown in the graphs on the right. The apparent discrepancy noted in the shape of the EBEB data agree well with the predictions of the model.

to the helical axis of the DNA (16), and that insertion of a dye molecule unwinds the DNA helix by 20°, and expands it by 3.4 Å. These two parameters, along with the Förster's radius are the only variables used in the modeling. For the dimer molecules, the length of the linker between the dye molecules is such that there are two base pairs separating the intercalators. The results of these models are shown in Figure 2. The Monte Carlo results have been adjusted to account for the different binding affinities of the six dyes to allow a direct comparison to the experimental data. This model accurately predicts much of the observed data, particularly for the monomers. As the model has no adjustable parameters, these ab initio predictions are a strong indicator of energy transfer as the dominant mechanism for the fluorescence depolarization observed. Compared to the other dyes, the shape of the fluorescence polarization curve for EB appears enigmatic. Surprisingly, the Monte Carlo model accurately predicts the shape, but not the scale of the fluorescence depolarization for EB. This effect may be due an inaccurate estimation of the binding constant, or to an affinity for cooperative binding to the DNA between two molecules.

Another mechanism of fluorescence depolarization, reabsorption of fluorescence, must also be considered. At dye concentrations around 1 μmol, reabsorption of fluorescence photons is not a significant factor when the dye is homogeneously distributed. However, when bound to cell nuclei, the dye molecules are localized to a small sphere, providing a significant opportunity for reabsorption. A nucleus contains about 6×10^9 bp of DNA. At an incorporation ratio of one dye molecule every 10 bp, 6×10^8 dye molecules will be contained in a sphere approx-

imately 5 μm across, which is equivalent to a local dye concentration of 0.0152 mol. The maximum absorption coefficient of our dye molecules is in the order of 1×10^5 $mol^{-1}cm^{-1}$ for YO and TO, and 7×10^3 $mol^{-1}cm^{-1}$ for EB (18). For a photon exiting a sphere with radius r, the average distance traversed for a random emission location and direction is $3/4r$. Applying Beer-Lambert's law, we conclude that over a distance of approximately 2 μm, the absorption probability of a photon at the maximum emission wavelength is 0.14. These numbers indicate that for the spectral range in which fluorescence emission and photon absorption overlap, reabsorption and emission of secondary fluorescence photons may take place. Similar to direct energy transfer, the fluorescence resulting from these secondary emissions is expected to lead to fluorescence depolarization and to chromatic shifts to longer wavelengths.

The observation that tandem dyes show depolarization at very low incorporation densities identifies energy transfer as the dominant mechanism. Nevertheless, in the higher concentration range, fluorescence reabsorption can be expected to occur as well, leading to spectral shifts towards longer wavelengths. Dichromatic staining properties have been reported for a number of DNA dyes (8,19–21). These spectral shifts are often interpreted as evidence for the existence of different classes of binding sites. The data presented here indicate that an alternative explanation, the occupation density of identical binding sites, also needs to be considered.

A major purpose of flow cytometry is to relate fluorescence intensity to the number of binding sites. Kerker et al. (22) pose the rhetorical question whether it is justified to consider fluorescence as a linear process which increases proportional with dye concentration. There are many mechanisms that may lead to a nonlinear relationship between excitation and fluorescence. Nevertheless, Kerker et al. (22) conclude that a linear treatment is justified as long as the dye molecules are bound to the substrate and are measured under similar conditions. This notion seems to be generally held by the flow cytometry community. In virtually all publications, fluorescence intensity is considered to be proportional to the number of attached dye molecules. Our experiments show that under identical binding conditions, the character of the fluorescence varies with the density of the fluorescent molecules. Flow cytometers and the associated optical detectors are particularly sensitive to signal polarization. The observed fluorescence depolarization could significantly influence the comparison of relative fluorescence intensities.

DNA measurements for cell cycle analysis require a linearity and precision within a few percent of full scale (23). In sharp contrast, polarized excitation sources introduce a degree of fluorescence anisotropy whose effects are much larger than the desired precision of 1 to 2%. For instance, in the direction perpendicular to the laser propagation vector and the sample stream, maximally polarized fluorescence ($P = 0.5$), will appear 20% brighter than light from an unpolarized source (3). In absolute terms, this difference is an order of magnitude larger than most cytometrists would find acceptable. We have shown that for an identical number of intercalated dye molecules, the polarization can vary by as much as 50% for the dimeric and monomeric forms of the dye. These differences in polarization will result in concomitant changes in the measured fluorescence intensity. To improve the robustness of DNA fluorescence measurements, it advisable to make sure that the dye-to-binding site ratio is the same for all samples. This operational detail may be difficult when studying samples with varying numbers of cells. If variations in cell numbers are expected, the samples could be stained with a range of dye concentrations. After the measurements are completed, only those samples that yield approximately the same absolute fluorescence intensities should be compared. Another solution is to measure fluorescence signals in flow cytometry at the "magic angle." When a detector is placed at an angle of 54.7° to the polarization vector of the excitation light, the measured fluorescence intensity becomes independent of the degree of polarization of the fluorescence emission (3). This scheme may accomplished either by rotating the detector relative to the polarization axis, or by rotating the laser's axis of polarization relative to the detector system. Although magic angle measurements do not abolish all nonlinear phenomena— differential bleaching, spectral shifts, etc., will still contribute to measurement errors—a significant improvement of accuracy can be expected.

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