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Identification of Nucleotides with Identical Fluorescent Labels Based on Fluorescence Polarization in Surfactant Solutions

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A solution-phase steady-state polarization-based method for discriminating among the four DNA nucleotides labeled identically with tetramethylrhodamine is described and demonstrated. Labeled nucleotides were dissolved in buffered surfactant solutions. In room temperature 4.5 mM Triton X-100 solutions at neutral pH, the measured steady-state polarizations of tetramethylrhodamine-labeled dATP, dCTP, dGTP and dUTP were 0.261 ± 0.003, 0.112 ± 0.003, 0.288 ± 0.003, and 0.147 ± 0.003, respectively. A blind test of 40 samples showed no errors in classification based on polarization. The reproducibility obtained during this study demonstrates that the four dye-labeled nucleotides can be discriminated with more than 99.8% confidence.

DNA sequence analysis plays an important role in our understanding of gene evolution and variation, gene regulation, and diseases related to genetic variation. Two approaches that are widely used to sequence DNA are the dideoxy chain termination method of Sanger[1,2] and the chemical degradation method of Maxam and Gilbert.[3] Of these, the former dominates modern high-speed sequencing technologies because of its facile ability to incorporate fluorescent labels for sequence determination. Between approximately 300 and 700 bases can be sequenced in a single run with modern high-speed methods. Consequently, many overlapping subsequences must be determined to construct a "consensus" sequence of a larger DNA segment. Despite dramatic increases in speed over the past decade, existing procedures for sequencing remain labor intensive and time-consuming.

The recent upsurge in efforts at the measurement and characterization of single molecules has created new opportunities for advanced sequencing.[4−22] A single-molecule-based alternate approach to sequencing large segments of DNA seeks to overcome resolution and registration problems by identifying bases cut sequentially from a single DNA molecule by an exonuclease.[23−34] This approach suffers at present from the limited number of dyes

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that are available for high-density labeling of the DNA bases and the limited range of available wavelengths, although progress continues in both areas.[34−37] A number of groups have investigated methods for identifying single-dye-labeled mononucleotide units on the basis of factors other than excitation or emission wavelength. Fluorescence lifetimes,[40−44] fluorescence burst intensities,[45] and time-resolved fluorescence anisotropy[46] are methods that have been explored with some success for their potential in single-molecule sequencing. Multiparameter methods such as spectral/temporal[38] and spectral/temporal/fluorescence polarization methods[39] have also been used. Most of the other common methods for discriminating between molecules having similar or identical (isochromatic) fluorescence spectra, for example, HPLC,[47−49] HPLC/MS,[50−52] and GC/MS[53,54] would be difficult to adapt for rapid sequencing on the single-molecule scale.

A drawback of current concepts for single-molecule sequencing is the need to use DNA nucleotides that are labeled prior to nucleolytic cleavage. This is because the chemical modification needed for labeling may reduce or eliminate the efficiency of nucleolytic cleavage compared to native DNA,[32] and the need for synthetic DNA restricts the achievable sequence length. An approach to single-molecule sequencing based on native DNA could have advantages over the current conception if detection of native nucleotides were possible at the single-molecule level. Such detection could be achieved by postnucleolytic-cleavage derivitization with a common fluorescent label. Discrimination of such labeled nucleotides would require an analytical method based on a spectroscopic property that is dependent on the chemistry of the nucleotide.

Our research group (among others) has investigated steady-state fluorescence polarization as a tool for the measurement and discrimination of small molecules in surfactant solutions.[55] In these studies, the surfactant solution provided a high-viscosity microenvironment for small-molecule fluorophores. The resulting decrease in molecular rotation of the fluorophores permitted them to exhibit a nonzero steady-state fluorescence polarization.[56−61] While the interaction of the surfactant with the fluorophore is usually nonspecific, it is governed by a variety of chemical factors such as Coulombic and hydrophobic forces.[62,63] These factors vary with the chemical nature of the fluorophore, leading us to inquire whether steady-state fluorescence polarization can be used to discriminate between identically labeled mononucleotides.

This report describes an investigation into the potential feasibility of room temperature fluorescence polarization as a means of discriminating between mononucleotides with identical labels. Our laboratory explored three common surfactants (Triton X-100, sodium dodecyl sulfate and cetyltrimethylammonium bromide) as hosts for the four tetramethylrhodamine-labeled nucleotides shown in Figure 1. Results indicate that the conventional fluorescence polarization at neutral pH in Triton X-100 can provide unambiguous identification (at >99.9% confidence level) of the labeled nucleotides. The following sections detail these studies and provide a simple analysis suggesting that polarization-based discrimination of labeled DNA nucleotides could be applied to single-molecule studies, albeit with difficulty.

EXPERIMENTAL SECTION

Reagents. Tetramethylrhodamine-6-dNTP (dNTP = dATP, dCTP, dGTP, and dUTP) were purchased from NEN Life Products, Inc. (Boston, MA) and were used as received. These labeled nucleotides are provided as mixtures of two isomers, as illustrated in Figure 1, with attachment at the 5 and 6 positions of the fluorophore's benzoic acid group.

Buffer solutions were VWR brand and were obtained from VWR Scientific. The pH 2 buffer contained 0.05 M glycine adjusted to the buffer pH with hydrochloric acid. The pH 3, pH 4, pH 5 and pH 6 buffers contained 0.05 M potassium hydrogen phthalate adjusted to the buffer pH. The pH 7 and pH 8 buffers contained a mixture of dibasic sodium phosphate and monobasic potassium phosphate. The pH 9 buffer contained 0.05 M boric acid and 0.05 M potassium chloride, and was adjusted to the buffer pH. The pH 10 buffer contained a mixture of 0.025 M sodium carbonate and 0.025 M sodium bicarbonate. All buffers contained an antimicrobial preservative, CAS 7732-18-5. All other reagents and solvents were obtained in analytical purity or better from Aldrich Chemical Co. and were used without further purification. Stock solutions for 0.02 M cetyltrimethylammonium bromide (CTAB), 0.2 M sodium dodecyl sulfate (SDS) and 0.01 M Triton X-100 (TX-100) were prepared in a 100-ml flask using distilled water and were used in the experiments directly.

Fluorescence Measurements. Fluorescence emission and polarization was measured using a Hitachi F-4500 spectrofluo-

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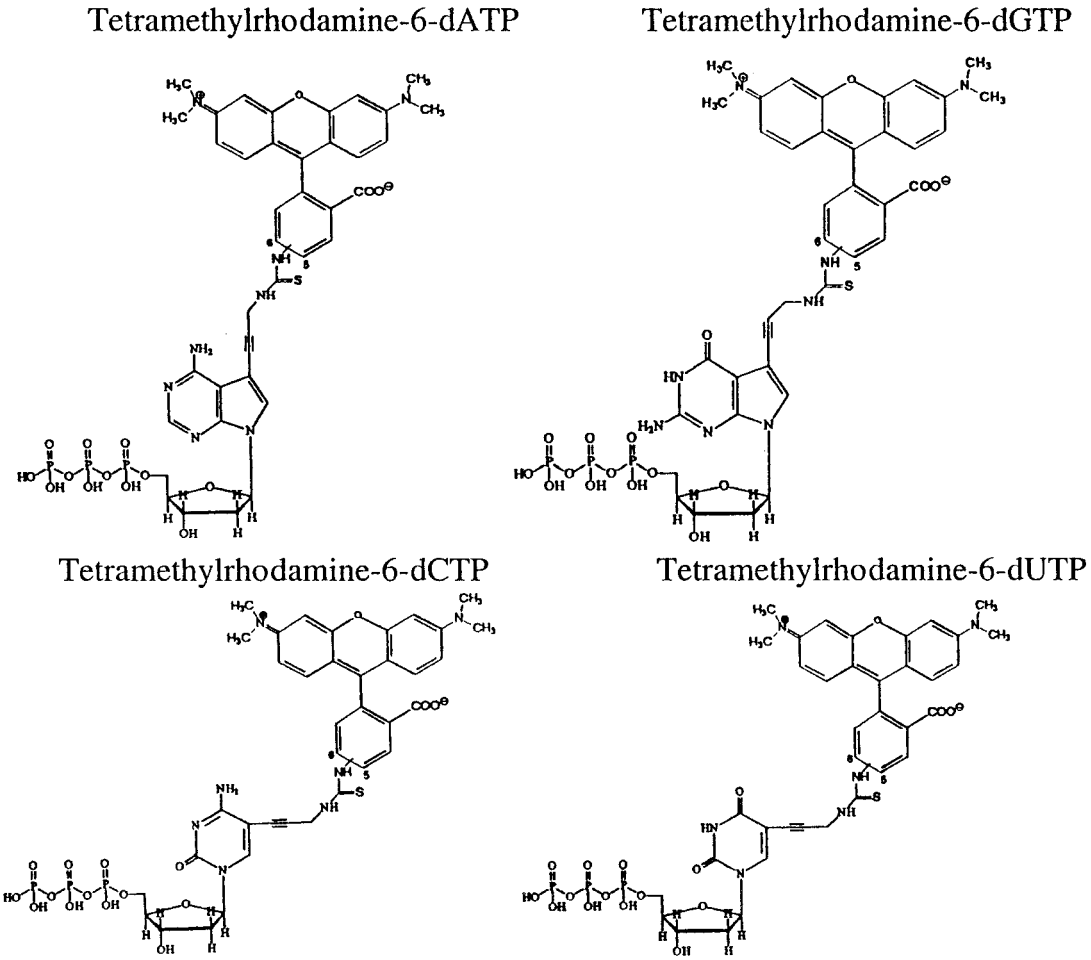

Figure 1. Structures of the tetramethylrhodamine-6-dNTPs used in this study. All compounds were commercially available as a mixture of the 5 and 6 isomers.

rometer (Hitachi Co., Tokyo, Japan). Fluorescence polarization was calculated from

$$P = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}} \quad (1)$$

where I_{vv} and I_{vh} represent the vertically and horizontally polarized emission intensity following excitation with vertically polarized light, respectively, and G ($=I_{hv}/I_{hh}$) is a correction factor for detector sensitivity to the polarization direction of emission, where I_{hv} and I_{hh} represent the vertically and horizontally polarized emission intensity excited by horizontally polarized light. Spectral resolutions of 5 and 10 nm were used for excitation and emission channels, respectively. All measurements were made with 10 s integration times. Fluorescence intensities were recorded with no polarization correction. Fluorescence intensities were measured at selected locations on the basis of the maxima observed in excitation and emission spectra recorded for the samples. All polarization measurements used 515 and 570 nm as excitation and emission wavelengths, respectively, to minimize scattering of excitation light into the detector and to avoid the polarized Raman scattering of water in the samples.

RESULTS AND DISCUSSION

Table 1 gives results for the measurement of fluorescence intensity and polarization for all four labeled nucleotides in all three surfactants at neutral pH and 25 C. Surfactant concentrations were set well above their critical micelle concentration (cmc). The wavelengths listed in Table 1 represent the maximums of excitation and emission spectra of the labels. These wavelengths were used to record the effective intensity of the fluorescence of each sample listed in the table.

Excitation and emission at 515 and 570 nm, respectively, were used to record the fluorescence polarization for each sample. Table 1 shows that different excitation and emission maximums were found for dye-labeled nucleotides in the three different media, even though tetramethylrhodamine was the chromophore in each case. However, the maximums for the labeled nucleotides varied by no more than 2−4 nm within a given surfactant. Because this variation is much less than the apparent resolution of the excitation or emission spectrum of tetramethylrhodamine, we can neglect the wavelength-dependence of polarization as a factor in the observed behavior of the different labeled nucleotides in any single medium to a first approximation. That is, we can assume the same optical transitions are being excited in, and detected from, the tetramethylrhodamine labels of each nucleotide. This implies that each labeled nucleotide exhibits the same large positive polarization in the absence of rotational depolarization to a first approximation. Differences in measured polarization between the labeled nucleotides can be attributed almost entirely to differences in rotational depolarization rates.

Table 1. Fluorescence Excitation and Emission Wavelengths, Fluorescence Intensity, and Fluorescence Polarization for Tetramethylrhodamine-6-dNTP (N = A, C, G, U)

	dATP	dCTP	dGTP	dUTP
$\lambda_{ex}/\lambda_{em}$, nm	546/566	546/568	546/566	546/564
		CTAB, 4.5 mM		
intensity	484 ± 16	295 ± 11	145 ± 3	334 ± 9
polarization	0.284 ± 0.001	0.280 ± 0.002	0.327 ± 0.002	0.281 ± 0.001
$\lambda_{ex}/\lambda_{em}$, nm	552/570	550/570	554/572	552/570
		SDS, 16 mM		
intensity	515 ± 13	491 ± 7	195 ± 2	452 ± 5
polarization	0.226 ± 0.001	0.188 ± 0.002	0.226 ± 0.001	0.199 ± 0.001
$\lambda_{ex}/\lambda_{em}$, nm	548/570	550/568	546/568	548/568
		TX-100, 4.5 mM		
intensity	332 ± 5	318 ± 9	124 ± 3	274 ± 12
polarization	0.261 ± 0.003	0.112 ± 0.003	0.288 ± 0.003	0.147 ± 0.003

All solutions are 3.0×10^{-7} M tetramethylrhodamine-6-dNTP in pH 7.0 phosphate buffer. All measurements were made at room temperature (25 °C). Spectral resolutions of 5 and 10 nm for excitation and emission, respectively, were used throughout. $\lambda_{Ex}/\lambda_{Em}$ wavelengths were determined from the maxima of excitation and emission spectra. Fluorescence intensities were measured using the maximum excitation and emission wavelengths. All polarization measurements were made with 515 nm excitation and 570 nm emission to reduce scattering effects. Standard deviations were based on six replicates of each measurement.

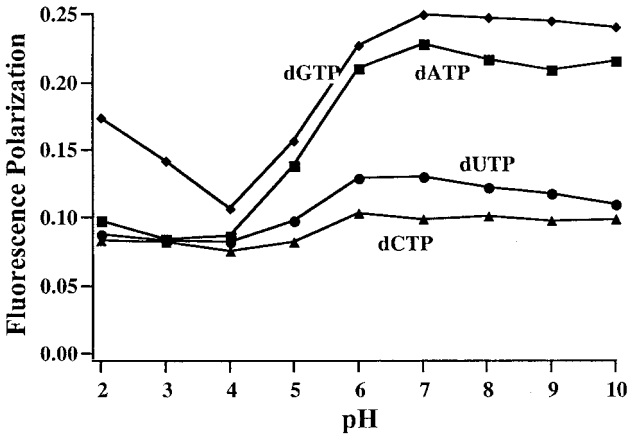

Figure 2. Effect of pH on fluorescence polarizations of tetramethylrhodamine-6-dNTPs in TX-100 surfactant solutions. Concentrations of each labeled nucleotide were 0.45 μM. The surfactant concentration was 2 mM. Excitation was at 515 nm (5 nm bandwidth), with emission detected at 570 nm (10 nm bandwidth). dGTP, ♦; dATP, ■; dUTP, ●; and dCTP, ▲.

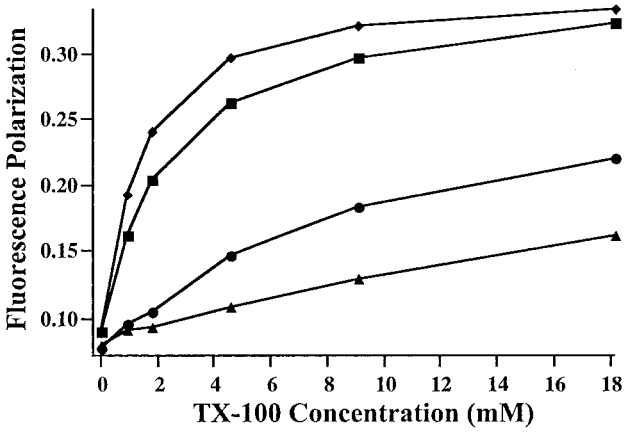

Figure 3. Effect of TX-100 concentration on fluorescence polarizations of tetramethylrhodamine-6-dNTPs in pH 7.0 buffer solutions. Concentrations of each labeled nucleotide were 0.30 μM. Excitation was at 515 nm (5 nm bandwidth), with emission detected at 570 nm (10 nm bandwidth). dGTP, ♦; dATP, ■; dUTP, ●; and dCTP, ▲.

Comparison of the observed polarizations of the labeled nucleotides in the different media suggests that, of the three surfactants tested, TX-100 is the most promising for the purpose of discriminating between the nucleotides. For stable measurements, however, we felt it important to explore the effects of TX-100 concentration and pH on the observed polarization. These measurements were made first to determine whether conditions can be chosen whereby small errors in concentration of the surfactant or the solution pH do not dramatically affect the reproducibility of the measurement. A second reason for these additional measurements was to locate optimum conditions for the identification of the nucleotides.

In a recent report,[55] we showed that the net charge of the fluorophore was one of the factors that determine affinity of fluorophores for micellar media. Solution pH is, thus, an important consideration in promoting selectivity between different species in this environment. Figure 2 shows the effects of varying the pH of the surfactant for a fixed TX-100 concentration of 2.0 mM. Concentrations of the labeled nucleotides in this experiment were fixed at 0.45 μM. This figure shows that pH 7 measurements are in a suitable plateau region for the polarization values of each of the labeled nucleotides. Significantly lower pH values degraded the separation of the different nucleotides. Higher pH values had relatively little effect on our ability to distinguish the nucleotides from one another.

Figure 3 shows, for neutral pH, the effects on the observed polarization of varying the TX-100 concentration. Labeled nucleotide concentrations were fixed in this experiment at 0.30 μM. The effect of surfactant concentration on the polarization of the labeled nucleotide fluorescence is due to the formation of micelles in the solution when the critical micelle concentration (cmc) for TX-100 is exceeded. The value of the cmc of TX-100 is reported to be 0.22−0.24 mM.[64] Below this concentration, the observed polarizations reach zero as a result of rapid small-molecule rotational depolarization of the fluorescence of the labeled nucleotides. Above this level, the observed polarization increases rapidly. At very high concentrations of TX-100, the observed

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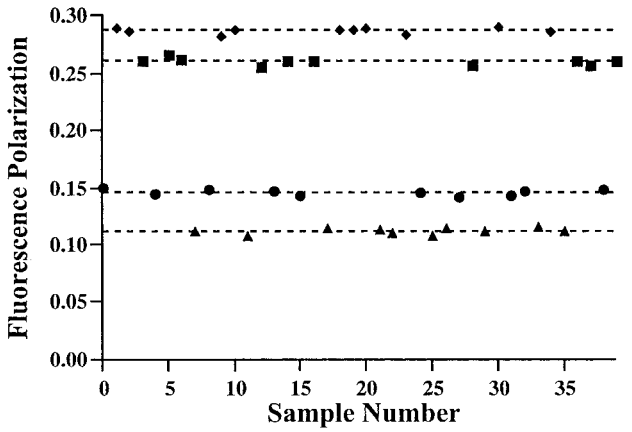

Figure 4. Results of a blind test to classify labeled nucleotides only on the basis of fluorescence polarization. All samples were 0.10 μM tetramethylrhodamine-6-dNTP and 4.5 mM TX-100 in pH 7.0 buffer solution. Excitation was at 515 nm (5 nm bandwidth), with emission detected at 570 nm (10 nm bandwidth). Samples assigned as dGTP, ♦; as dATP, ■; as dUTP, ●; and as dCTP, ▲.

fluorescence polarization of the labeled nucleotides begins to approach $P \approx 0.3$, apparently the ultimate value associated with complete partitioning of the dyes into surfactant micelles. Between these limits, however, a wide range of TX-100 concentrations exist over which the nucleotides partition differently and exhibit differing values of polarization.

On the basis of the results in the preceding tables and figures, solutions with neutral pH and a TX-100 concentration of 4.5 mM were selected as test media for discriminating between the nucleotides based on steady-state polarization.

A blind test was arranged to determine whether discrimination of the nucleotides based on steady-state polarization in room-temperature fluid media could be demonstrated. In our test, a member of another research laboratory at University of South Carolina volunteered to prepare 40 random blind samples for measurement. The samples were composed of 0.3 μM of a randomly selected labeled nucleotide and 4.5 mM TX-100 in a pH 7 buffer solution. These samples were labeled with sample numbers to facilitate handling and record-keeping and were transferred to the authors for testing. No indication of the identity of the nucleotides in the samples was provided to the authors until after the polarization-based classification was completed.

Results for the measurement of the random blind samples versus the sample number are shown in Figure 4. Dashed lines have been added to the figure to show the values of polarization expected for the four nucleotides. Assignments of the samples were determined by which expected value was closest to the sample measurement. Samples assigned as dGTP are represented by diamonds in Figure 4, and samples assigned as dATP, dUTP, and dCTP are represented by squares, circles, and triangles, respectively. Once measurement and assignment was completed, the identity of the samples was made known, and no errors in assignment had been made. The greatest deviation of any point in Figure 4 from the average for its class is an error of 0.0062 for sample 5, which belongs to the dATP class. The average values and 95% confidence limits determined for the four classes was found to be 0.288 ± 0.006, 0.261 ± 0.007, 0.147 ± 0.006, and 0.112 ± 0.006 for dGTP, dATP, dUTP, and dCTP, respectively. The confidence limits on these values are small when compared with the differences between them. Because the minimum separation of two average values is between dGTP and dATP (a difference of 0.0267), and because the measurement of dATP was subject to the maximum standard deviation ($\sigma_{\mathrm{dATP}} = 0.00329$), we can obtain an estimation of the maximum level of confidence that can be attributed to these measurements. The maximum confidence level will be defined by a Student's t value of

$$t_{\max} = \frac{0.0267}{2\sigma_{\mathrm{dATP}}} = 4.06 \qquad (2)$$

The level of confidence with which this value of Student's t can be associated (given 9 degrees of freedom in our measurements) is[65]

$$F(4.06) = \int_{-\infty}^{4.06} \left(\frac{\Gamma(5)\,\mathrm{d}x}{\Gamma(9/2)\sqrt{9\pi}\left(1 + \frac{x^2}{9}\right)^5} \right) = \int_{-\infty}^{4.06} \frac{0.388035\,\mathrm{d}x}{\left(1 + \frac{x^2}{9}\right)^5} = 0.998 \qquad (3)$$

where F is the included fraction of the measurement population and Γ is the Euler γ function. This calculation indicates at best a 99.8% confidence level in the assignment of samples to the dATP classification; this is the lowest level of confidence of any classification in the system.

Despite the high level of confidence that can be expressed for the macroscopic solutions used for the preceding studies, it remains to be determined whether single-molecule-level detection and discrimination can be accomplished using fluorescence polarization. The major uncertainties in extrapolating from these results to the single molecule level are (a) the contribution of isomers to the results and (b) estimating how the diminished signal levels inherent in single molecule detection would affect the confidence with which samples could be classified.

The first issue, that of the contribution of isomers to the previous results, arises because a single molecule is also a single isomer and, therefore, cannot be characterized by the behavior of an isomeric mixture. For a pure sample, we could reasonably expect the ergodic hypothesis to hold true, that is, the time-averaged behavior of a single molecule should approach the ensemble behavior of many molecules. This is generally untrue for mixtures. At best we can assert that our results show that isolated isomers are likely to exhibit differential polarization. A more unequivocal statement requires specific information about the isomers. An effort was made in our laboratory to separate the isomers of the labeled nucleotides that comprise the commercially available mixture (see Figure 1) using high-performance liquid chromatography. Unfortunately, no separation could be obtained with either reverse phase (octadecyl-modified column) or normal phase chromatography in the solvent systems we chose. This suggests that both of the isomers may show similar partitioning behavior to one another in the TX-100 surfactant solutions, but it is by no means certain. Without explicit separate measurements

(65) *CRC Handbook of Chemistry and Physics*; Weast, R. C., Ed.; CRC Press: BocaRaton, FL, 1986.

of each isomer of the labeled nucleotides, no valid extrapolation of these results directly to the single-molecule level is feasible.

The question of signal levels is, however, one that can be addressed. In our measurements, the active chromophore is tetramethylrhodamine. Rhodamine 6G (R6G) has been used in single-molecule studies by others, some reports of which have provided information on the distribution of the numbers of photoelectrons characterizing single molecule events. Reference 45, for example, provides direct measurements of burst intensities for R6G in an optical system designed for single-molecule detection. In this case (see Figure 3b of ref 45), the most probable number of photoelectrons detected was reported as approximately 60 for a single molecule of R6G. The fwhm for this distribution can be estimated from the plot as approximately 20 photoelectrons, nearly the value of 18 expected for Poisson statistics in photon counting. We make the assumptions that (a) all photons detected in a normal fluorescence measurement can be detected by the two polarization channels in a polarization measurement, and (b) the standard deviation of each measurement is given the by square root of the average number of counts on that channel. Under these assumptions, we can derive the following expression for the standard deviation of the polarization measurement,

$$\sigma_p = \sqrt{\frac{1+P}{N}} \tag{4}$$

where P is the polarization, N is the average number of photoelectrons detected, and σ_P is the standard deviation of the polarization. The expected standard deviations of P for hypothetical single molecules whose time averaged polarizations are the same as for the isomeric mixtures presented here can be calculated from the average polarizations reported here and the estimated average number of photoelectrons in a single measurement. For an average photoelectron count of only 60, as reported in ref 45, eq 4 gives $\sigma_P \approx 0.13$ for all of the labeled nucleotides. This standard deviation would be too great for polarization to be used in classification on the single-molecule scale. Eq 4 also provides a means of estimating the minimum signal level that would be required for single-molecule measurement via fluorescence polarization. This estimate is made by determining the maximum standard deviation for a measurement that would give adequate 95% confidence limits for the most difficult classification (dATP vs dGTP). Using this criterion, we estimate signal levels of at least 23 000 photoelectrons detected on average per molecule would be necessary. Although not a mathematical or physical impossibility, this signal level must be considered challenging to obtain in practice.

Schaffer et al.[46] report the successful polarization-based discrimination of rhodamine 123 and a variant of green fluorescence protein. These molecules have grossly different rotational depolarization rates, such that the steady-state polarization for the protein was 0.414, but the steady-state polarization of the rhodamine was 0.015. The average burst size measured in ref 46 was >200. The result in ref 46 is, therefore, consistent with a measurement standard deviation from eq 4 on the order of 0.08 for the protein and 0.07 for rhodamine. These values are very close to the standard deviations reported in Table 2 of ref 46. Combining these with the observed polarization difference of 0.4, the two species should be discriminated with about 96% efficiency by our analysis, which is consistent with the results in the reference. Although the method of the reference works for grossly different rotating fluorophores, the similarity of the four labeled nucleotides would render the treatment ineffectual for the present analysis. Only the surfactant medium provides sufficient selectivity toward rotational depolarization to make discrimination potentially feasible. Unfortunately, the discrimination is low for one of the determinations (dATP vs dGTP).

The estimated requirement for 95% confidence in discriminating between dUTP and dCTP is 7000 photoelectrons, but only 700 are needed to discern dATP from dUTP. Thus, discrimination between the classes dATP/dGTP and dUTP/dCTP appears feasible via fluorescence polarization in surfactant media in the near term. If detection capabilities increased to the 7000-photoelectron level (or if a surfactant with improved discriminating ability were found), true sequencing would be feasible by this route by sequencing the complementary strands of a single DNA molecule against one another. That is, if the dUTP/dCTP class could be discriminated, it would be possible to use information from the complementary strand to determine the specific identities of nucleotides in the dATP/dGTP class.

CONCLUSIONS

In this report we have demonstrated the feasibility of classifying the four DNA nucleotides solely on the basis of fluorescence polarization in the presence of a surfactant when they are labeled with identical dyes. Although this measurement works very well in bulk solutions, comparison with the signal levels that are expected in single-molecule sequencing efforts leads us to believe that fluorescence polarization does not currently have a role in this effort. Signals greater than 2 orders of magnitude more intense than those observed in ref 45 would be necessary to change this conclusion. In that report, a laser power of only 20 mW was employed to excite fluorescence; increasing the laser power and dwell time of the molecules in the beam waist and optimizing the optics and detector beyond those of ref 45 could provide such a signal increase. Even so, the expected additional losses inherent in polarization measurement, the increased probability of photobleaching, and the potential for laser-induced heating to affect the partitioning of molecules into the surfactant phase would all tend to counter the positive effects of increased laser power.

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