

Stable fluorescent complexes of double-stranded DNA with bis-intercalating asymmetric cyanine dyes: properties and applications

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ABSTRACT

The synthesis, proof of structure, and the absorption and fluorescence properties of two new unsymmetrical cyanine dyes, thiazole orange dimer (TOTO; 1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide) and oxazole yellow dimer (YOYO; an analogue of TOTO with a benzo-1,3-oxazole in place of the benzo-1,3-thiazole) are reported. TOTO and YOYO are virtually non-fluorescent in solution, but form highly fluorescent complexes with double-stranded DNA (dsDNA), up to a maximum dye to DNA bp ratio of 1:4, with > 1000-fold fluorescence enhancement. The dsDNA-TOTO (λ_{\max} 513 nm; λ_{\max}^F 532 nm) and dsDNA-YOYO (λ_{\max} 489 nm; λ_{\max}^F 509 nm) complexes are completely stable to electrophoresis on agarose and acrylamide gels. Mixtures of restriction fragments pre-labeled with ethidium dimer (EthD; λ_{\max}^F 616 nm) and those pre-labeled with either TOTO or YOYO were separated by electrophoresis. Laser excitation at 488 nm and simultaneous confocal fluorescence detection at 620–750 nm (dsDNA-EthD emission) and 500–565 nm (dsDNA-TOTO or dsDNA-YOYO emission) allowed sensitive detection, quantitation, and accurate sizing of restriction fragments ranging from 600 to 24,000 bp. The limit of detection of dsDNA-TOTO and YOYO complexes with a laser-excited confocal fluorescence gel scanner for a band 5-mm wide on a 1-mm thick agarose gel was 4 picograms, about 500-fold lower than attainable by conventional staining with ethidium bromide.

INTRODUCTION

In 1990, Glazer et al. (1) showed that double-stranded DNA (dsDNA) formed stoichiometric, highly fluorescent complexes with the bis-intercalator ethidium homodimer (EthD;

5,5'-[4,7-diazadecamethylene]-bis[3,8-diamino-6-phenylphenanthridinium] dichloride diHCl, ref. 2) that were stable to electrophoresis on agarose gels in the absence of free dye. Utilizing a laser-excited confocal fluorescence gel scanner, it was possible to detect bands of prestained DNA restriction fragments after electrophoretic separation on agarose gels with a sensitivity about two orders of magnitude higher than by conventional fluorescence-based procedures (1). These observations, as well as other reports of the stability of complexes between dsDNA and other appropriately linked dimers or oligomers of intercalating compounds (3, 4), suggested that this phenomenon could be exploited to generate a family of highly fluorescent, stable dsDNA-dye complexes with distinctive properties. Such complexes could then be exploited for multiplex detection of dsDNA fragments, as well as for many analytical applications in which appropriately derivatized dsDNA fragments labeled non-covalently with different dyes could be used as a unique family of fluorescent probes.

In a quest for additional stably intercalating fluorophores, we turned our attention to asymmetric cyanine dyes. Lee et al. (5) showed that the cyanine dye thiazole orange (TO; 4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium iodide) bound to dsDNA with a stoichiometry characteristic of monointercalators: one dye per two base pairs at saturation. Whereas the free TO was very weakly fluorescent, a 3,000-fold enhancement of fluorescence was observed on binding to RNA. Rye et al. (6) reproduced the observations of Lee et al. (5) and showed that the binding of TO to dsDNA was readily reversible. These observations suggested that unsymmetrical cyanine dyes like TO or its analogues could be bridged through a bis-cationic linker similar to that linking the chromophores in ethidium homodimer to produce dimeric dyes which would be excellent candidates for compounds which would form stable, highly fluorescent complexes with dsDNA.

We show here that two such compounds 1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-

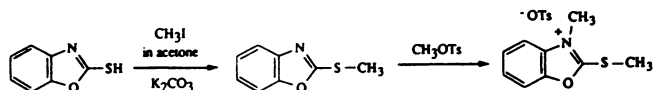
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(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide (TOTO) and its benzoxazole analogue, 1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium tetraiodide (YOYO) (see Fig. 1, for structures) form stable highly fluorescent complexes with dsDNA with fluorescence enhancements greater than a 1,000-fold. We describe the synthesis of these compounds and NMR studies confirming the assigned structures, as well as the absorption and fluorescence properties of the dimeric compounds, TOTO and YOYO, and the monomeric dyes, TO and oxazole yellow (YO), in the free form and when bound to dsDNA. These dyes allow much more sensitive fluorescence detection of dsDNA on gels than has heretofore been possible. Moreover, laser excitation at 488 nm and simultaneous confocal detection at 620–750 nm (dsDNA-EthD emission) and 500–565 nm (dsDNA-TOTO or dsDNA-YOYO emission) allowed detection, quantitation, and accurate sizing of restriction fragments ranging from 600 to 24,000 bp at picogram sensitivity per band.

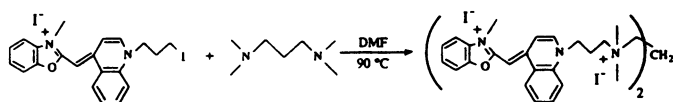
MATERIALS AND METHODS

Synthesis of oxazole and thiazole dyes

Oxazole yellow (YO) and thiazole orange (TO) were prepared according to Brooker et al. (7). 2-Methylthio-N-methylbenzoxazolium tosylate was used for the synthesis of the oxazole-based compounds while the corresponding 2-methylthio-N-methylbenzothiazolium derivative was used for the synthesis of the thiazole compounds. 2-Methylthio-N-methylbenzoxazolium tosylate was prepared by heating equal equivalents of methyl tosylate and commercially available 2-methylthiobenzothiazole. The corresponding benzoxazolium tosylate was prepared by S-methylation of commercially available 2-mercaptobenzoxazole and treatment of the S-methyl derivative with methyl *p*-toluenesulfonate.



1'-(3'-Iodopropyl)-3-methyl-oxa-4'-cyanine iodide was prepared by the method of Brooker et al. (7). The synthesis of oxazole yellow homodimer (YOYO) is described below. Thiazole orange homodimer (TOTO) was synthesized in an analogous manner.



1'-(3'-Iodopropyl)-3-methyl-oxa-4'-cyanine iodide (1.25 g) and 0.17 ml (0.5 eq) of N,N,N',N'-tetramethylpropanediamine in 30 ml of dimethylformamide was kept at 90°C for 3 days. At the end of that period, 120 ml of methanol was added and the mixture allowed to stand at 0°C overnight. The precipitate was filtered to yield 0.66 g of an orange-red solid which was analyzed for purity by thin layer chromatography and NMR.

Preparation of stock dye solutions

Ethidium dimer (EthD; Molecular Probes, Inc., Eugene, OR), dissolved in filtered, deionized water at ≥ 1.1 mM, gave a solution stable for up to 1 year at -20°C . Diluted stock solutions of EthD in 40 mM Tris-acetate, 1 mM EDTA (TAE), pH 8.2

were stable for up to 3 months at 4°C . Stock solutions of TOTO, YOYO, and YO (≤ 8 mM) were prepared by dissolving the solid dyes in dimethylsulfoxide (DMSO). These solutions were then divided into small aliquots and stored under N_2 at 4°C . The dye solutions were stable when stored in this manner for several months. Repeated thawing and re-freezing of the same DMSO stock solution eventually resulted in gradual loss of TOTO and YOYO fluorescence. Stock solutions of TO, stable for at least 4–5 months at 4°C , were prepared by dissolving the dye in methanol. Working stock solutions of all dyes, except EthD, were prepared fresh immediately prior to use by dilution into 4 mM TAE–0.1 mM EDTA buffer, pH 8.2 (referred to below as 4 mM TAE). Spectroscopic measurements on TOTO solutions in this buffer indicated an apparent half-life of 5 hours at 4°C , and thus were used immediately after dilution of the TOTO/DMSO solution into the aqueous buffer. All dye-containing solutions were stored in the dark and working stock solutions were kept on ice during use.

DNA preparations

DNA sizing ladders were obtained from GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD). Stock solutions of the λ DNA/*Hind*III digest (718 μg DNA/ml), 1 kb sizing ladder (958 μg DNA/ml), and ϕ X174 DNA/*Hae*III digest (714 μg /ml), were stored at -20°C . A stock solution of the High Molecular Weight Ladder (53 μg /ml) was stored at 4°C . All of these solutions were in 10 mM Tris-HCl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA. Calf thymus DNA (Type I) was obtained from Sigma. The calf thymus DNA was first sheared by repeated passage through a small gauge needle and then purified by phenol:chloroform extraction and sodium acetate/ethanol precipitation. Both M13mp18 and pBR322 plasmids (3–5 μg) were cut at a single site with *Hind*III (6–10 units) using the Boehringer-Mannheim (Indianapolis, IN) restriction kit. The linear dsDNA was then extracted with phenol:chloroform and recovered by precipitation with sodium acetate/ethanol. Analysis by agarose gel electrophoresis showed nearly complete single cutting of the plasmids.

Absorbance and fluorescence emission spectra

For determination of the absorption and fluorescence emission spectra of dsDNA-dye complexes, a stock solution of calf thymus DNA in 4 mM TAE, pH 8.2, was added to each dye in 4 mM TAE buffer, to a final volume of 1 ml. In each instance, the final DNA concentration was 1.9×10^{-4} M bp and the final dye concentration was 1.9×10^{-6} M, yielding a molar ratio of DNA bp to dye of 100:1. The mixtures were incubated in the dark at room temperature for 1.5 hours prior to spectroscopic measurement. Absorption spectra of free dyes were determined in each instance on 5.5×10^{-6} M solutions in 4 mM TAE, pH 8.2. Because of the very low fluorescence quantum yield of the free dyes, their fluorescence was determined at higher concentrations as follows: EthD, 5.6×10^{-5} M; TO, 2.2×10^{-4} M; TOTO, 2.4×10^{-5} M; YO, 2.0×10^{-5} M; and YOYO, 7.6×10^{-5} M, all in 4 mM TAE, pH 8.2. Absorption spectra were acquired on a computer-controlled dual-beam Perkin-Elmer (Norwalk, CN) Lambda 6 UV/Vis Spectrophotometer. Fluorescence measurements were performed with a Perkin-Elmer MPF44B spectrofluorimeter connected to a Perkin-Elmer Hitachi 057 plotter. Since for some of the samples the Stokes shift was small, λ_{exc} was set in every instance on the blue side of the absorption band at the wavelength

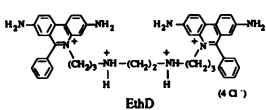
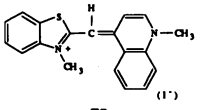
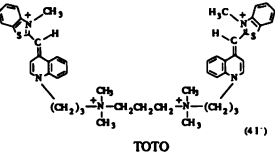
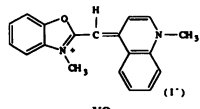
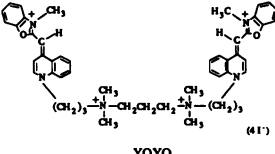
Dye Structures	$\frac{\lambda_{\text{max}}^{\text{A}} \text{ (nm)}}{F_{\text{Free}} \text{ Bound}}$		$\epsilon \times 10^{-3}$ ($\text{M}^{-1} \text{cm}^{-1}$)	$\frac{\lambda_{\text{max}}^{\text{F}} \text{ (nm)}}{F_{\text{Free}} \text{ Bound}}$		$F_{\text{Bound}}/F_{\text{Free}}$	DNA-Dye Complex Relative Fluorescence Intensity
	Free	Bound		Free	Bound		
 EthD	492	534	8.9 (492)	626	616	35	1.0
 TO	501	509	77.0 (502)	640	527	18,900	2.5
 TOTO	481	513	131.7 (507)	630	532	1,100	1.2
 YO	475	486	75.7 (476)	575	505	700	5.6
 YOYO	458	489	118.6 (482)	564	509	3,200	4.4

Figure 1. Structures of fluorescent dyes and their spectroscopic properties in free solution and when complexed to dsDNA. In column 3, ϵ for EthD was measured in water, all other ϵ measurements were in methanol. The wavelength at which these ϵ values were determined are given in parentheses. In column 5, the fluorescence enhancement on binding to dsDNA, $F_{\text{Bound}}/F_{\text{Free}}$, is the ratio of the fluorescence intensities at the peak emission for the dsDNA-bound and the free dye in the same buffer normalized to the same absorbance at the excitation wavelength. Gaugain et al. (15) reported the enhancement in EthD fluorescence quantum yield on binding to dsDNA to be 40. Measurements on dsDNA-dye complexes were at 100 DNA bp per dye. For other details see 'Materials and Methods'.

where the absorbance was one-half of that at the maximum, with the objective of minimizing stray light contributions at the emission maximum.

NMR spectroscopy

NMR experiments on TOTO were carried out on a GN-500 NMR spectrometer with the dye in DMSO- d_6 . DQF-COSY, DQF-RELAY and NOESY spectra were collected in TPPI mode, collecting 512 blocks in t_1 , each with 1024 complex points. The data were transferred to a VAX computer and processed using the program FTNMR (Hare Research Inc., Woodinville, WA).

YO and YOYO gave the following proton NMR spectra in DMSO- d_6 :

YO: 8.71 (d, 1 H, $J=8.4$ Hz), 8.41 (d, 1 H, $J=7.2$ Hz), 8.0–7.92 (m, 2 H), 7.87 (d, 1 H, $J=7.1$ Hz), 7.75–7.68 (m, 2 H), 7.55 (d, 1 H, $J=7.9$ Hz), 7.50 (d, 2 H, $J=8.0$ Hz), 7.43 (t, 1 H, $J=7.8$ Hz), 7.35 (t, 1 H, $J=8.1$ Hz), 7.09 (d, 2 H, $J=7.9$ Hz), 6.19 (s, 1 H), 4.12 (s, 3 H), 3.81 (s, 3 H), 2.28 (s, 3 H).

YOYO: 8.77 (d, 2 H, $J=8.0$ Hz), 8.60 (d, 2 H, $J=7.4$ Hz), 8.25 (d, 2 H, $J=8.7$ Hz), 7.99 (t, 2 H, $J=7.6$ Hz), 7.92 (d, $J=7.3$ Hz), 7.78 (d, $J=8.0$ Hz), 7.73 (t, 2 H, $J=7.7$ Hz), 7.62 (d, 2 H, $J=7.8$ Hz), 7.47 (t, 2 H, $J=7.6$ Hz), 7.38 (t, 2 H, $J=7.6$ Hz), 6.27 (s, 2 H), 4.70 (t, 4 H, 7.5 Hz), 3.87 (s, 6 H), 3.76–3.65 (m, 4 H), 3.45–3.35 (m, 4 H), 3.19 (s, 12 H), 2.45–2.30 (m, 6 H).

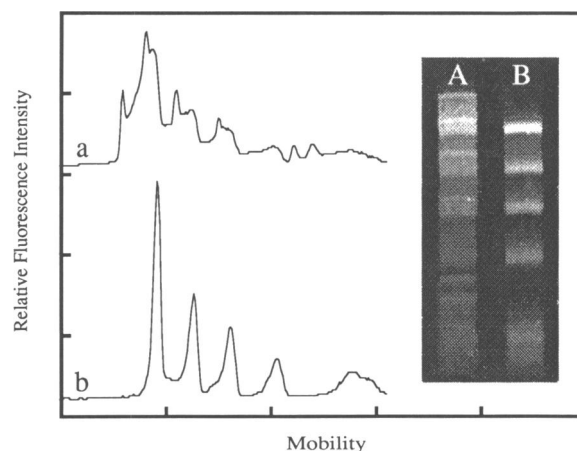


Figure 2. Effect of the order of addition of DNA and YOYO on the λ DNA/HindIII restriction band pattern. For lane (A), 25.2 μ l of a 1×10^{-7} M YOYO solution was added to a 49.8 μ l sample of λ DNA/HindIII (2.5×10^{-7} M bp) in 4 mM TAE buffer, pH 8.2. For lane (B), 8 μ l of a 1.6×10^{-6} M bp λ DNA/HindIII solution was added to a 67 μ l solution of YOYO (3.8×10^{-8} M) in 4 mM TAE, pH 8.2. For both samples, the ratio of DNA bp to YOYO was 5:1. Both samples were well mixed and then incubated in the dark at room temperature for 30 minutes. A 25 μ l aliquot of 15% (w/v) Ficoll was then added to each mixture and 5 μ l (400 pg of DNA) subjected to electrophoresis on a 0.9% agarose gel as described in 'Materials and Methods.' The fluorescence intensity plots shown to the left illustrate the band resolution obtained in each lane. Trace (a) corresponds to lane (A) of the inset, and trace (b) corresponds to lane (B).

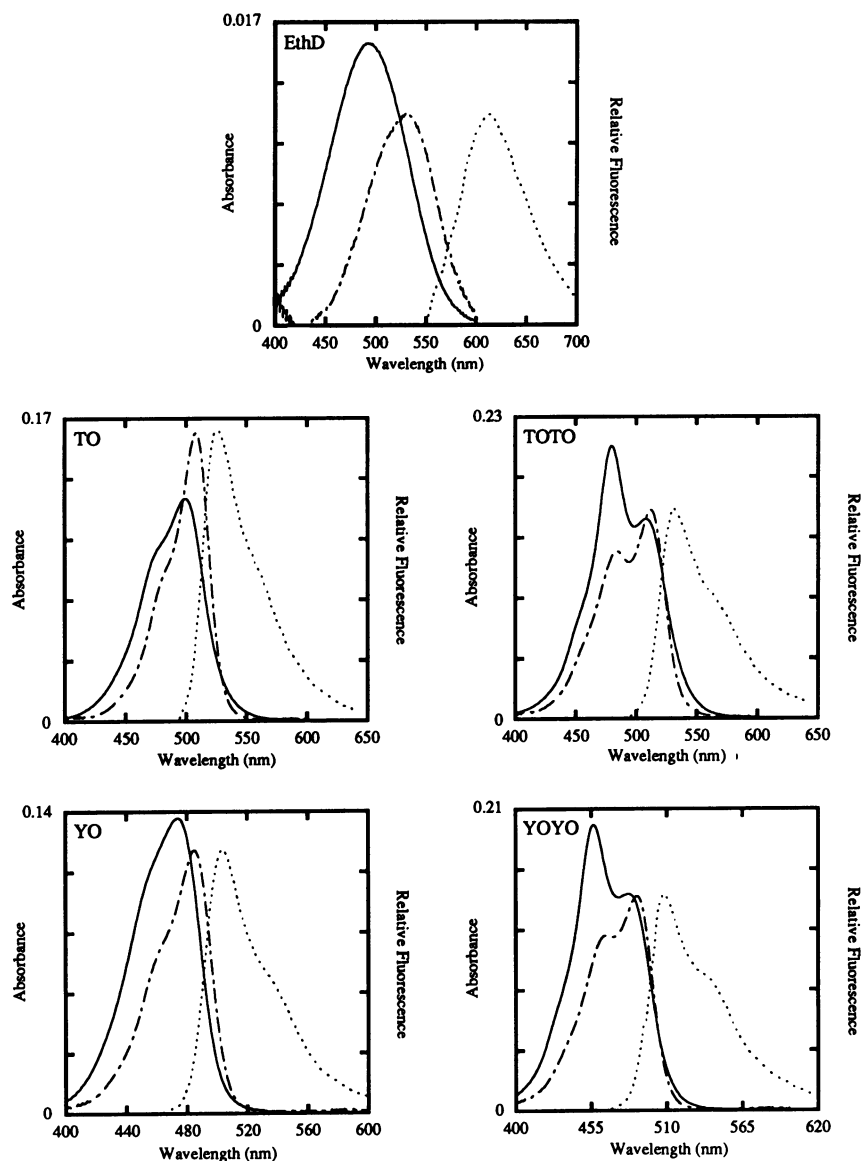


Figure 3. Absorption spectra of free dyes and absorption and fluorescence emission spectra of dsDNA-dye complexes in 4 mM TAE buffer, pH 8.2. For dsDNA-dye complexes, spectra were recorded 1.5 hours after mixing of calf thymus DNA with dye. (—) indicates the absorption spectra of free dyes. For the absorption (---) and fluorescence emission (·····) spectra of the dsDNA-dye complexes, the final concentrations were 1.9×10^{-4} M bp DNA and 1.9×10^{-6} M dye, to give a molar ratio of DNA bp: dye of 100:1. For additional experimental details, see 'Materials and Methods.'

These spectra were consistent with the structures proposed for YO and YOYO on the basis of the synthetic route.

Complex formation with dsDNA and agarose gel electrophoresis

The following protocol was used for the formation of the dsDNA-dye complex. All operations were performed at room temperature. The dsDNA and the dye solutions were individually diluted into either 4 or 40 mM TAE buffer prior to use. Mixing was typically performed in a total volume of 75–150 μ l in sterile plastic Eppendorf tubes. The DNA-dye solutions were well-mixed by re-pipeting and the samples incubated for a minimum of 30 minutes in the dark. For two-color applications, different DNA samples were initially labeled separately with EthD and either TOTO or YOYO as described above. Following an initial

incubation, the different DNA samples were mixed together and incubated in the dark for an additional 30 minutes. Ficoll (Sigma, St. Louis, MO; type 400; 15% (w/v) in deionized, filtered, sterile water) was mixed with the DNA-dye solution to a final concentration of 3.75%. A 5 μ l sample of this mixture was then immediately loaded onto an agarose gel. Vertical gels were pre-run for 1–2 hours at 10V/cm and then run for 1 hour following sample application. Horizontal slab gels were pre-electrophoresed for 3 hours at 10 V/cm. After loading the horizontal gel was run for 5 min at 6.7 V/cm and then for 18 hours at 1.4 V/cm.

Agarose (ultrapure; GIBCO-BRL) gel electrophoresis of all DNA-dye samples was conducted in a vertical gel format, with the exception of the High Molecular Weight sizing experiment which was run on a horizontal submarine gel. The vertical gels

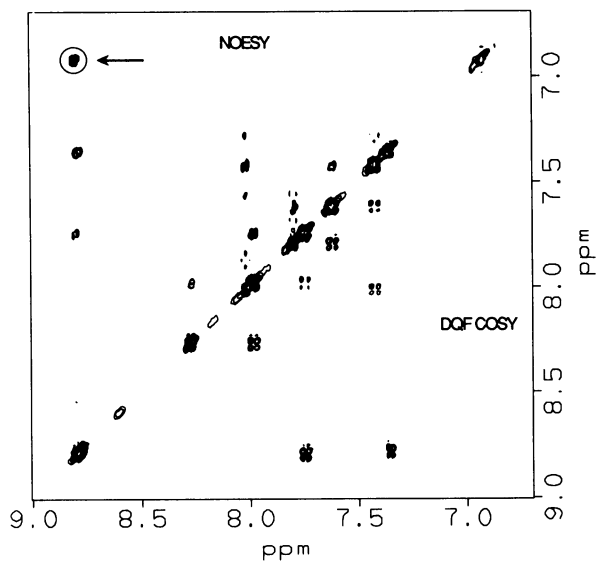


Figure 4. A combined NOESY (upper left from diagonal) / DQF-COSY (lower right from diagonal) of the aromatic portion of TOTO in DMSO- d_6 solution, 25°C. The circled NOESY peak is between the aromatic singlet on the bridge between the rings of the chromophores to a doublet on the quinoline ring, establishing the geometry of this linkage.

(6 cm long, 8 cm wide, 1 mm thick, with 5 mm wide wells) were cast in a Bio-Rad Mini-Protean II gel apparatus (Bio-Rad, Richmond, CA). All gels were made in 40 mM TAE, pH 8.2 and electrophoresis was conducted in the same buffer.

For detection of DNA bands with YO, the dye was added to the electrophoresis running buffer at $1.6 \times 10^{-7} M$. The gels were pre-run at 10V/cm for 1.5 hours, and then run for 1 hour at 10V/cm after sample application without changing the buffer.

Comments on the preparation dsDNA-dye complexes

In the experiments described here and earlier (1, 6, 8, 9), the quality of the electrophoretic separations and the resulting fluorescence images was found to be a sensitive function of the order of mixing of dye and DNA, of the DNA concentration, and of the molar ratio of DNA to dye. In general, for EthD, DNA concentrations between 50 pg/ μl and 1.5 ng/ μl and a DNA bp:dye ratio of 5:1 are optimal. For TOTO, DNA concentrations between 20 pg/ μl and 400 pg/ μl and a DNA bp:dye ratio of 5:1 are appropriate. Samples of complexes suitable for electrophoretic fractionation can be formed with these dyes at higher DNA concentrations if the ratio of DNA bp:dye is raised, e.g., to 10:1 to 40:1. With YOYO, suitable samples are obtained over a more restricted range of DNA concentrations and DNA bp:dye ratios. DNA-YOYO complexes, which produce sharp bands on electrophoretic separation, are formed at DNA bp:dye ratio of 5:1 and DNA concentrations below 100 pg/ μl . Suitable DNA-YOYO complexes can be formed at DNA concentrations as high as 500 pg/ μl if the DNA bp:dye ratio is raised to 10:1 or higher. Departure from appropriate DNA:dye ratios and concentrations usually results either in precipitation of the DNA-dye complex, or severe streaking and/or band splitting on electrophoresis.

The order of mixing of DNA and dye can also affect the resolution of bands in DNA restriction fragment patterns. This phenomenon, particularly evident with YOYO, is illustrated in

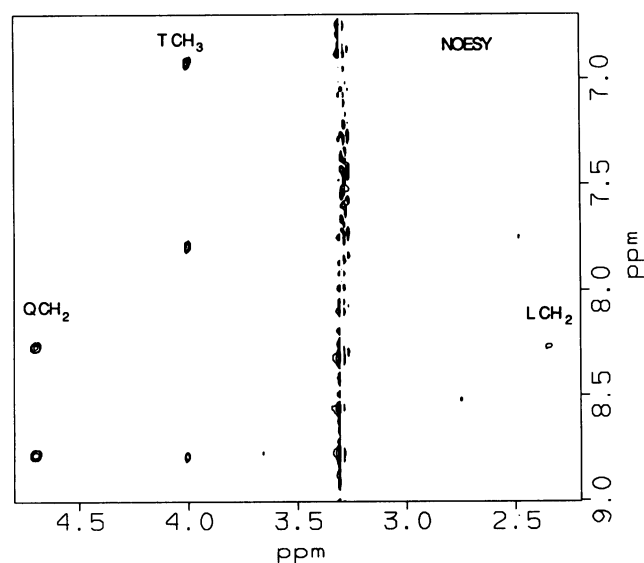


Figure 5. The upfield to aromatic crosspeak region of the NOESY for TOTO. The labels are: QCH₂ for the methylene group attached to the quinoline ring, with NOEs to the quinoline aromatic protons; TCH₃ for the methyl group on the thiazole ring, with NOEs to the thiazole aromatic ring, the bridging singlet, and one of the quinoline protons; and LCH₂ for the other protons of the polyalkylamine linker, with a weak NOE to quinoline ring protons.

Fig. 2. The inset in Fig. 2 shows two lanes of a gel, each loaded with the same amount of λ DNA/*Hind* III complexed with YOYO at a ratio of 5 bp per dye. The only differences between the samples were the order of mixing of the DNA and YOYO solutions and the concentrations of these components at the time of mixing. The final concentrations were the same for both samples. The sample in which YOYO was added to a solution of DNA is shown in lane A, the sample with the reverse order of mixing is shown in lane B. The pattern in lane A shows poor band resolution and complicated band splitting patterns, whereas that in lane B is satisfactory.

Two-color confocal fluorescence imaging system

Following electrophoresis, gels were scanned with the two-color, confocal fluorescence gel imaging system as previously described (9). Laser light (488 nm; 35 mW) was reflected from a long pass dichroic beam splitter, passed through a microscope objective and brought to a focus within the agarose gel. The resulting fluorescence was collected by the objective in an epi-illumination format, passed back through the beam splitter and directed at a longpass dichroic beam splitter (reflects 500–565 nm at 45° incidence and transmits ≥ 566 nm; Omega Optical, Brattleboro, VT) to achieve color separation between the EthD and thiazole/oxazole channels. The fluorescence emission from the respective detection channels was subjected to spatial and spectral filtration and detected with a photomultiplier (Hamamatsu, Bridgewater, NJ). For EthD detection, two long pass 610 nm sharp cutoff filters (Schott) were placed in front of the photomultiplier tube. For TOTO detection, a bandpass discrimination filter with a transmission window of 530 \pm 30 nm (Omega Optical) and a long pass 495 nm sharp cutoff filter (Schott, Oakridge, NJ) were placed in front of the photomultiplier. These filters were exchanged for a 488 nm rejection band filter for YO and YOYO detection. A computer-

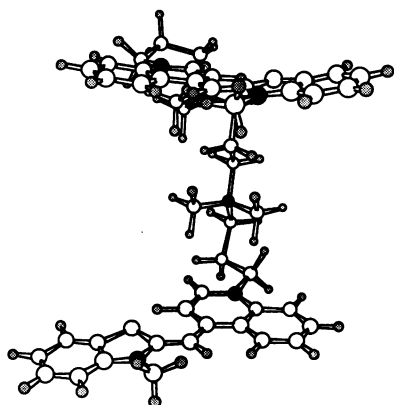


Figure 6. A structure, created using the programs ChemDraw and Chem3D, is shown with the chromophores in positions such as they might be when bisintercalated into DNA. The positions of the two rings of the chromophore are as experimentally determined. The relative positions of the chromophores and the conformation of the linker chains are hypothetical. Nitrogen atoms are shaded darkest, sulfur is shaded the lightest and hydrogens are intermediate.

controlled dc servo motor-driven XY translation stage was used to move the gel past the laser beam.

The resulting unprocessed binary data files were composed of 12-bit pixels arrayed in FTS file format with alternating pixels representing the thiazole or oxazole and the EthD channels. These IBM-DOS files were then separated into their respective arrays and converted to 8-bit Macintosh TIFF files. The resulting images were processed with the NIH image processing program Image 1.29 on a Macintosh IIfx computer. Density plots of the gel lanes were generated by averaging the pixel values across columns of a defined section of the image. These values were then plotted as a function of the column position. Fluorescence intensity values for the detected bands were calculated by integrating the peaks generated in the density plots. Migration distances were measured to the peak center of the fluorescence intensity profiles of the density position plots.

RESULTS

Spectroscopic properties of free dyes and of the dsDNA-dye complexes

Data on the absorbance and fluorescence emission spectra of the dyes, in their free and DNA-bound forms, is presented in Figs. 1 and 3. All of the dyes show a red shift ($\sim 8-10$ nm for the monomeric and $30-40$ nm for the dimeric dyes) of their absorption maxima, and a blue shift ($\sim 50-113$ nm for the thiazole and oxazole dyes and 10 nm for EthD) of their fluorescence emission maxima upon binding to dsDNA. Of these dyes, EthD has the largest Stokes shift, but the lowest molar extinction coefficient, lowest fluorescence enhancement upon DNA binding, and the weakest fluorescence intensity in the dsDNA-bound form. All four thiazole and oxazole derivatives exhibit very large fluorescence enhancements upon binding to dsDNA, with those for TO and YOYO being particularly large (Fig. 1). Lee et al. (5) determined a 3000-fold fluorescence quantum yield enhancement for TO upon binding to RNA. The $\sim 19,000$ -fold enhancement seen here on TO binding to dsDNA could be an overestimate since a relatively high concentration of TO (2.2×10^{-4} M) had to be employed to measure the

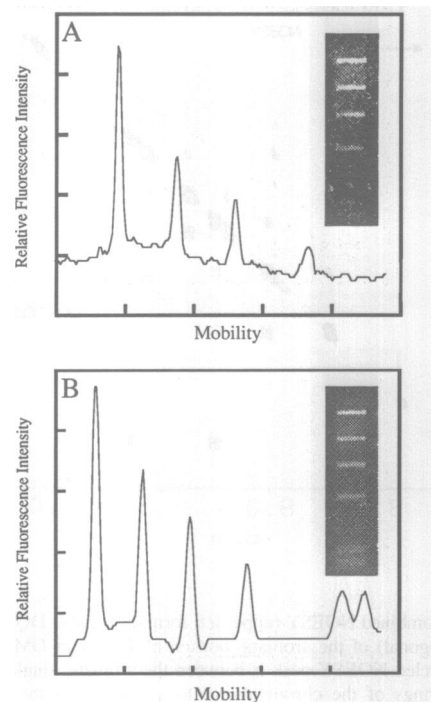


Figure 7. Detection of (A) λ DNA/HindIII-YO and (B) λ DNA/HindIII-YOYO complexes. Panel A shows a fluorescence image and a fluorescence intensity plot of a lane loaded with 400 pg of λ DNA/HindIII-YO complex at a ratio of 2 bp per YO and separated in the presence of 1.6×10^{-7} M YO in the running buffer. The lowest band contains ~ 40 pg DNA. Panel B shows the corresponding data for a lane loaded with 100 pg of λ DNA/HindIII-YOYO complex at a ratio of 5 bp per YOYO. The lowest band contains ~ 4 pg DNA. For A, λ DNA/HindIII (1.7×10^{-7} M bp) was incubated at room temperature for 30 min in the dark with YO (8.4×10^{-8} M) in 4 mM TAE buffer, pH 8.2. For B, λ DNA/HindIII (4.2×10^{-8} M bp) was incubated at room temperature for 30 minutes in the dark with YOYO (8.2×10^{-9} M) in 4 mM TAE buffer, pH 8.2. After incubation, a 25 μ l aliquot of 15% (w/v) Ficoll was then added to 75 μ l of each sample and a 5 μ l aliquot applied to a vertical 0.9% agarose gel in 40 mM TAE, pH 8.2. Electrophoresis was performed as described in 'Materials and Methods.'

fluorescence of the free dye, and any self-quenching of TO fluorescence at this concentration would lead to a lowered value for the fluorescence intensity of the unbound dye.

As shown in Fig. 3, for EthD, TOTO, YO, and YOYO, binding to dsDNA results in an absorbance decrease of $\sim 10-25\%$ at the absorption maxima of the chromophores. For TO, however, binding to dsDNA leads to an absorbance increase of $\sim 20-25\%$ at its λ_{max} .

NMR structural analysis of TOTO

The solution structure of TOTO was verified by 1 H NMR spectroscopy as shown in Figs 4-6. The identification of coupled protons, both in the aromatic ring system and in the polyalkylamino connecting linker, was established through DQF COSY and DQF RELAY spectra, taken in DMSO- d_6 solution at 25° C. The proximity of functional groups substituting the rings was established through a NOESY spectrum, taken under the same conditions, with a mixing time of 0.5 sec. Clear connectivities were observed linking the N-CH $_3$ on the thiazole ring to the attached aromatic ring, and also to the only singlet in the downfield region, arising from the methine proton bridging the thiazole and quinoline rings. This singlet in turn shows an

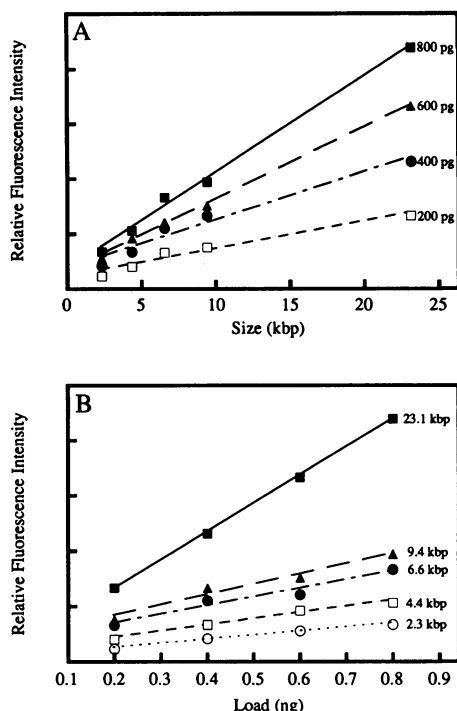


Figure 8. Dependence of the fluorescence intensity of λ DNA/*Hind* III-YOYO complexes in 0.9% agarose gels (A) on the size of the DNA fragments and (B) on the amount of sample loaded per lane. Curve fitting was performed by a least-squares analysis. All samples contained a molar ratio of 10 DNA bp per YOYO and were prepared by incubating λ DNA/*Hind* III (3.4×10^{-7} to 8.4×10^{-8} M bp) with YOYO (3.4×10^{-8} to 8.4×10^{-9} M) in 40 mM TAE, pH 8.2 at room temperature for 30 minutes in the dark. A 25 μ l aliquot of 15% (w/v) Ficoll was then added to each 75 μ l sample and 5 μ l samples of these mixtures were subjected to electrophoresis as described in 'Materials and Methods'.

NOE to a downfield proton, which by analysis of the couplings, belongs to the benzenoid ring of the quinoline. The methylene group of the linker, attached to the quinoline nitrogen, shows NOEs to one proton of a coupled pair, and to one of the protons of the benzenoid ring of the quinoline. NOEs are also observed between the N-methyl groups of the linker and the neighboring methylene protons. Integrations of protons show clearly that there are three propyl groups in the linker, and two quaternary ammonium ions.

Although NOEs may be averaged by molecular motion, the patterns and intensities of the NOEs observed indicate that the predominant conformer is that shown in Fig. 6, with the quinoline and thiazole rings in a *trans*-like geometry. Molecular modeling indicated that there is some steric interaction between one of the quinoline ring protons and the sulfur of the thiazole. The alternate rotamer about the bridge linking the thiazole and quinoline rings would lead to a more significant steric conflict with the thiazole, and should lead to an NOE between the methine proton and one of the isolated pair of protons on the quinoline, which is not observed. This indicates that the predominant conformer is shown.

Interaction of YO with dsDNA

We have previously shown that preformed TO-dsDNA complexes dissociated upon electrophoresis in the absence of free dye, but

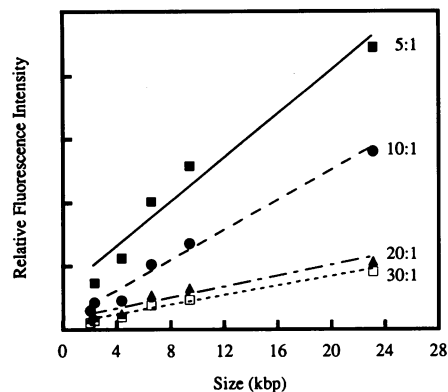


Figure 9. Dependence of the fluorescence intensity λ DNA/*Hind*III-YOYO complexes on the DNA:dye ratio. dsDNA:dye ratios in bp:dye are given at the right of each line. Curve fits for the data were determined by least-squares analysis. λ DNA/*Hind*III (1.6×10^{-6} M bp) was mixed with various amounts of YOYO to generate the DNA:dye ratios indicated. All mixtures were in 40 mM TAE buffer, pH 8.2. Each mixture was incubated for 30 min in the dark at room temperature. A 25 μ l aliquot of 15% (w/v) Ficoll was then added to each 75 μ l sample and 5 μ l (600 pg of DNA) of each mixture applied to a 0.9% agarose gel. For electrophoresis conditions, see 'Materials and Methods'.

that addition of TO the running buffer allowed the fluorescence detection of as little as 20 pg of dsDNA in a 5 mm wide band on a 1 mm thick agarose gel (8). Analogous results were obtained with dsDNA-YO complexes, except that the detection limit was ~ 40 pg of dsDNA per band (for details, see Fig. 7A).

Interaction of YOYO and TOTO with dsDNA

The TOTO and YOYO homodimers (Fig. 1), synthesized with a bis-cationic linker similar to that used to join ethidium monomers in EthD (2), formed complexes with DNA stable to electrophoresis in the absence of free dye. With the detection system employed in this study, at a molar ratio of dsDNA bp to dye of 5:1, the detection limit for 5 mm wide, 1 mm thick bands of either TOTO- or YOYO-dsDNA complexes is ~ 4 pg of DNA (e.g., Fig. 7B). This detection sensitivity is roughly 10-fold higher than that attainable with EthD and over 500-fold higher than that achievable with conventional ethidium bromide staining.

The fluorescence of EthD-DNA and TOTO-DNA complexes has been shown to be insensitive to variation in the DNA base composition, and the fluorescence intensity of DNA fragments labeled with these dyes in agarose gels is directly proportional to the amount of DNA present over a 6-fold range of DNA-dye ratios (1, 9). Similarly, the fluorescence intensity of YOYO-DNA complexes in agarose gels is directly proportional to the amount of DNA present (for details, see Fig. 8). As shown in Fig. 9, this linear correlation also holds over a range of molar ratios of DNA bp: YOYO from 5:1 to 30:1. It was observed empirically that to obtain a linear correlation between DNA concentration and fluorescence of the DNA:YOYO bands, the initial mixing of dye with DNA had to be performed in 40 mM TAE rather than 4 mM TAE. As noted in earlier experiments with EthD and TOTO (1, 9), binding of YOYO results in a slight retardation in the mobility of the DNA fragments. Taking the mobilities of the DNA fragments at 30 DNA bp per YOYO as 1, the mobilities at other ratios were 0.99 for 20:1, 0.97 for 10:1, and 0.94 for 5:1.

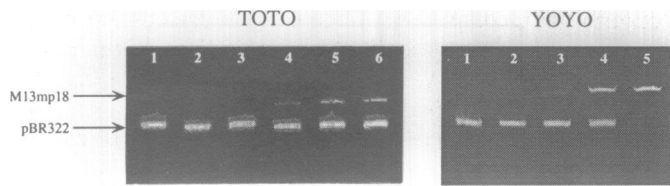


Figure 10. Migration of TOTO and YOYO between dsDNA molecules. (Left) Fluorescence image of a gel loaded in each lane with 250 pg of pBR322 DNA pre-mixed with TOTO at DNA bp:dye of 5:1, with increasing amounts of uncomplexed M13mp18 DNA added to the mixtures in lanes 2–6 (lane 2, 250 pg; lane 3, 500 pg; lane 4, 5 ng; lane 5, 50 ng; lane 6, 100 ng). Lane 1 contains only pBR322-TOTO. In each case, linear pBR322 DNA (1.6×10^{-6} M bp) was mixed with TOTO (1.0×10^{-7} M) and incubated for 30 min at room temperature in the dark. Subsequently, an appropriate aliquot of a linear M13mp18 DNA stock (either 1.6×10^{-6} M bp or 8.0×10^{-5} M bp) was added to each mixture to give a final volume of 75 μ l and the desired M13mp18 DNA excess. These mixtures were then incubated for another 30 min in the dark at room temperature. (Right) Fluorescence image of a gel which was loaded in lanes 1–4 with 100 pg of pBR322 DNA pre-mixed with YOYO at DNA bp:dye of 5:1, with increasing amounts of unlabeled M13mp18 DNA added to the mixtures in lanes 2–4 (lane 2, 200 pg; lane 3, 1 ng; lane 4, 200 ng) Lane 1 contains 100 pg of pBR322-TOTO at 5 bp per dye and lane 5 contains 200 ng of M13mp18 DNA at 10,000 bp per dye. The DNA-YOYO mixtures in lanes 2–4 were prepared in the same manner as described above for dsDNA-TOTO. Ficoll additions, loading and gel electrophoresis were as described in 'Materials and Methods' and Fig. 6.

TOTO and YOYO transfer between dsDNA molecules

Study of dsDNA-EthD complexes showed that addition of a large excess of unlabeled dsDNA led to a rapid transfer of some of the dye to the unlabeled DNA. No further transfer was observed once the ratio of DNA bp to dye in the donor complex decreased to 15–20 bp:dye (1). The same phenomenon was observed with the dsDNA complexes of TOTO and YOYO (see Fig. 10). However, no further transfer of dye from these donor complexes to large excess unlabeled dsDNA was seen once the ratio of DNA bp to dye in the dsDNA-TOTO or dsDNA-YOYO complex decreased to 7–8 bp:dye. The amount of dye transferred did not change with time over a 24 hour period at room temperature.

Two-color detection and sizing of dsDNA fragments with EthD and either YOYO or TOTO

dsDNA complexes of EthD, YOYO, and TOTO can all be efficiently excited by the 488 nm argon ion laser line. The marked differences in the positions of the fluorescence emission maxima of dsDNA-bound EthD (616 nm), YOYO (509 nm), and TOTO (532 nm) allow ready discrimination between the emissions of appropriate pairs of complexes, e.g., dsDNA-EthD and dsDNA-YOYO. Simultaneous two-color detection of two different families of restriction fragments in a single lane, utilized for restriction fragment size determination, is illustrated in Fig. 11. Panels A and B show the output of the 620–750 nm detector (recording the fluorescence of EthD-stained 1 kb ladder used as a size standard) and of the 500–565 nm detector (recording the fluorescence of YOYO-stained λ DNA/*Hind* III fragments used as unknowns), respectively. Panel C shows the fluorescence intensity plots derived from the multiplexed samples (lane 2) on panels A and B. In panel D, the plot of mobilities (peak positions) of standard fragments plotted against $1/\log(\text{fragment size in bp})$ provides the calibration line for the conversion of the mobilities of unknown fragments to sizes. As shown in Table I, the combination of EthD-dsDNA and either dsDNA-YOYO or dsDNA-TOTO complexes led to accurate determination of sizes of DNA fragments ranging from 600 to 23,130 bp.

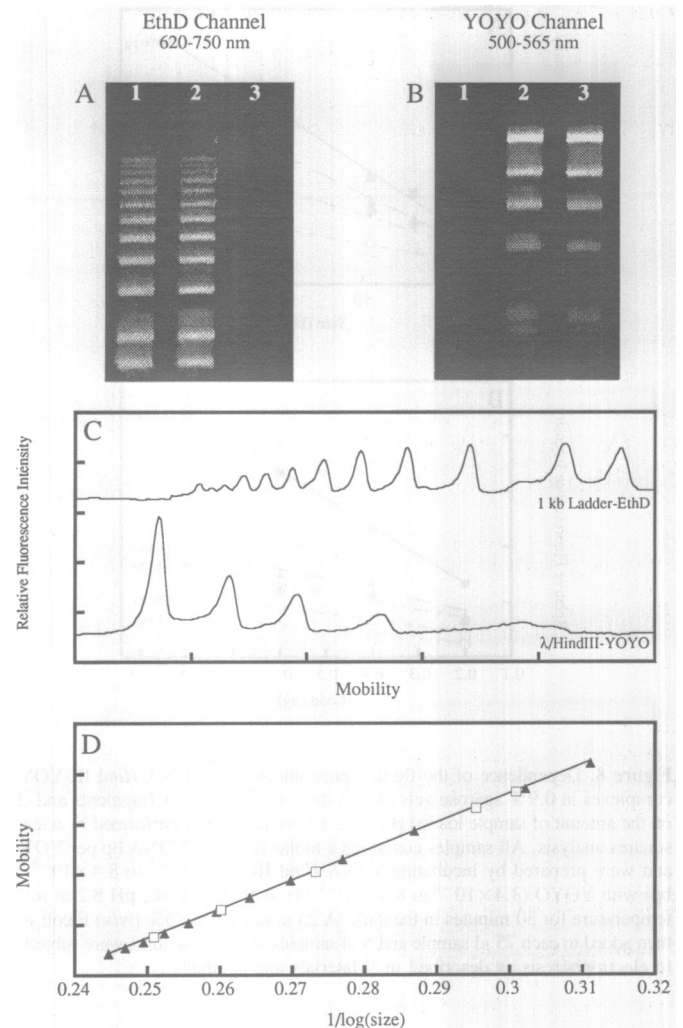


Figure 11. Determination of sizes of dsDNA restriction fragments by simultaneous two-color detection of EthD and YOYO complexes. Panels (A) and (B) show the fluorescence images of a vertical 0.9% agarose gel at 620–750 nm and 530–565 nm, respectively. DNA:dye ratios are given as moles bp per mole dye. Lanes 1, 8 ng of 1 kb ladder DNA-EthD complex (10 bp per EthD). Lane 2, 'two-color' mixture containing 8 ng of 1 kb ladder DNA-EthD complex (10 bp per EthD) and 2 ng of λ DNA/*Hind*III-YOYO (20 bp per YOYO). Lane 3, 2 ng of λ DNA/*Hind*III-YOYO (20 bp per YOYO). Panel (C) illustrates the fluorescence intensity plots derived from lane 2. The mobilities of the DNA-dye fragments derived from this graph are plotted in panel (D). To determine fragment sizes, the mobilities of the λ DNA/*Hind*III-YOYO fragments are plotted onto the least squares plot for the 1 kb ladder DNA-EthD fragments. Data from such an experiment are given in Table I. The mixture applied to lane 2 was prepared by mixing separately 1 kb ladder DNA (6.0×10^{-6} M bp) with EthD (6.0×10^{-7} M) and λ DNA/*Hind*III (1.9×10^{-6} M bp) with YOYO (9.5×10^{-8} M). These samples were then mixed together to yield a solution containing: 3.3×10^{-6} M bp 1 kb ladder DNA, 3.3×10^{-7} M EthD, 8.4×10^{-7} M λ DNA/*Hind*III, and 4.2×10^{-8} M YOYO. The 1 kb ladder sample in lane 1 was a mixture of 1 kb ladder DNA (3.3×10^{-6} M bp) with EthD (3.3×10^{-7} M). The λ DNA/*Hind*III-YOYO sample in lane 3 was generated by diluting the initial λ DNA/*Hind*III-YOYO mix to yield a λ DNA/*Hind*III concentration of 8.4×10^{-7} M bp and a YOYO concentration of 4.2×10^{-8} M. All mixtures and dilutions were in 4 mM TAE buffer, pH 8.2. For electrophoresis conditions, see 'Materials and Methods' and Fig. 6.

The order of mixing of the bis-intercalators with DNA was found to be a critical parameter in obtaining reproducible, well-resolved DNA fragment patterns in gels (e.g., Fig. 2; see 'Materials and Methods'). Sharp patterns were obtained when

Table 1. Two colour DNA size determination with EthD, TOTO, and YOYO.

Target DNA Stain	Target DNA ^a	Band ^b	Actual Size (bp) ^c	Calculated Size (bp)
TOTO ^d	ΦX174/HaeIII	1	1,353	1,299
		2	1,078	1,004
		3	872	805
		4	603	570
YOYO	λ/HindIII	2	9,416	9,689
		3	6,557	6,963
		4	4,361	4,556
		5	2,322	2,431
TOTO ^d	λ/HindIII	6	2,027	2,105
		1	23,130	23,788
		2	9,416	9,570

^aThe calibration DNA ladder for the ΦX174/HaeIII pattern and bands 2–6 of the λ/HindIII pattern (stained with YOYO) was a 1 kb Sizing Ladder. Calibration for bands 1 and 2 of the λ/HindIII pattern (stained with TOTO) was a High Molecular Weight Sizing Ladder. In each case, the sizing standards were stained with EthD.

^bFragments numbered from largest to smallest.

^cBased upon literature from commercial vendors (BRL and New England Biolabs).

^dData from ref. 9.

the dsDNA solution was added to the dye solution, but not if the order of mixing was reversed. The underlying reasons for the difference in the electrophoretic separation of otherwise identical mixtures are unclear.

DISCUSSION

Cyanine dyes are a valuable family of fluorophores. A variety of cyanine based chromophores, with absorption and emission maxima ranging from 500 nm to 750 nm or higher, can be readily synthesized by linking the appropriate quaternized, heteroaromatic nuclei through a methine (polymethine) bridge (10–12). Cyanine dyes typically have high quantum yields (0.1–0.4 in ethanol), large extinction coefficients (approaching or greater than 100,000 M⁻¹ cm⁻¹), and moderate photostabilities (11–13). The asymmetric cyanine dyes TO, TOTO, YO and YOYO described in this report display the useful spectroscopic characteristics of cyanine dyes. The monomeric dyes, TO and YO, have large extinction coefficients ($\epsilon > 70,000$ M⁻¹ cm⁻¹), while those of the dimeric dyes, TOTO and YOYO, exceed 100,000 M⁻¹ cm⁻¹. In aqueous solution, all four dyes are virtually non-fluorescent. Complex formation with dsDNA results in a very large increase in the fluorescence quantum yield (Fig. 1). Interestingly, the Stokes shifts demonstrated by the free thiazole and oxazole dyes (100–150 nm) far exceed the typical shifts noted for cyanine dyes (15–25 nm; refs. 11, 12), while the DNA-bound forms of the dyes possess more typical cyanine-like separations of absorption and emission (18–20 nm). Neither TO nor YO form complexes with dsDNA stable to electrophoresis. However, the enormous enhancement of fluorescence of the intercalated dyes can still be exploited to detect dsDNA on gels. Addition of TO (6) or YO to the running buffer allows detection of bands of restriction fragments on agarose gels containing as little as 20–40 pg of dsDNA.

As noted earlier, the complexes of ethidium bromide with dsDNA are not stable to electrophoresis, whereas EthD, in which the two ethidium chromophores are bridged by bis-cationic linker

does form stable complexes (1). Similarly bridged dimers of the asymmetric cyanine dyes, TOTO and YOYO, form stable complexes with dsDNA. Earlier studies have shown that the length, flexibility and character of the linker critically affect the binding of polyfunctional ligands to DNA, and that the charge on the linker can also be extremely important (2–4, 14–20). Our observation support the conclusion that bis-cationic linkers of appropriate lengths are likely to be generally useful in the preparation of tight binding bis-intercalators.

The detection sensitivity obtainable with TOTO and YOYO (e.g., Fig. 7A) is over 500 times greater than that achievable with conventional ethidium bromide staining. This sensitivity is the outcome of a combination of the following factors: (a) the high sensitivity of the laser-excited confocal fluorescence gel scanner, (b) the many thousand-fold enhancement of the fluorescence of the bound dye dimers, and (c) the ability to pre-stain the DNA with a fluorochrome which remains stably associated with the DNA during electrophoresis, thus eliminating the interfering background dye fluorescence inevitably associated with staining the entire gel. The binding of EthD, TOTO and YOYO to DNA and the resulting fluorescence appear to be largely independent of the DNA base pair composition. As illustrated for dsDNA-YOYO complexes in Figs. 8 and 9, the fluorescence is linearly dependent on the amount of DNA present in the individual bands of a λDNA/HindIII restriction pattern, and this relationship holds across a 6-fold range of DNA to dye ratios. A similar relationship has also been shown to hold for both EthD (1) and TOTO (9). This direct dependence of fluorescence signal on the quantity of DNA in stained bands makes possible the quantitation of DNA with either EthD, TOTO or YOYO.

The great stability of the complexes of TOTO and YOYO with dsDNA is illustrated further by the experiments shown in Fig 10. Competition of a dsDNA-TOTO and YOYO complexes formed at a molar bp to dye ratio of 5:1 with a large excess of unlabeled DNA results in a transfer of only 15–20% of the originally bound dye from the initial complexes to the unlabeled DNA. The stability of the complexes formed between dsDNA and EthD, TOTO and YOYO has allowed us to extend the two-channel detection of DNA molecules we first described for EthD and TO (6) to the multicolor detection and sizing of dsDNA fragments. By staining a dsDNA sizing ladder with EthD and an 'unknown' sample of DNA fragments with TOTO or YOYO, same-lane detection of the DNA samples can be readily achieved by separating the distinct fluorescence of the dsDNA-EthD and dsDNA-TOTO (or YOYO) complexes. The fragment sizes calculated from TOTO and YOYO stained samples compare quite well with actual DNA fragment sizes (within 1–7% error), as shown in Table I. Multiplex sizing methods reduce the error of restriction fragment sizing by running the sizing ladder and unknown DNA fragments in the same lane of the gel. Alternate approaches to this method have been developed for both denaturing polyacrylamide (21) and non-denaturing agarose slab gel (22) formats. Unlike the technique we describe in this report, these methods depend upon ligation of fluorescently labeled oligonucleotide fragments onto the DNA sizing standards and unknown DNA samples. In addition to requiring much simpler pre-staining manipulation of unknown samples and no additional enzymatic chemistry, the DNA staining methods we describe above should be considerably more sensitive than approaches which depend upon ligation, since more sensitive fluorochromes can be employed and each DNA molecule can carry more than one or two fluorescent labels.

In conclusion, this report documents the stability of the TOTO and YOYO complexes with dsDNA and describes their application to the labeling, detection, and sizing, of dsDNA fragments. As pointed out earlier (1, 6), such highly fluorescent complexes containing many stably bound chromophores should prove useful as probes in immunoassays, in fluorescence *in situ* hybridization on chromosomes, and in many other areas where simultaneous labeling with several distinct, intense fluorophores is necessary.

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