Top Heterocycl Chem (2008) 14: 11–29
DOI 10.1007/7081_2007_109

Published online: 23 February 2008

Cyanine Dye–Nucleic Acid Interactions

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Abstract Cyanine dyes are widely used in biotechnology due to their ability to form fluorescent complexes with nucleic acids. This chapter describes how the structure of the dye determines the mode in which it binds to nucleic acids as well as the fluorescence properties of the resulting complexes. Related dyes, such as hemicyanines and styryl dyes, are briefly described as well. In addition, covalent conjugates of cyanines with nucleic acids or with nucleic acid-binding ligands allow fluorescent labeling and probing of DNA/RNA structure and function. Several examples of different types of conjugates and their applications are described.

Keywords Cyanines · Dyes · Fluorescence · Intercalation · Nucleic Acids

Abbreviations

A	Adenine
C	Cytosine
DNA	Deoxyribonucleic acid
FRET	Förster resonance energy transfer
G	Guanine
HOMO	Highest occupied molecular orbital
LUMO	Lowest unoccupied molecular orbital

PNA Peptide nucleic acid
RNA Ribonucleic acid
T Thymine

1 Introduction

The cyanines are among the oldest synthetic dyes commonly used today. The name "cyanine" comes from the first member of this class, which was reported by Williams in 1856 and was blue in color [1]. In the ensuing 150+ years, thousands more cyanines have been synthesized due to demand based on diverse applications of these versatile dyes [2]. Their ease of synthesis and rich palette of colors initially motivated the use of cyanines in the textile industry. Later, the cyanines became important components of photographic films, making possible the development of vibrant color images. Both of these applications rely on the ability of cyanines to efficiently absorb light at specific wavelengths in the visible region. Most recently, the cyanines have found a third home in the biotechnology sector that relies on their ability to emit light, i.e., fluoresce. In particular, cyanine dyes have become widely used as fluorescent labels and sensors for bioimaging and detection applications. Many of these applications involve the interaction between the dyes and nucleic acids (DNA and RNA), which is the subject of this chapter. A brief summary of the structural and spectroscopic properties of the cyanines is in order.

The general structure for a cyanine is shown in Fig. 1. In these dyes, two nitrogen-containing heterocycles are linked by a polymethine bridge containing an odd number of carbons, which allows resonance delocalization of a positive charge between the two nitrogens. Variation of the length of the polymethine bridge and the identity of the heterocycles allows tuning

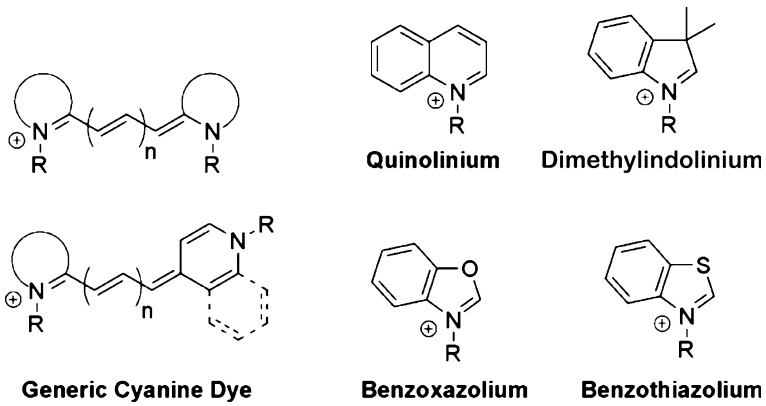


Fig. 1 Generic structures of cyanine dyes and typical heterocyclic components

of the absorption and emission spectra throughout the visible and near-infrared regions of the electromagnetic spectrum. For example, addition of two extra methine units typically causes a ca. 100 nm spectral shift to a longer wavelength. Meanwhile, dyes based on dimethylindole and benzothiazole heterocycles have comparable absorption and emission wavelengths, whereas benzoxazole-containing dyes absorb at shorter wavelengths. Adding substituents to one or both rings allows further fine-tuning of the spectra.

Cyanines can be categorized structurally as being either *symmetrical* or *unsymmetrical*, examples of which are shown in Fig. 2. This chapter will consider these two structural classes separately, since they often exhibit vastly different spectral and nucleic acid binding behavior, which leads to distinct applications. For example, the fluorescent DNA labels **Cy3** and **Cy5** are symmetrical cyanines, while the commonly used fluorescent DNA stain thiazole orange (**TO**) and the dimeric analogue **TOTO-1** are unsymmetrical cyanines (the latter with respect to the chromophore, not the overall structure).

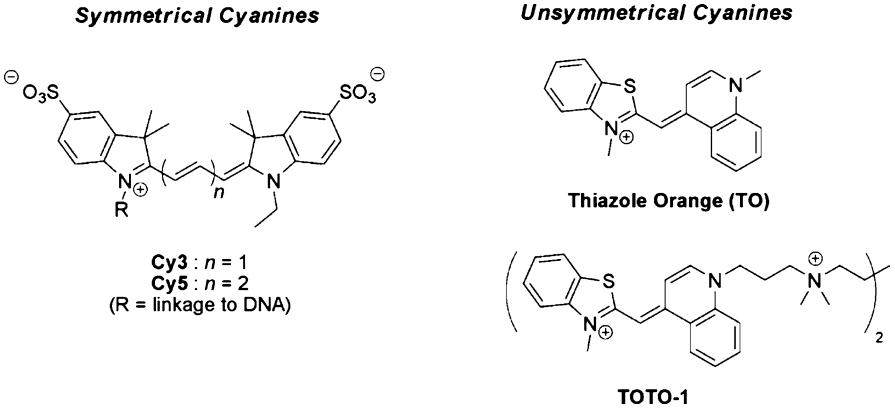


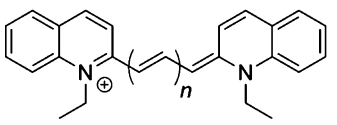
Fig. 2 Commonly used symmetrical and unsymmetrical cyanine dyes. (Counterions are not shown; these are typically iodide, bromide, or tosylate.)

This introduction concludes with a brief description of nucleic acid binding modes. Cyanine dyes typically associate noncovalently with double-helical DNA in one of two ways: (1) intercalation, in which the dye inserts between two adjacent base pairs resulting in a π-stacked sandwich complex, or (2) minor groove binding, in which the dye inserts lengthwise into the narrower of the two grooves present in the DNA structure. Methods for determining DNA binding modes are reviewed elsewhere [3] and so will not be discussed here. For additional information, interested readers are also referred to recent detailed reviews of the interactions between cyanine dyes and DNA [4, 5]. This chapter will focus on how the dye structure relates to nucleic acid binding mode and photophysical behavior, with emphasis on both fundamental properties and applications.

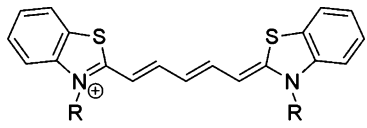
2
Symmetrical Cyanines

2.1
Noncovalent Binding

The earliest report of a cyanine dye–DNA interaction was published by Lerman in his classic paper proposing the intercalation binding mode for planar, cationic dyes that were commonly used to stain DNA for microscopic observation [6]. The dye, pinacyanol, caused a decrease in the viscosity of a DNA solution, in contrast to acridine dyes, which increased the viscosity of the same solutions. Intercalation necessarily causes the DNA to lengthen, leading to the observed increased viscosity, so Lerman concluded that pinacyanol bound in a nonintercalative manner and this conclusion was supported by later studies. However, simply decreasing the bridge length of the dye by two methines yields pseudoisocyanine, which unequivocally binds to DNA by intercalation at low dye to DNA ratios [7]. Another example is provided by the tricationic dye **DiSC$_{3+}$(5)**, which binds to various types of DNA by intercalation as well as by minor groove binding as either a monomer or a cofacial dimer [8].

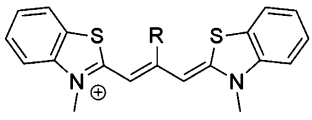


***n* = 1: Pinacyanol**
***n* = 0: Pseudoisocyanine**

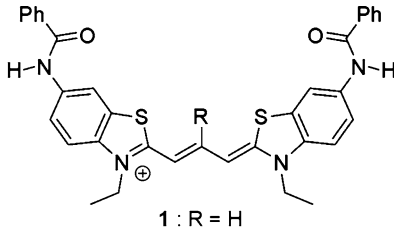


DiSC$_{3+}$(5) : R = $(CH_2)_3\overset{\oplus}{N}(CH_3)_3$

An interesting class of symmetrical cyanines features substitution of the methine bridge. For example, Yarmoluk and coworkers synthesized **Cyan2**, an analogue of **DiSC$_1$(3)** bearing a methyl group at the beta (i.e., central) carbon of the trimethine bridge [9]. Under identical conditions, the unsubstituted dye exhibits a <5-fold increase in fluorescence in the presence of DNA while the β-methyl analogue displays a ca. 100-fold fluorescence enhancement (Table 1) [9, 10]. The difference can be traced to the background fluorescence



DiSC$_1$(3) : R = H
Cyan2 : R = CH_3



1 : R = H
2 : R = CH_3

Table 1 Fluorescence enhancements for symmetrical cyanines in the presence of DNA

Dye	*F* (Buffer)	*F* (DNA)	Enhancement
DiSC$_1$(3)	560	2570	4.6
Cyan2	24.5	4350	178
1	8.6	610	71
2	5.4	1120	207

of the two dyes in the absence of DNA. A similarly large enhancement is observed for the β-methyl dye in the presence of RNA as well. The origin of this behavior has been attributed to a change in the stereochemistry of the trimethine bridge [11]. In the unsubstituted dye, the strongly fluorescent all-*trans* isomer should be favored. However, substitution on the bridge is expected to lead to *cis* isomers, which are nonfluorescent (Fig. 3). Whether binding of **Cyan2** to DNA or RNA induces isomerization to the all-*trans* form or promotes fluorescence by another mechanism is unclear. Regardless of the mechanism, the fact that the β-methyl dye has a low fluorescence quantum yield in solution allows it to exhibit a large enhancement upon nucleic acid binding. The idea of using fluorogenic dyes as indicators for the presence of nucleic acids will also be an important theme for the unsymmetrical cyanines described later in this chapter.

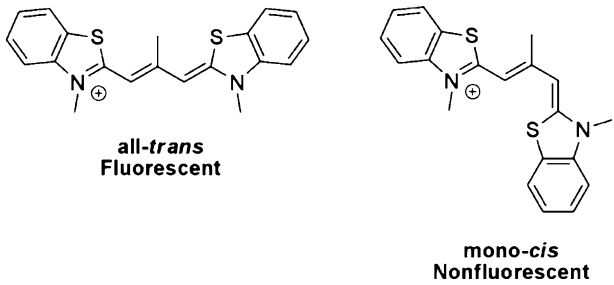
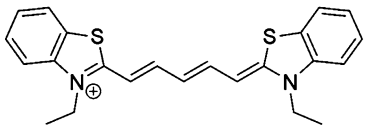


Fig. 3 Effect of a *meso* alkyl substituent on conformation and fluorescence of a symmetrical cyanine dye

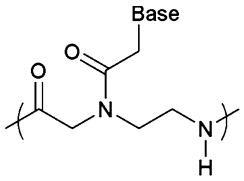
The same group studied the impact of substituents at carbon 6 of the benzothiazole ring system for trimethine dyes bearing unsubstituted or β-substituted bridges [10]. Bulky benzoylamino groups at these positions (e.g., dyes **1** and **2**) led to improved fluorescence enhancements in the presence of DNA, relative to **DiSC$_1$(3)** and **Cyan2** (Table 1). In both cases, the larger fluorescence enhancement is due to lower fluorescence in buffer rather than higher fluorescence in DNA. The likely explanation for these results is enhanced formation of nonfluorescent aggregates by the 6,6′-substituted dyes

in the absence of DNA rather than to a change in the inherent photophysical properties of the dye. The peripheral amide substituents also increase the selectivity for DNA over RNA and bovine serum albumin, a protein that is known to bind organic dyes via hydrophobic interactions.

The complexity of noncovalent DNA binding by symmetrical cyanines has led to very few applications. One exception is the monocationic dye **$DiSC_2(5)$**, which was found to assemble into helical aggregates in the presence of a hybrid duplex composed of complementary DNA and peptide nucleic acid (PNA) strands [12]. (Like many symmetrical cyanines [13, 14], **$DiSC_2(5)$** also aggregates in water, but only at higher concentrations than were used in the assay.) Aggregation of **$DiSC_2(5)$** in the presence of PNA–DNA duplexes leads to a 120-nm shift to shorter wavelength of the absorption spectrum, resulting in a visible blue-to-purple color change. Thus, the PNA oligomer and cyanine dye constitute a simple qualitative sensor for the presence of specific DNA sequences. Several subsequent reports described improvements on the original assay [15–17].



$DiSC_2(5)$



Peptide Nucleic Acid (PNA)
Base = G, A, C or T

2.2 Covalent Binding

In contrast to the dearth of applications involving noncovalent binding of symmetrical cyanines to DNA or RNA, covalently bound versions of these dyes, particularly **Cy3** and **Cy5** [18], are among the most widely used fluorescent labels in biotechnology. For example, in DNA/RNA microarray experiments, captured target strands are usually detected using **Cy3** (green) or **Cy5** (red) fluorescence, where the dye is introduced either in the form of a Cy-labeled secondary probe strand that hybridizes to the captured target or by enzymatic synthesis of a Cy-labeled DNA strand complementary to the target, with subsequent capture of the fluorescent strand on the array.

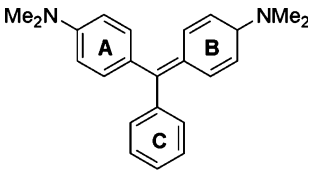
Two structural features of **Cy3** and **Cy5** are evident (Fig. 2): the dimethylindole heterocycles and the peripheral anionic sulfonate substituents are present to suppress intercalation into DNA as well as dye aggregation, which is a concern for multiply labeled probes. These properties help to give the "CyDyes" high fluorescence quantum yields (and, therefore, sensitive detection) without interfering with DNA hybridization.

3
Unsymmetrical Cyanines

3.1
Noncovalent Binding

3.1.1
Insights into Photophysics

The unsymmetrical cyanines such as thiazole orange (**TO**, Fig. 2) are best known for their *fluorogenic* behavior in the presence of DNA and RNA. These dyes exhibit very low fluorescence quantum yields in aqueous solution but 10^2–10^3-fold enhancements upon binding to nucleic acids [19]. The fluorescence response has long been attributed to restricted torsional motion of the dye excited state when intercalated into DNA [20]. Recent ultrafast time-resolved experiments by Ernsting and colleagues were consistent with a twisting motion leading to nonradiative relaxation of **TO** in fluid solution [21]. In addition, calculations by Yaron and coworkers indicate that twisting beyond an interplanar angle of 60° is required before nonradiative relaxation becomes efficient [22]. Thus, **TO** and presumably other unsymmetrical cyanines need not be planar in order to exhibit a large fluorescence enhancement: rigidification with an interplanar angle less than 60° should also lead to strong fluorescence from the dye. This phenomenon has also been observed in the case of the fluorogenic dye malachite green bound to a specific RNA aptamer: the interplanar angle between the A and B rings is 57° [23], yet the fluorescence is increased more than 1000-fold upon binding to the RNA [24].



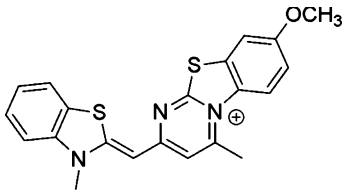
Malachite Green

The unsymmetrical cyanines typically bind to DNA with K_d values in the low to mid micromolar range, depending on the ionic strength of the medium and the charge state of the dye [25]. This means that nucleic acids present at nanomolar or lower concentrations will not be detected by the fluorogenic dye. In order to improve the affinity and, therefore, sensitivity of DNA/RNA detection, a series of bis-cyanine dyes such as **TOTO-1** (Fig. 2) have been synthesized [26]. The presence of two intercalating groups as well as four positive charges leads to binding at nanomolar concentrations.

Vauthey and coworkers showed that the maximum fluorescence enhancements observed for bis-intercalating cyanines are lower than those for the corresponding monointercalating analogues [27]. This is not due to differences in the quantum yields of the bound dyes, which are very similar. Rather, in the absence of DNA, the two chromophores of a bis-intercalating cyanine dye can π-stack, yielding an intramolecular dimer. The authors propose that stacking of the two chromophores actually suppresses the twisting motion that leads to deactivation of the dye excited state. Thus, where fluorophore dimerization is often associated with reduced fluorescence, in the case of the bis-cyanines dimerization enhances fluorescence, albeit only slightly. Since the quantum yield in the absence of DNA is higher for the bis dyes, they exhibit smaller enhancements upon intercalating into DNA. In practice, the significantly higher affinity of the bis-intercalators usually outweighs the lower fluorescence enhancement, so dyes such as **TOTO-1** are more widely used than monointercalating analogues.

3.1.2 New Unsymmetrical Dyes

Synthetic efforts are continuing to focus on new fluorogenic dyes for detecting DNA and/or RNA. Deligeorgiev and coworkers recently reported several interesting new unsymmetrical cyanines [28]. The novel feature of these dyes is an expanded heterocyclic system that includes the benzothiazole normally found in **TO** derivatives, such as in compound **3**. These dyes also substitute a nitrogen atom for one of the CH groups in the polymethine bridge. Dye **3** exhibited a 360-fold fluorescence enhancement in the presence of double-stranded DNA.



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Silva and coworkers synthesized several **TO** analogues bearing electron-withdrawing fluorine and trifluoromethyl groups on the benzothiazole or quinoline ring systems (Fig. 4, [22]). These substituents significantly alter the HOMO–LUMO gap, leading to predictable spectral shifts. For example, fluorination of the benzothiazole system results in blue-shifted absorption and emission spectra. This is because the HOMO is spread over both rings but the LUMO is localized on the quinoline. Therefore, electron-withdrawing fluor-

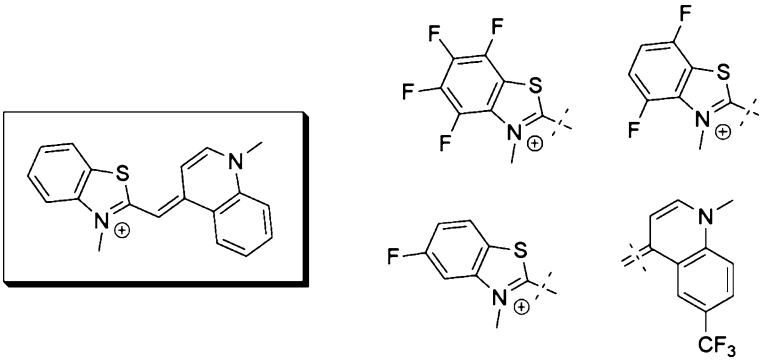
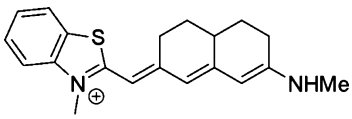


Fig. 4 Fluorinated thiazole orange derivatives

ine substituents on the benzothiazole will stabilize the HOMO more than the LUMO, leading to the observed blue-shift. These authors also reported that while increased fluorination resulted in improved photostability (as observed previously for a symmetrical cyanine dye [29]), DNA binding affinity was reduced.

The *hemicyanines* can be classified as unsymmetrical dyes due to the fact that only one of the two nitrogens is part of a heterocyclic system. Hemicyanine **4** was synthesized and characterized by Kostenko and coworkers [30]. This dye is similar to **TO** in that it has a benzothiazole group and a single methine in the bridge between the two ring systems, although the longer conjugation in **4** leads to absorption and emission at longer wavelengths. The dye exhibits a 10–15-fold fluorescence enhancement in the presence of double-stranded DNA. The weaker fluorogenic behavior compared to **TO** appears to be due to stronger fluorescence of **4** in the absence of DNA. Based on the computational study by Yaron described above [22], it would be interesting to determine whether the torsional barrier in the excited state is lower for **4** than for **TO**.



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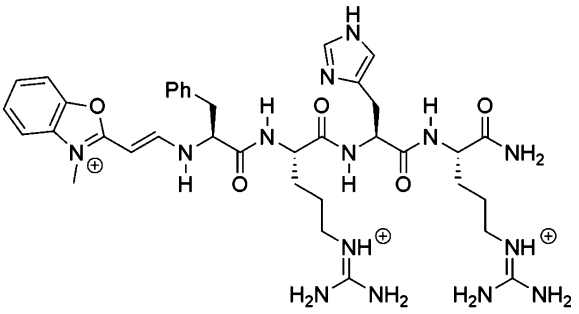
Another mechanism for enhancing fluorescence in hemicyanines was described in the same paper by Kostenko [30]. A precursor dye possessing an exocyclic methyl ether instead of an amine was synthesized and exhibited ca. 100–150-fold lower fluorescence than **4**, both in aqueous buffer and in the presence of DNA. However, in situ reaction with a primary amine led to substitution of the methoxy group and concomitant enhancement of fluores-

cence. This reaction was used to label a protein with the dye, but could also be extended to labeling of other biomolecules and probes.

A hemicyanine dye has also found use as a reporter for guanine (G) quadruplex DNA. Quadruplexes are the subject of increasing scrutiny due to their suspected involvement in regulating gene expression at multiple levels, including transcription, splicing, and translation [31]. Dye–peptide conjugate **5** was discovered by screening of a combinatorial library for binding to a DNA quadruplex structure modeled on the human telomeric DNA sequence [32]. The dye bound to the quadruplex at low micromolar concentrations and exhibited greater than 600-fold lower affinity for double-stranded DNA. The same dye was subsequently used to monitor the unfolding kinetics of a DNA quadruplex [33]. The DNA was covalently labeled with tetramethylrhodamine, which served as the energy acceptor in a Förster resonance energy transfer (FRET) assay where noncovalently bound **5** was the donor. Unfolding of the quadruplex and trapping by a complementary DNA oligonucleotide resulted in release of the hemicyanine donor and loss of FRET. The results are significant because the dynamics of quadruplex folding/unfolding as well as the interconversion between different quadruplex polymorphs are likely to be intimately associated with the biological functions of these intriguing structures.



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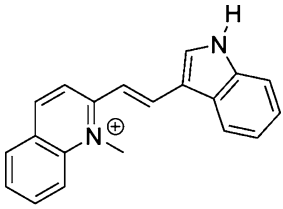
The styryl dyes are similar to unsymmetrical cyanines in that they have two heterocyclic nitrogen atoms and are cationic, but the charge is not delocalized as effectively by resonance as in the cyanines. Nevertheless, these dyes can exhibit substantial shifts and enhancements in fluorescence in response to environmental changes. Recently, Chang and coworkers screened a combinatorial library of styryl dyes for selective RNA staining and obtained three useful compounds [34]. The best dye **6** exhibits more than 50-fold enhancement of fluorescence upon binding to ribosomal RNA from *Escherichia coli*. Both green and red fluorescence were observed, depending on the dye structure, and effective staining of intracellular RNA was observed by confo-



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cal microscopy. The dearth of selective labels for RNA makes this work highly significant and it will be interesting to learn the specific interactions the dye uses to recognize RNA.

3.1.3 Applications

An interesting application for the intercalating unsymmetrical cyanines was recently reported by Benvin and coworkers [35]. Whereas most applications of these dyes involve detection of the DNA or RNA into which they intercalate, these investigators used a DNA–dye assembly to fluorescently label other things, such as mammalian cells and polystyrene beads. The concept is shown in Fig. 5: intercalating dyes bind to the DNA template at high densities, creating very bright fluorescent objects (where "brightness" is defined as the product of the molar extinction coefficient and the fluorescence quantum yield). By virtue of having so many dyes bound to the same DNA template, the overall assembly has a very large extinction coefficient. (A similar concept was proposed by Glazer in a patent [36]; the main innovation in the pub-

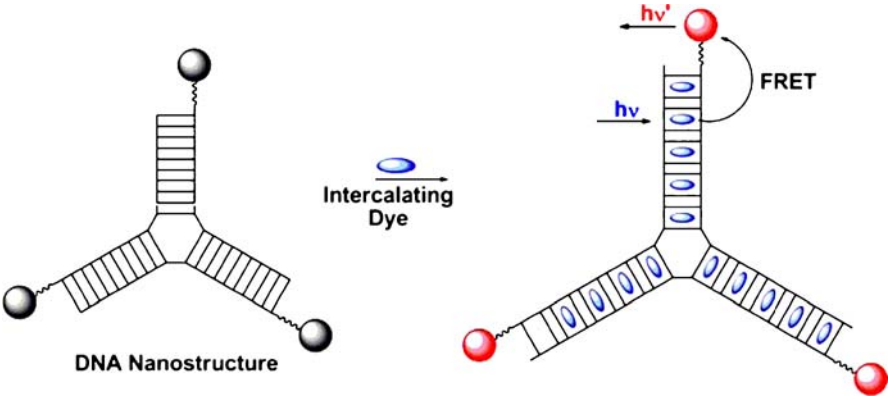
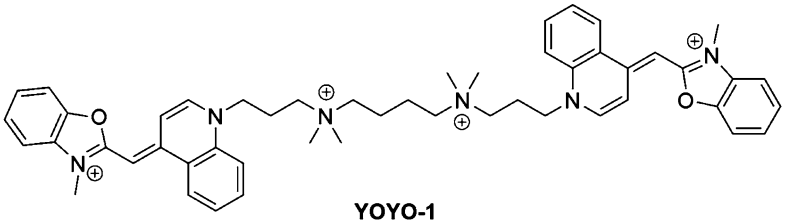


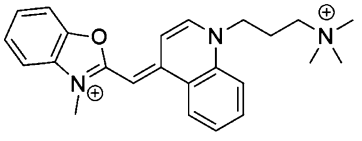
Fig. 5 Illustration of the DNA nanotag concept. Intercalating dye is an unsymmetrical cyanine such as a high-affinity thiazole orange or oxazole yellow analogue. Branched DNA substrates allow assembly of high-density fluorophore arrays

lished work is the use of a branched DNA template to increase the density of fluorophores.) This supramolecular fluorophore, referred to as a "DNA nanotag", can be functionalized with various groups that allow its attachment to a wide variety of substrates, imparting an intensely fluorescent label. Furthermore, additional attachment of longer-wavelength energy acceptor dyes leads to efficient FRET. This allows large separation between the excitation and emission wavelengths, which should improve image quality due to reduced background fluorescence from the sample. The main drawback to the system is that the dyes are noncovalently bound to the DNA template, meaning they are free to dissociate and bind to other molecules in the sample. Nevertheless, straightforward chemistry is available that allows dyes to be covalently attached to DNA, so this problem should be readily addressed.

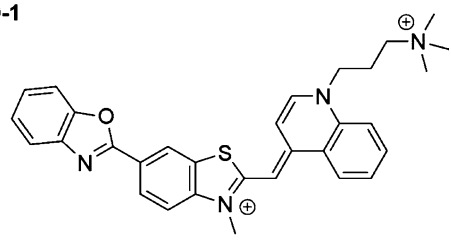
The binding of various small molecules to DNA is typically studied under decidedly nonbiological conditions with respect to the nucleic acid. Genomic DNA generally exists in highly compacted states when packaged into cellular nuclei, and an important question concerns how this affects DNA binding of dyes that are used for microscope imaging. Åkerman and coworkers approached this question by studying the association of four different unsymmetrical cyanines with genomic DNA packaged into protein capsids of bacteriophage T5 [37]. The dyes varied with respect to overall charge (+1 to +4) and DNA binding mode (intercalation vs minor groove binding). Binding of the dyes to the packaged capsid DNA was compared to the same DNA extracted from the capsid. The binding kinetics were considerably slower, particularly for the tetracationic bisintercalator, **YOYO-1**. In addition, the affinities of the dyes were ca. fivefold lower for the capsid DNA. Possible explanations for these results include conformational constraints imposed by packaging of the DNA into the capsid, which is expected to im-



YOYO-1



YO-PRO-1



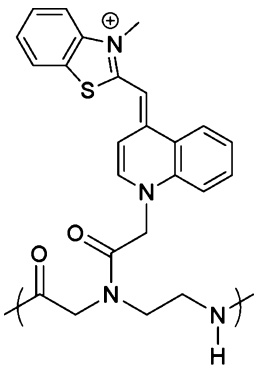
BOXTO-PRO

pact intercalation because of the structural distortion imposed on the DNA by the dye, and competition with multivalent cations that are present inside the capsid to help balance the high negative charge density compared with extended DNA in solution. Another intriguing result from this study was the tendency of the dication intercalator (**YO-PRO-1**) and groove binder (**BOXTO-PRO** [38]) to induce aggregation of the bacteriophage. The authors suggest that this is due to association of the dyes with the phage tails by electrostatic attraction, resulting in cross-linking of the phage and assembly into clusters.

3.2 Covalent Binding

The large fluorescence enhancements exhibited by **TO** and related dyes upon binding to DNA and RNA has led to their widespread use in the laboratory over the past two decades. While much is known about the photophysics of these dyes, a lingering question concerns the sequence dependence of fluorescence. Early work by Netzel and coworkers revealed that both **TO-PRO-1** and **YO-PRO-1**, dicationic analogues of **TO** and **YO**, respectively, exhibited ca. 50–80% greater fluorescence in alternating G-C versus alternating A-T sequences [39]. Even in the simplified DNA copolymers used for those experiments, uncertainty exists about the dye binding site since there are two distinct intercalation sites, e.g., 5′-A-T-3′ or 5′-T-A-3′.

The challenge in further refining our understanding of how sequence determines fluorogenic response is controlling where the dye binds. In an attempt to address this, Seitz and coworkers synthesized a series of PNA oligomers in which the central base was replaced by **TO** and was flanked by various combinations of bases [40]. The PNA–**TO** conjugates were then hybridized with their corresponding perfectly matched and mismatched single-



PNA-TO Conjugate

stranded DNA oligonucleotides. (A thymine was always placed directly across from the **TO**.) In contrast to the earlier report by Netzel et al., the highest quantum yields (ϕ_f = 0.20–0.27) were observed when at least one of the flanking base pairs was A-T/T-A. When both flanking base pairs were G-C or C-G, ϕ_f = 0.13–0.16. Whether the lower quantum yields in G-C/C-G intercalation sites reflect a less restrictive environment or partial quenching by a competitive mechanism such as electron transfer is unknown. Similar experiments in which **TO** is forced to intercalate at a specific site within DNA–DNA duplexes would also be interesting because the different helical parameters for DNA–DNA and PNA–DNA could lead to different fluorogenic responses by the dye in homologous sequence contexts.

3.2.1 DNA-Conjugated Dyes

The fluorogenic properties of unsymmetrical cyanines have been exploited in several different conjugation formats, where a dye is covalently attached to a nucleic acid, protein, or other molecule. If the dye conjugate binds to another molecule and the local environment of the dye is substantially changed in the resulting complex, a change in fluorescence might be observed allowing sensing of the binding event. The first example of this concept was reported by Ishiguro and coworkers, who attached oxazole yellow to a DNA oligonucleotide at an internal position [41]. (The dye was linked to the phosphorus in the internucleotide linker, so no base was replaced.) Hybridization of the **YO**–DNA conjugate to a complementary DNA resulted in a ca. 20-fold fluorescence enhancement. This effect was attributed to intercalation of the dye into the DNA double helix.

More recently, Lartia and Asseline investigated a large number of cyanine–DNA conjugates [42]. Several different monomethine dyes were attached to either internal or terminal positions on DNA oligonucleotides. Surprisingly, most of the conjugates exhibited *lower* fluorescence after hybridization to complementary DNA strands. This insensitivity to hybridization could arise from stacking of the dye with the nucleobases in the single-stranded conjugate, leading to substantial fluorescence even in the absence of the complementary strand. A similar effect has been noted for conjugates of these dyes with PNA, described in more detail in the next section.

The same research group also reported on the behavior of DNA probes labeled at the 5′ end with **TO** [43]. Once again, the hybridized probes exhibited lower fluorescence than the single-stranded probe, but the significant finding was that hybridization to oligonucleotides having single mismatches resulted in greater fluorescence than for perfectly matched complements. The reason for this discrimination is unclear, although one possibility is that the mismatched base pair allows the DNA to adapt better to the **TO** intercalator, creating a more effective binding site for the dye.

3.2.2 PNA-Conjugated Dyes

The high affinity and sequence selectivity of PNA for complementary DNA and RNA targets [44] has generated great interest in its use in a wide variety of hybridization-based applications [45]. Thiazole orange, oxazole yellow, and other analogues have been conjugated to the ends of PNA oligomers [46]. The resulting PNA–**TO** conjugates have been commercialized as "LightUp® probes" and have found use as reporters in real-time PCR assays that measure viral loads in patients infected by cytomegalovirus and SARS coronavirus [47].

LightUp® probes have been used primarily to detect specific DNA or RNA target sequences. A slightly different application involves using the fluorescence enhancement of the conjugated **TO** to report on successful hybridization of a PNA to a desired nucleic acid. For example, Peteanu and coworkers developed a FRET assay for RNA splicing, a posttranscriptional process that deletes internal regions of RNA (introns) and connects the flanking regions (exons) [48]. PNA probes labeled with fluorescent donor and acceptor dyes were designed to hybridize to the exons on either side of an intron. Splicing deletes the intron and links the exons, bringing the donor- and acceptor-labeled PNAs into close proximity and leading to a large increase in FRET efficiency (Fig. 6). **TO**-labeled probes were used initially to demonstrate that both PNAs bound to the large, folded RNA target. In the FRET experiments, **TO** was retained as the donor fluorophore while an Alexa dye was conjugated to the acceptor PNA. Splicing resulted in >75% FRET efficiency and was detected both in bulk solution and by using single molecule microscopy.

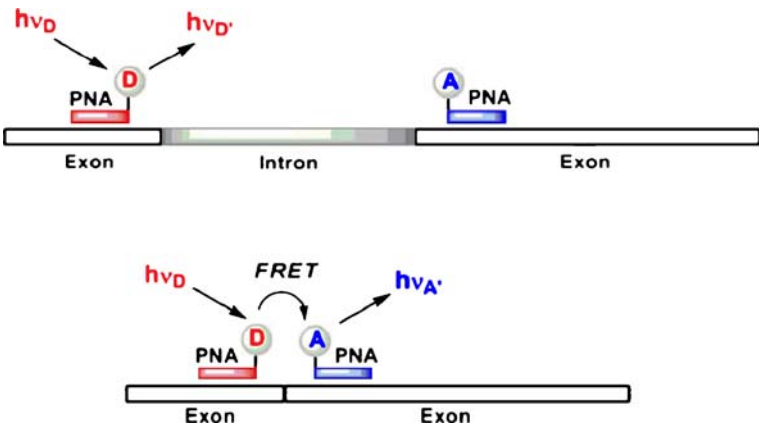


Fig. 6 Use of fluorescent PNA probes to assay RNA splicing. Unsymmetrical cyanine dyes exhibit fluorogenic responses upon hybridization, verifying RNA labeling. Increased FRET indicates splicing

The fluorogenic dye used in hybridization probes need not be attached to a terminal position. As described earlier, Seitz and coworkers have synthesized PNA probes in which **TO** was attached directly to the backbone at an internal site [40]. The resulting PNA–**TO** conjugates are called "forced intercalation" (FIT) probes due to the fact that **TO**, like other intercalators such as ethidium bromide [49], binds only weakly to PNA–DNA hybrids. These probes can be hybridized to complementary DNA targets having any base opposite the **TO** [50]. Good mismatch discrimination was observed when the dye was attached to the PNA via a short linker extending from the quinoline ring. Fluorescence enhancements of 10–20-fold were observed upon hybridization to perfectly matched targets, whereas lower enhancements result from targets having single mismatches directly adjacent to the **TO** site. Comparison of a series of linker lengths and conjugation site revealed that an acetyl linker to the quinoline ring gave the best results, with tenfold greater fluorescence than the same linker to the benzothiazole ring.

3.2.3 Peptide- and Protein-Conjugated Dyes

The fluorogenic properties of the unsymmetrical cyanines have also been exploited for studying DNA-binding peptides and proteins. For example, Kelley and coworkers synthesized a series of **TO**-conjugated cationic peptides and studied their binding to calf thymus DNA [51]. Substantial fluorescence enhancements were seen in most cases, with conjugation of the peptide to the quinoline ring leading to ca. threefold greater quantum yields than conjugation to the benzothiazole ring. The better performance of the quinoline-linked conjugate is similar to that reported for the FIT probes described in the preceding paragraph, and it will be interesting to see if similar results are obtained for other types of **TO** conjugates.

Along similar lines, Thompson synthesized conjugates where **YO** was attached to sequence-specific DNA binding peptides [52]. In the presence of double-stranded DNA containing a binding site for the peptide, the peptide-conjugated dye exhibited a fluorescence quantum yield of 0.37 and an estimated enhancement factor of 770, values that compare well with those of DNA-bound **YO** (i.e., without the peptide): 0.44 and 920, respectively. However, the peptide enhances the affinity of the dye for the DNA by ca. 200-fold (K_d = 10 nM for the conjugate versus 2 μM for the free dye).

While the two preceding examples demonstrate that peptide–dye conjugates can bind to and signal the presence of DNA via fluorescence enhancement, they lack the ability to recognize any predetermined sequence. To achieve this goal, a general DNA recognition module is needed. The minor groove binding polyamides developed over the past decade by Dervan's group are capable of high affinity and sequence-specific recognition of virtually any target sequence [53]. Conjugation of **TO** to three different polyamides

did not alter their specific recognition sequences [54]. Fluorescence enhancement values of >1000 were observed for specific targets and these were at least tenfold greater than for mismatched sequences. The affinities were in the expected range for the polyamides (K_d = ca. 10 nM). In addition, DNA footprinting experiments verified that the polyamide domain bound in the minor groove while plasmid relaxation assay demonstrated unwinding of the DNA, indicating that the **TO** chromophore intercalates into the DNA. Thus, the polyamide–**TO** conjugates could be useful for detecting specific sequences of DNA in the low nanomolar concentration regime.

Genomic DNA is highly compacted into nucleosome particles consisting of double-helical DNA wrapped around cationic histone proteins. A fluorogenic reporter dye could allow study of the structure and dynamics of nucleosome assembly. Woodbury and coworkers reported the conjugation of **TO** to a cysteine residue in the histone H3 protein to provide a sensor for the protein–DNA interactions [55]. Assembly of the labeled histone with DNA to form reconstituted nucleosomes resulted in a ca. 20-fold enhancement of fluorescence when performed at low salt concentration. Reconstitution at higher ionic strength leads to somewhat lower enhancement and the reason for this difference is unclear. The probe also exhibits different enhancements based on the sequence and length of the DNA used to reconstitute the nucleosomes.

In summary, conjugating cyanine dyes to various oligonucleotides, peptides, proteins, or their analogues can yield useful fluorescent labels, probes, and sensors for biological imaging and detection. The desired application dictates the dye selection, with symmetrical dyes such as **Cy3** or **Cy5** ideally suited for labeling because their fluorescence quantum yields are relatively insensitive to changes in environmental conditions. The unsymmetrical dyes such as **TO** are more useful in sensing schemes due to their fluorogenic behavior.

4 Conclusions

The popularity of cyanine dyes has never been greater than it is now, with applications in nucleic acid detection leading the way. A strong foundation of fundamental knowledge concerning how the dye structure influences DNA binding and the mechanisms for fluorogenic behavior assists in the design of new dyes with improved properties. An area for future growth lies in the development of RNA-specific dyes. The need for such probes grows seemingly on a daily basis, with each new discovery of RNA involvement in regulating gene expression. The ability to detect and track these short RNAs as they maneuver through the cell could dramatically increase our knowledge of these critical biological pathways.

Acknowledgements The author gratefully acknowledges the hard work and intellectual contributions of an outstanding group of students and collaborators who have studied the fundamentals and developed applications of cyanine dye–nucleic acid interactions. Research in the author's lab was made possible by generous financial support from the US National Science Foundation and National Institutes of Health, the American Chemical Society, and Carnegie Mellon University.

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