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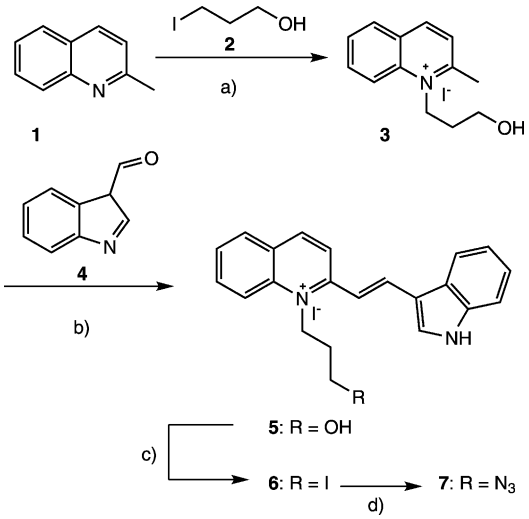
A "Clickable" Styryl Dye for Fluorescent DNA Labeling by Excitonic and Energy Transfer Interactions

Moritz M. Rubner, Carolin Holzhauser, Peggy R. Bohländer, and Hans-Achim Wagenknecht*[a]

Fluorescent bioanalytics and fluorescent cell imaging of nucleic acids demands the design and synthesis of new dyes that exhibit potentially interesting optical properties particularly in combination with other dyes.[1] Dual covalent labeling of nucleic acids has turned out to be very useful for creating tailor-made fluorescent probes for such applications.[2] For instance, hybridization-sensitive probes can be created by two covalently attached thiazole orange (TO) fluorophores that interact excitonically only in the single strand.[3] On the other hand, observable fluorescence color changes can be achieved by applying an energy transfer that occurs between two adjacent fluorescent labels that are forced into close proximity by the surrounding DNA architecture.[4] With respect to the preparation of such dual labeled oligonucleotides recent developments of the "click"-type Huisgen–Sharpless–Meldal cycloaddition for nucleic acids offers great advantages since (unexpectedly) complicated and time-consuming syntheses of DNA building blocks for potentially interesting dyes can be avoided.[5]

The cyanine-styryl-type dyes represent very promising candidates for nucleic acids based primarily on their recently reported property as bright noncovalent binders to RNA.[6] Herein, we present the synthesis of a "clickable" CyIQ (*cy*anine *i*ndole *q*uinoline) fluorophore that can be easily attached covalently to any desired 2′-position in nucleic acids. Moreover, the optical properties of the CyIQ dye can be tuned by excitonic interaction with a second adjacent label, or by energy transfer processes with thiazole red (TR)[7] as a second fluorescence base surrogate.

The CyIQ dye consists of a quinoline part that is conjugated with an indole by a styryl-type bridge. The dye can be prepared in a four-step synthesis including the introduction of the "clickable" azide functionality (Scheme 1). It starts with commercially available 2-methyl quinoline (**1**) that is alkylated with 3-iodopropanole (**2**) as the short linker between the dye and the azide group. Condensation of the methyl group of **3** in the presence of piperidine yields dye **5**. In the next steps, an Appel reaction leads to iodide **6**, and the azide is introduced by nucleophilic substitution to yield **7**, ready for postsynthesis DNA modification.



Scheme 1. Synthesis of the CyIQ azide **7**: a) dioxane, 101 °C, 19 h; 67%; b) piperidine, EtOH, reflux, 24 h; 87%; c) CBr_4, Ph_3P, dichloromethane, room temperature, 18 h; 70%; d) NaN_3, DMF, room temperature 18 h; 65%.

Due to its positive charge the CyIQ dye **5** is sufficiently water soluble for titration experiments with a random-sequence double-stranded DNA (Figure 1). The absorption spectra of this titration reveal significant changes; especially the absorption shift from 465 nm (without DNA) to 472 nm (with DNA) indicates excitonic dye–dye interactions of **5** (in the absence of DNA), which are interrupted by the interaction of the dye with an increasing amount of DNA. These dye–dye interactions cause a significant fluorescence quenching of **5** in aqueous solution without DNA. With increasing amounts of DNA the fluorescence intensity of the dye is recovered and finally enhanced to the 17-fold when the saturation plateau is reached. This fluorescence enhancement is typical for cyanine dyes and higher than standard ethidium bromide; with the latter dye a tenfold increase was observed in similar titrations.[8]

With CyIQ azide **7** in hand, we performed "click"-type reactions according to our published protocols.[9] The dye was attached to 2′-propargylated uridines in oligonucleotides in

[a] Dipl.-Ing. M. M. Rubner, Dipl.-Chem. C. Holzhauser, Dipl.-Chem. P. R. Bohländer, Prof. H.-A. Wagenknecht
Karlsruhe Institute for Technology, Institute for Organic Chemistry
Fritz-Haber-Weg 6, 76131 Karlsruhe (Germany)
Fax: (+49) 721-608-44825
E-mail: Wagenknecht@kit.edu

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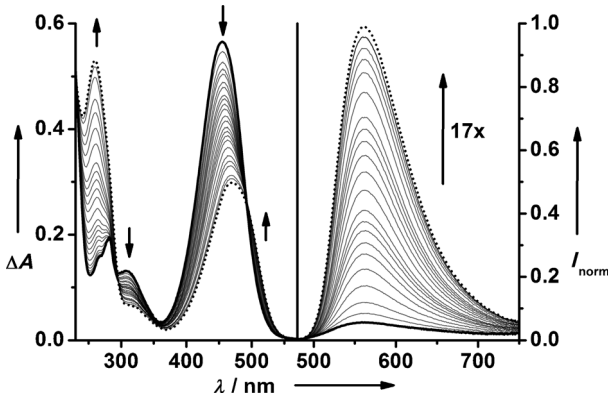
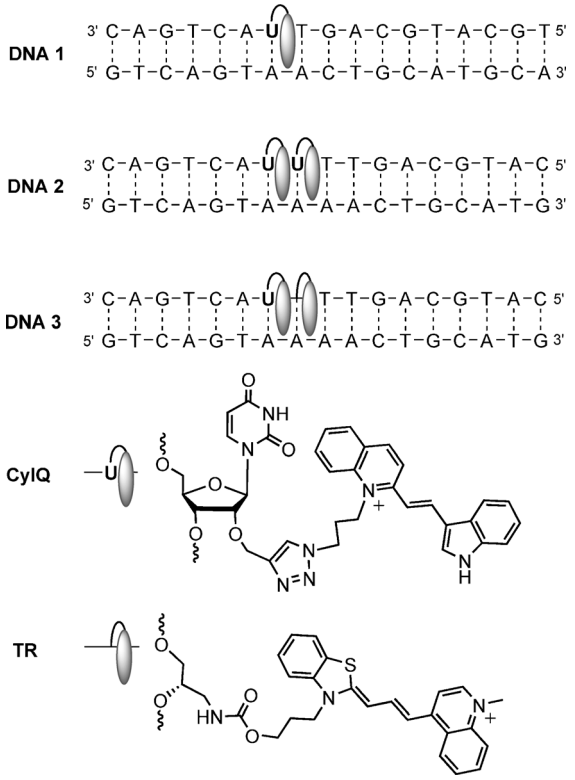


Figure 1. UV/Vis absorption spectra (left) and fluorescence spectra (right) of the titration of **5** with DNA. Solid line: free dye (24.0 μM) without DNA; gray line: intervening titration steps; dotted line: DNA (13.2 μM) with **5** (24.0 μM), at the beginning of saturation (50 mM Na-P_i-buffer; pH 7; λ_{exc} = 495 nm).

60–70% yield. The corresponding phosphoramidite is commercially available. This structural approach has the major advantage that it places the CyIQ dye into close proximity of the DNA duplex while potentially keeping the Watson–Crick-type base pairing of the uridine at the site of modification intact.[10] **DNA1** represents a reference duplex that bears a single CyIQ modification in the middle (Scheme 2). We studied the optical properties of the single CyIQ modification also in different base pair environments (see **DNA4**–**DNA7** in the Supporting Information). In **DNA2**, two CyIQ dyes are combined in the same strand adjacent to each other. Finally, in **DNA3** the CyIQ dye is combined with the red emitter TR, again in the same strand. The latter dye was incorporated into oligonucleotides as a DNA base substitution by using the corresponding synthetic phosporamidite as a DNA building block.[7]



Scheme 2. Sequences of modified **DNA1**–**DNA3**.

The CyIQ dye as a single modification in oligonucleotides displays solid optical properties that make this dye a suitable candidate for fluorescent bioanalytics and imaging. CyIQ exhibits an absorption maximum at 495 nm that lies in an excitation range comparable to well-known dyes, such as thiazole orange, BODIPY, fluoresceine and Cy3. The fluorescence at 565 nm occurs with a quantum yield of 0.07 in the duplex; together with an extinction coefficient of 41 500 $M^{-1}cm^{-1}$ a brightness of 2900 $M^{-1}cm^{-1}$ is achieved. These values are comparable to single thiazole orange labels in DNA and therefore show the applicability of CyIQ for bioanalytical applications. The melting temperatures (T_m, see Table S1 in the Supporting Information) reveal representatively for two duplexes that there is no significant decrease of thermal stability as a result of a single CyIQ 2′-modification: **DNA4** (bearing the dye in a T–A environment) has a T_m of 62.0 °C, which is only 0.5 °C lower compared to a completely unmodified one (T instead of U),[9a] and **DNA7** (bearing the dye in a G–C environment) has a T_m of 69.7 °C, which is even 1.7 °C higher than a comparable unmodified duplex (see the Supporting Information).[9a]

The photostability of fluorescent dyes, however, is an increasingly critical parameter for applications especially with respect to fluorescent cell imaging. Hence, we tested the photochemical stability of CyIQ as a single modification in double stranded DNA in comparison with the standard dyes thiazole orange, fluoresceine, BODIPY and Cy3, which absorb in a similar wavelength range. The irradiation was performed with a 75 W Xe arc lamp and a $<$305 nm cut-off filter. The photobleaching was followed by UV/Vis absorption spectroscopy and fluorescence spectroscopy at the dye-typical wavelengths between 490 and 520 nm. The results show that the CyIQ dye is significantly more stable compared to thiazole orange, BODIPY and fluoresceine (Figure 2). Extensive photobleaching to approximately 10% remaining fluorescence intensity was observed with BODIPY as an internal DNA modification[11] in less than 10 min, and with thiazole orange[7] and fluoresceine in less than 2 h. Remarkably, after 2 h irradiation of CyIQ-modified **DNA1** approximately 75% fluorescence was maintained. Extensive photobleaching of the CyIQ dye in DNA was observed only after irradiation for a significantly prolonged time (more than 15 h). Only the Cy3 dye was more stable.

Nevertheless, together with the above-mentioned fluorescence properties it becomes clear that CyIQ represents an important alternative to the routinely applied dyes thiazole orange, BODIPY and, most importantly, fluoresceine as an internal and covalent label for DNA. In contrast to the more photostable Cy3 dye it is important to point out, that the 2′-modification with the CyIQ dye has the advantage of

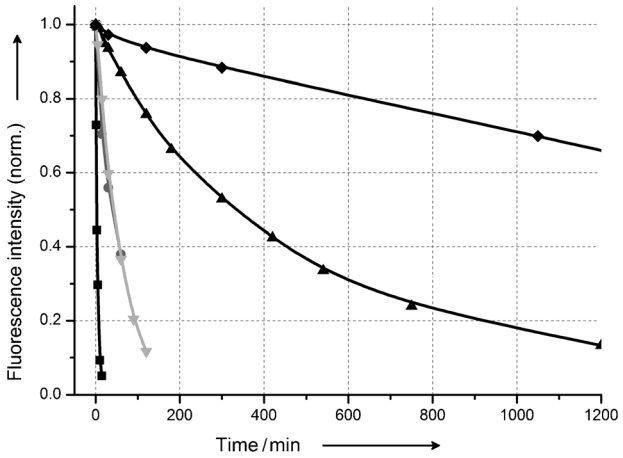


Figure 2. Photobleaching of different dyes covalently attached to DNA (2.5 μM in 10 mM Na-P_i-buffer, pH 7.0, 250 mM NaCl) carried out with a 75 W Xe arc lamp and a 305 nm cut-off filter (for the sequences of **DNA8**–**DNA12**, see the Supporting Information). Double stranded **DNA9** (BODIPY; ■), **DNA10** (fluorescein; ●); **DNA1** (CyIQ, ▲); **DNA8** (TO; ▼); **DNA12** (Cy3, ◆).

maintaining Watson–Crick base pairing (based on similar experiments published recently)[9] and thereby keeps the DNA duplex intact.

In another set of experiments, the CyIQ dye was combined with itself or thiazole red as second adjacent fluorophore in **DNA2** and **DNA3**, respectively (Figure 3). The absorption spectrum of single stranded **DNA2** bearing the intrastrand CyIQ–CyIQ fluorophore pair exhibited a significant hypsochromic shift to 463 nm. Based on the absorption changes that were observed in titration experiments with the CyIQ dye **5** (Figure 1) this result supports the idea that the CyIQ dye undergoes excitonic interactions. Typically, such H aggregates of DNA-interacting dyes exhibit significant fluorescence quenching,[12] that is, in fact, observed for single-stranded **DNA2**. The fact that the fluorescence is not quenched quantitatively can be attributed probably to the conformational dynamics of DNA as a biopolymer and the presence of conformational subensembles with unstacked CyIQ dyes. Nevertheless, the excitonic interactions of the stacked ensemble can be interrupted by annealing **DNA2** with the corresponding complementary counterstrand. As a result an approximately sevenfold fluorescence enhancement is observed from the doubly CyIQ-labeled single to the double strand. Accordingly, the absorption maximum is shifted back to 495 nm.

On the other hand, the fluorescence of CyIQ as a covalently attached label in DNA shows a pretty good overlap with the absorption of TR as a base substitution in DNA.[7] Hence, it is reasonable to assume that energy transfer might take place between the two dyes. Indeed, if the doubly labeled single strand **DNA3** is excited at the CyIQ-typical wavelength 495 nm a significant amount of the thiazole red fluorescence at approximately 660 nm is obtained. Interestingly, this energy transfer process is blocked nearly completely when single-stranded **DNA3** is annealed with the

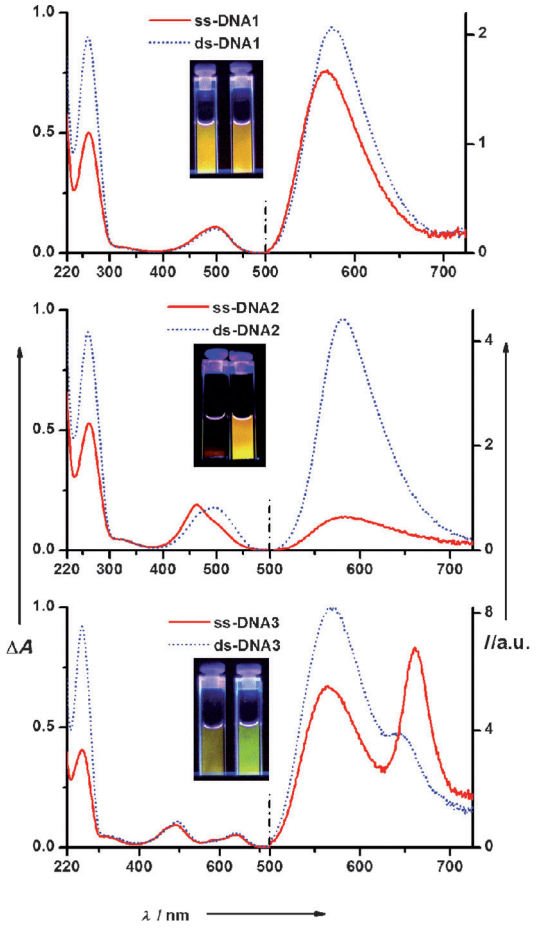


Figure 3. UV/Vis absorption (left) and fluorescence (right) of **DNA1** (top), **DNA2** (middle) and **DNA3** (bottom); solid red lines represent single strands, dotted blue lines are double strands, each 2.5 μM in 10 mM Na-P_i-buffer, pH 7.0, 250 mM NaCl, λ_{exc} = 495 nm.

corresponding counterstrand. Accordingly, the red fluorescence vanishes and turns into a green color.

In conclusion, we have shown that the CyIQ dye represents a promising covalently attached fluorescent label for DNA, since it shows good brightness and excellent photostability. In particular, the photochemical stability of CyIQ is significantly enhanced compared to the routinely applied fluoresceine. The dye is "clickable", that means it can be incorporated postsynthetically and therefore easily into oligonucleotides by using the cycloaddition between azides and acetylenes. In this study, the "click" reaction was performed representatively to 2′-propargylated uridines in DNA. This structural approach has the advantage that the preference for pairing between the modified uridine and adenine in the counterstrand is potentially maintained and thereby the DNA duplex conformation stays stable.[9a] One could envision that this experimentally simple labeling method can be extended to other acetylene-containing building blocks in RNA and DNA, which are either commercially or synthetically available. Beside the application as single internal DNA marker, the CyIQ label can be combined with itself and TR, all chromophors adjacent to each other as intra-

strand fluorophore pairs. Excitonic interactions between the CyIQ dyes and energy transfer interactions between CyIQ and thiazole red provide interesting alternatives for fluorescence readouts for DNA hybridization, which are either fluorescence enhancement or fluorescence color change, respectively.

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Keywords: click chemistry · cyanine · DNA · energy transfer · exciton

[1] a) B. A. D. Neto, A. A. M. Lapis, F. S. Mancilha, I. B. Vasconcelos, C. Thum, L. A. Basso, D. S. Santos, J. Dupont, *Org. Lett.* **2007**, *9*, 4001–4004; b) Y. N. Teo, J. N. Wilson, E. T. Kool, *J. Am. Chem. Soc.* **2009**, *131*, 3923–3933; c) H. Kobayashi, M. Ogawa, R. Alford, P. L. Choydke, Y. Urano, *Chem. Rev.* **2010**, *110*, 2620–2640; d) E. Socher, L. Bethge, A. Knoll, N. Jungnick, A. Herrmann, O. Seitz, *Angew. Chem.* **2008**, *120*, 9697–9701; *Angew. Chem. Int. Ed.* **2008**, *47*, 9555–9559; e) H. Kashida, K. Sekiguchi, X. Liang, H. Asanuma, *J. Am. Chem. Soc.* **2010**, *132*, 6223–6230; f) V. L. Malinowskii, D. Wenger, R. Häner, *Chem. Soc. Rev.* **2010**, *39*, 410–422; g) S. Berndl, M. Breunig, A. Göpferich, H.-A. Wagenknecht, *Org. Biomol. Chem.* **2010**, *8*, 997–999.

[2] a) S. Laib, S. Seeger, *J. Fluoresc.* **2004**, *14*, 187–191; b) Y. Okamura, S. Kondo, I. Sase, T. Suga, K. Mise, I. Furusawa, S. Kawakami, Y. Watanabe, *Nucl. Acids Res.* **2000**, *28*, e107; c) P. Conlon, C. J. Yang, Y. Wu, Y. Chen, K. Martinez, Y. Kim, N. Stevens, A. A. Marti, S. Jockusch, N. J. Turro, W. Tan, *J. Am. Chem. Soc.* **2008**, *130*, 336–342; d) R. Varghese, H.-A. Wagenknecht, *Org. Biomol. Chem.* **2010**, *8*, 526–528.

[3] a) S. Ikeda, T. Kubota, K. Kino, A. Okamoto, *Bioconjugate Chem.* **2008**, *19*, 1719–1725; b) S. Ikeda, T. Kubota, M. Yuki, A. Okamoto, *Angew. Chem.* **2009**, *121*, 6602–6606; *Angew. Chem. Int. Ed.* **2009**, *48*, 6480–6484.

[4] a) S. Sindbert, S. Kalinin, H. Nguyen, A. Kienzler, L. Clima, W. Bannwarth, B. Appel, S. Müller, C. A. M. Seidel, *J. Am. Chem. Soc.* **2011**, *133*, 2463–2480; b) R. Häner, S. M. Biner, S. M. Langenegger, T. Meng, V. L. Malinovskii, *Angew. Chem.* **2010**, *122*, 1249–1252; *Angew. Chem. Int. Ed.* **2010**, *49*, 1227–1230; c) K. Börjesson, S. Preus, A. H. El-Sagheer, T. Brown, B. Albinsson, L. M. Wilhelmsson, *J. Am. Chem. Soc.* **2009**, *131*, 4288–4293; d) S. Berndl, H.-A. Wagenknecht, *Angew. Chem.* **2009**, *121*, 2454–2457; *Angew. Chem. Int. Ed.* **2009**, *48*, 2418–2421.

[5] a) P. M. Gramlich, C. T. Wirges, A. Manetto, T. Carell, *Angew. Chem.* **2008**, *120*, 8478–8487; *Angew. Chem. Int. Ed.* **2008**, *47*, 8350–8358; b) M. Grotli, M. Douglas, R. Eritja, B. S. Sproat, *Tetrahedron* **1998**, *54*, 5899–5914; c) A. H. El-Sagheer, T. Brown, *Chem. Soc. Rev.* **2010**, *39*, 1388–1405.

[6] a) Q. Li, Y. Kim, J. Namm, A. Kulkarni, G. R. Rosania, Y.-H. Ahn, Y.-T. Chang, *Chem. Biol.* **2006**, *13*, 615–623; b) Q. Li, Y. T. Chang, *Nat. Protoc.* **2006**, *1*, 2922–2932.

[7] C. Holzhauser, S. Berndl, F. Menacher, M. Breunig, A. Göpferich, H.-A. Wagenknecht, *Eur. J. Org. Chem.* **2010**, 1239–1248.

[8] a) C. Prunkl, M. Pichlmaier, R. Winter, V. Kharlanov, W. Rettig, H.-A. Wagenknecht, *Chem. Eur. J.* **2010**, *16*, 3392–3402; b) C. Holzhauser, H.-A. Wagenknecht, *Angew. Chem.* **2011**, *123*, 7406–7410; *Angew. Chem. Int. Ed.* **2011**, *50*, 7268–7272.

[9] a) S. Berndl, N. Herzig, P. Kele, D. Lachmann, X. Li, O. S. Wolfbeis, H.-A. Wagenknecht, *Bioconjugate Chem.* **2009**, *20*, 558–564; b) D. Lachmann, S. Berndl, H. A. Wagenknecht, O. S. Wolfbeis, *Beilstein J. Org. Chem.* **2010**, *6*, 13.

[10] F. Seela, V. Sirivolu, P. Chittepu, *Bioconjugate Chem.* **2008**, *19*, 211–224.

[11] H. A. Wagenknecht, T. Ehrenschwender, *J. Org. Chem.* **2011**, *76*, 2301–2304.

[12] a) H. C. Hannah, B. A. Armitage, *Acc. Chem. Res.* **2004**, *37*, 845–853; b) B. A. Armitage, *Top. Curr. Chem.* **2005**, *253*, 55–76.

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