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DNA and RNA "Traffic Lights": Synthetic Wavelength-Shifting Fluorescent Probes Based on Nucleic Acid Base Substitutes for Molecular Imaging

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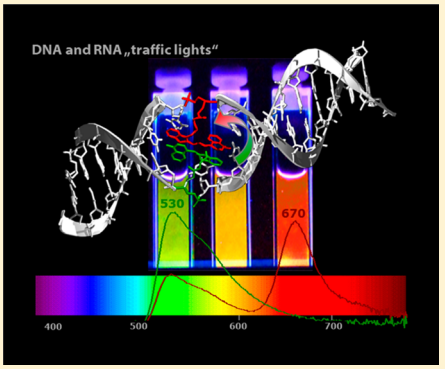
ABSTRACT: The DNA base substitute approach by the (*S*)-3-amino-1,2-propanediol linker allows placing two fluorophores in a precise way inside a given DNA framework. The double helical architecture around the fluorophores, especially the DNA-induced twist, is crucial for the desired photophysical interactions. Excitonic, excimer, and energy transfer interactions yield fluorescent DNA and RNA probes with dual emission color readout. Especially, our DNA and RNA "traffic light" that combines the green emission of TO with the red emission of TR represents an important tool for molecular imaging and can be applied as aptasensors and as probes to monitor the siRNA delivery into cells. The concept can be extended to the synthetically easier to access postsynthetic 2′-modifications and the NIR range. Thereby, the pool of tailor-made fluorescent nucleic acid conjugates can be extended.



Molecular imaging represents the most powerful technique not only to visualize subcellular structures but also to follow the action of biomolecules inside cells in real time.[1−3] A great variety of fluorescent probes and nanoparticles are available for biological imaging.[4−6] A complete toolbox for fluorescent tagging of protein locations and functions was created.[7] On the other hand, tailor-made fluorescent labeling of nucleic acids for molecular imaging has remained challenging.[8] This stands in contrast to the central importance of DNA and RNA in cellular functions. Hence, visualizing nucleic acids represents an important goal for chemical biology. The great advantage of nucleic acids—from an organic−synthetic point of view—is that they are synthesized by building blocks. In this bottom-up approach, fluorescent probes can be introduced synthetically by providing the corresponding DNA building blocks.[9] If such building blocks were synthetically not obtainable or fluorescent probes are not compatible with the broadly applied phosphoramidite chemistry, postsynthetic methodologies allowed the modification of oligonucleotides.[10−12] Moreover, polymerase-assisted biochemical syntheses of labeled oligonucleotides were achieved, first tested in primer extension experiments (PEX) and subsequently applied for amplification of DNA by PCR.[10,13] The latter approach has the advantage that longer and biologically relevant pieces can be prepared. Herein, we summarize our efforts over the past decade to modify nucleic acids chemically with organic chromophores by the DNA base substitution approach. This allowed us to develop wavelength-shifting fluorescent probes ("DNA/RNA traffic lights") as a powerful tool for molecular imaging.

CONCEPT OF DNA BASE SUBSTITUTION: LESSONS FROM ETHIDIUM AND INDOLE

In principal, fluorophores can be conjugated to DNA/RNA bases, conjugated to the sugar moieties of nucleosides, or incorporated as DNA/RNA base replacement.[9] As we expected that the DNA architecture will play a major role for fluorophore interactions (vide infra), we chose the DNA/RNA base replacement approach. In order to develop a generally and easily applicable way to substitute DNA bases synthetically by fluorophores, the 2′-deoxyribofuranoside was replaced by an acyclic linker between the phosphodiester bridges. This approach has several advantages: (i) These linkers lack hydrolytically labile glycosidic bonds and hence provide high chemical stability. This is important especially with respect to the positive charge of a variety of fluorophores (e.g., cyanines). Indeed, we observed a significant hydrolytic lability in the case of the ethidium nucleoside **1**.[14] (ii) Acyclic linkers are compatible with phosphoramidite chemistry since they provide two hydroxy functions with similar reactivity as those of 2′-deoxyribofuranosides. (iii) Separation of anomeric mixtures is not necessary. (iv) Acyclic linkers provide enough conformational flexibility to intercalate the fluorophore. We apply (*S*)-3-amino-1,2-propanediol that was similarly used by Meggers et al. for the design and synthesis of glycol nucleic acids (GNA)[15] and by Pedersen et al. for twisted intercalating nucleic acids (TINA).[16] In comparison to nucleoside **1**, the number of carbon atoms (between the phosphodiester bridges) in the ethidium DNA building block **2** is reduced from 3 to 2. The alternative ethidium DNA building block **3** contains (*S*)-threolinol as an acyclic linker, hence one carbon atom more, but exhibits similar destabilization in DNA as **2**.[17,18] Structurally similar linkers, threolinol and serinol, were heavily applied by Asanuma et al. to develop acyclic threolinol and serinol nucleic acids (aTNA and SNA).[19] In the meantime, this is an established chemistry for DNA base substitutions.

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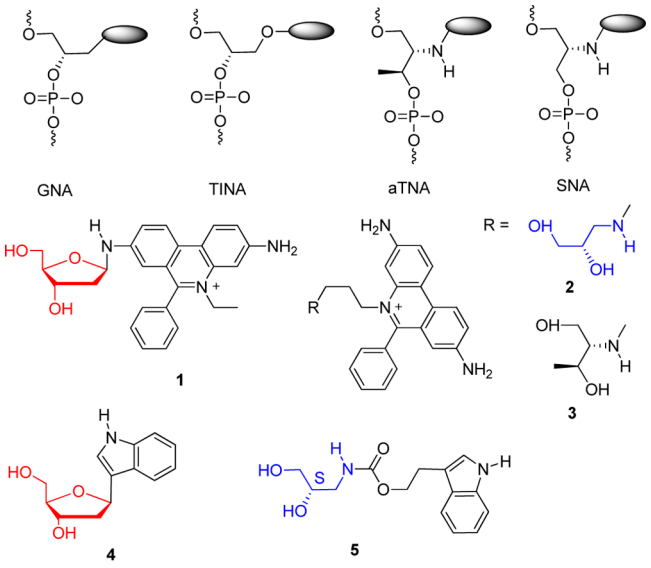
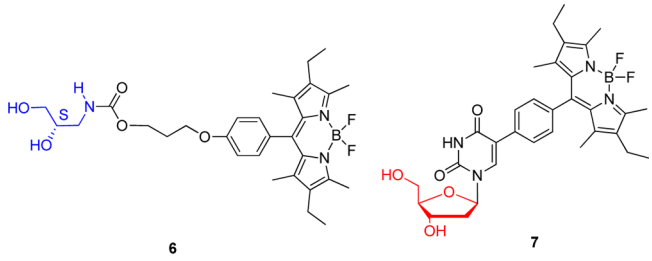


(Structure Block 1)

Two important questions need to be answered: (i) How do these non-nucleosidic DNA base substitutions influence the double-strand stability? The studies with indole as an artificial DNA base gave a surprising answer. The natural-like C-nucleoside **4** (pure β) destabilizes the DNA to a similar extent as the non-nucleosidic indole **5**.[20] Accordingly, destabilization needs to be assigned to both the poor stacking of the chromophore indole and structural influence of the non-natural linker. Chromophores (e.g., BODIPY,[21] cyanines[22]) that stack better will show smaller destabilization. (ii) How important is the *S*-configuration of the linker? Fluorescence quenching studies with nile blue revealed that the interactions between the fluorophore and the adjacent base pairs seem to be similar with the *S*- and *R*-configurated linker.[23]

COMPARISON WITH DNA BASE MODIFICATIONS: LESSONS FROM BODIPY

DNA base modifications represent a commonly applied alternative concept for DNA base substitutions to synthetically attach fluorophores.[24] It is generally assumed that the fluorophore gets Watson–Crick pairing properties when it is attached to the DNA base. We used representatively the well-known BODIPY and compared the optical properties of DNA base modification **6** with that of DNA base substitute **7**.[21] In fact, **6**[25] exhibits slightly preferred pairing with adenine in the counterstrand and is tolerated by DNA polymerases in primer extension experiments[26] but shows dramatically reduced quantum yields. In contrast, **7** exhibits high quantum yields but no preferential base-pairing properties. Most importantly, DNA strands modified with **7** show excellent brightness (compared to fluorescein), which makes this fluorescent label a promising tool for molecular imaging.



(Structure Block 2)

FLUORESCENCE SHIFTS WITH THIAZOLE ORANGE AND THIAZOLE RED: FROM TO DIMERS TO "TRAFFIC" LIGHTS

As living cells show strong intrinsic background fluorescence, it is of utmost importance to design molecular probes with excitation beyond 450 nm and large wavelength shift between excitation and emission to rule out the risk of wrong fluorescence readouts. This can be achieved by combination of two labels to increase fluorescence intensity upon binding to a complementary target sequence (A) or to induce a wavelength shift between excitation and emission, thus leading to a visual fluorescence color change (B).

(A) The chromophore of thiazole orange (TO), probably one of the best known representatives of the cyanine dyes, exhibits strong fluorescence intensity increase upon binding to duplex DNA.[27] This property has been very successfully used by Seitz et al. in the so-called forced intercalation TO–PNA probes.[28] We first incorporated TO into oligonucleotides (**DNA1**, **DNA2**) via the quinolinium nitrogen bound to our previously mentioned (*S*)-3-amino-1,2-propanediol linker. However, the sensitivity of TO as DNA base substitution **8** to environmental changes was not maintained. Hence, we combined **8** with 5-nitroindole (NI) as base surrogate **9** to monitor DNA hybridization by fluorescence enhancement (Figure 1).[29] **DNA1** bears only the TO modification **8**, whereas **DNA2** bears additionally NI in a distance of one base pair away from TO. Fluorescence quenching of **8** occurs only in single-stranded **DNA2** due to photoinduced short-range electron transfer from **8** to **9**. Upon hybridization of **DNA2** with the complementary strand, a fluorescence intensity increase can be observed that is caused by separation of TO from NI and thus interruption of

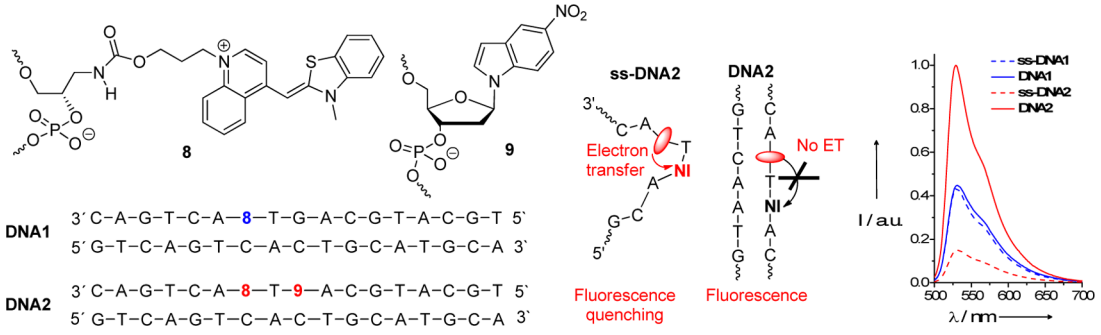


Figure 1. DNA building blocks **8** (TO) and **9** (NI) and sequences of **DNA1** and **DNA2** (left). Schematic illustration (middle). Fluorescence spectra, 2.5 μM DNA, 10 mM NaP_i buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 530 nm (right).

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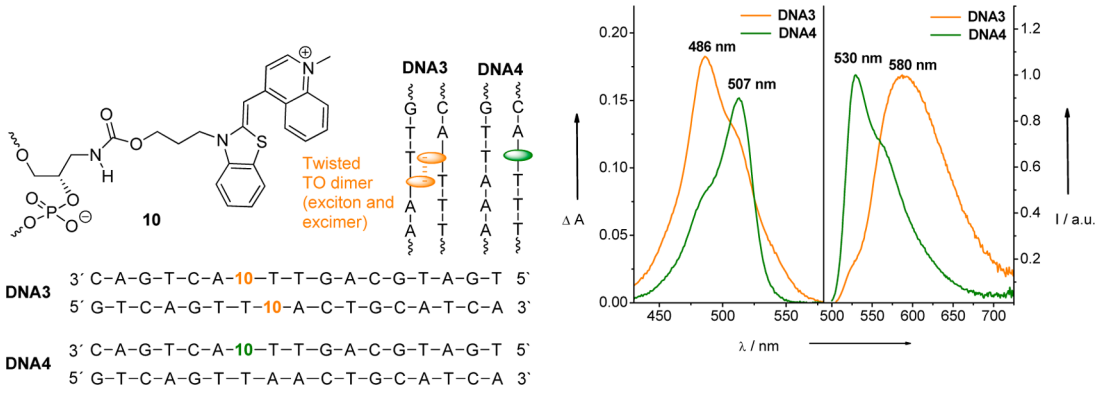

Figure 2. DNA building block **10** (TO) and sequences **DNA3** and **DNA4** (left). Schematic illustration (middle). UV/vis absorption and fluorescence spectra, 2.5 μM DNA, 10 mM NaP$_i$ buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 490 nm (right).

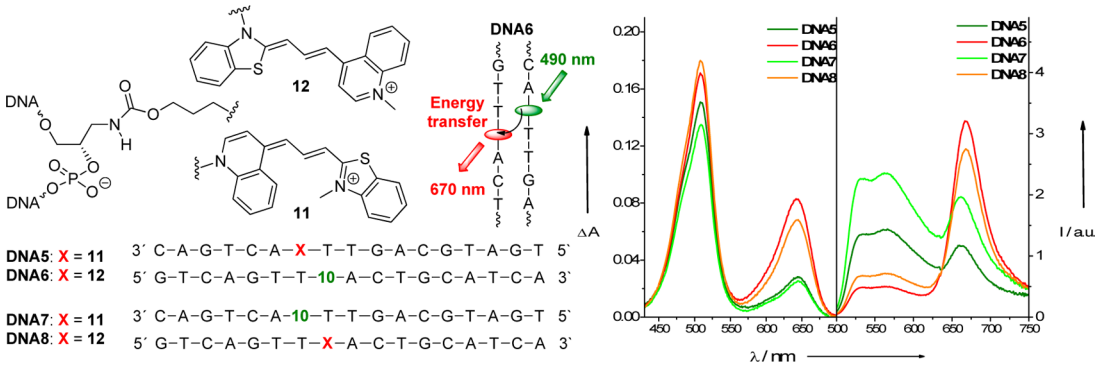

Figure 3. DNA building blocks **11** and **12** (TR) and sequences **DNA5**–**DNA8** (left). Schematic illustration (middle). UV/vis absorption and fluorescence spectra, 2.5 μM DNA, 10 mM NaP$_i$ buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 490 nm (right).

short-range electron transfer. This concept of hybridization-sensitive oligonucleotide probes can be transferred to the commercially available Cy3 dye. In the meantime, Seitz et al. developed a new TO base surrogate with a short tether that responds to hybridization without the use of a second label and electron transfer.[30]

(B) Although approach A showed an applicable fluorescence intensity enhancement, it relies on single fluorescent color readout, and thus undesired fluorescence quenching inside cells cannot completely be ruled out. Hence, dual labels that change their emission maximum upon hybridization represent important alternatives for imaging: (B) In order to develop wavelength-shifting DNA/RNA probes, two thiazole–cyanine-based fluorophores were combined as interstrand chromophore pairs. Both dyes are incorporated into the DNA/RNA backbone (the 3′- and 5′-termini remain unlabeled) and are forced in close proximity to each other by the surrounding double helical architecture. Due to photoelectronic interactions, the emission is shifted bathochromically, yielding a visual color change.

If two TO dyes are incorporated into **DNA3** via the benzothiazole nitrogen (**10**), the dimer shows differences that are characteristic for excitonic interactions (Figure 2). Similar results have previously been observed for dimeric dyes (like TOTO), which are used for noncovalent staining of nucleic acids.[31−33] Most recently, Okamoto et al. used TO and other cyanine dimers as covalent 2′-deoxyuridine labels in the so-called ECHO probes (exciton-controlled hybridization-sensitive fluorescent oligonucleotide).[34] ECHO probes show an emission intensity enhancement upon hybridization with their target sequence. In contrast, the TO dimer in **DNA3** does not primarily show fluorescence quenching (which would be typical for excitonically coupled dyes) but exhibits a remarkably red-shifted emission from the TO typical value of 530 nm in **DNA4** (green) to 580 nm (orange). This excimer-type emission is caused by the helical twist in the TO dimer, yielding an apparent Stokes' shift of 94 nm.[35] The antiparallel orientation of the two TO dyes in **DNA3** is crucial. We subsequently transferred this concept to RNA and showed that the interstrand TO dimer could be applied for imaging of transfected CHO cells. The yellow colored emission was distinguishable from the green TO monomer by confocal microscopy.[36]

The major disadvantage of the TO dimer in terms of selectivity is the partial overlay of excitation and fluorescence wavelengths (green and orange). Hence, we developed the concept further to generate an even larger wavelength shift. We replaced one TO dye by thiazole red (TR). The difference between DNA building blocks **11**/**12** (TR) and **9**/**10** (TO) is the longer methine bridge that shifts absorption and emission to longer wavelengths. As the absorption of TR overlaps pretty well with the emission of TO, both chromophores can be combined as an interstrand energy transfer pair in DNA (Figure 3).[37]

We combined **11** with **10** (in **DNA5** and **DNA7**) and **12** with **10** (in **DNA6** and **DNA8**) as interstrand chromophore pairs. It is important to point out that the melting temperature (T_m) of such doubly labeled DNA is reduced by less than 2 °C in comparison to natural A–T base pairs, although both dyes TO and TR are significantly larger than natural DNA bases. The sequence complementarity of the neighboring sequence is not altered. The energy transfer from TO to TR shows highest efficiencies in **DNA6** and **DNA8** with red-to-green ratios (R/G)

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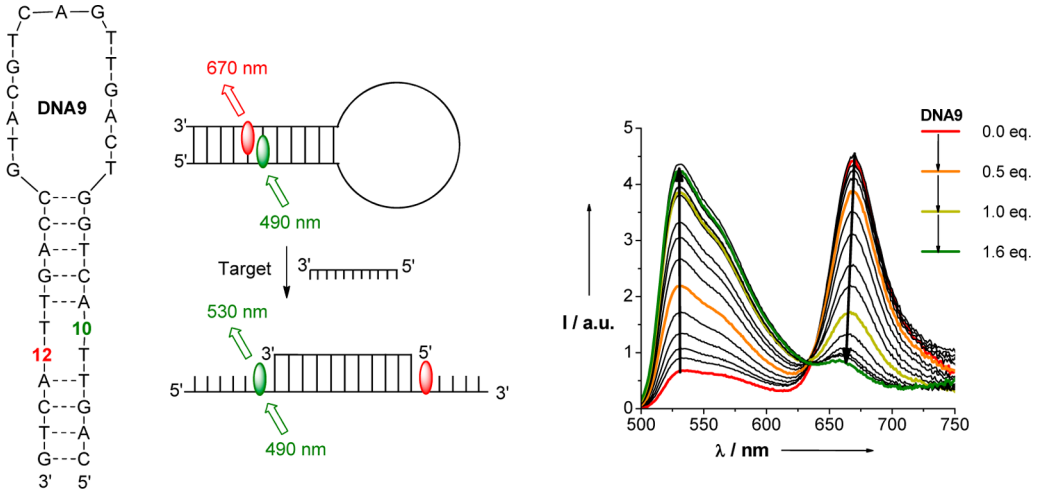

Figure 4. Sequence of **DNA9** (left). Schematic illustration of the MB (middle). Fluorescence spectra of the titration of **DNA9** with up to 1.6 equiv of target oligonucleotide, 2.5 μM DNA, 10 mM NaP_i buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 490 nm.

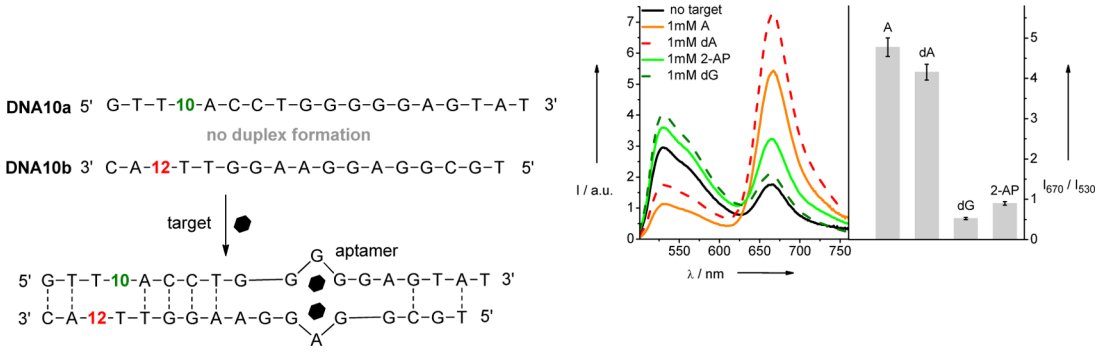

Figure 5. Sequences of **DNA1a** and **DNA10b** and schematic illustration of the aptasensor (left). Fluorescence spectra and target selectivity (right), 2.5 μM DNA, 10 mM NaP_i buffer (pH 7.0), 250 mM NaCl, 20 °C, λ_{exc} = 490 nm.

of up to 6. The linkage of the TR dye via its benzothiazole nitrogen (**12**) to the DNA backbone was crucial. **DNA6** was microinjected in CHO-K1 cells and imaged by confocal microscopy.[37] Remarkably, the R/G ratio of 6 persists even inside cells. Moreover, a systematic variation of orientations of **10** and **12** (see Figure 3) was evaluated and improved the R/G ratio to 20.[38] Alternatively, the distance dependence showed that a DNA duplex carrying one A–T pair as a spacer between the chromophores increased the R/G ratio to 16. Together, the latter results show that the TR fluorescence intensities (when excited at 490 nm = selective for **10**) vary due to different excitonic interactions that interfere with energy transfer. If ground-state TO/TR dimers were excited, they cannot undergo energy transfer, given the fact that energy requires the selective excitation of an uncoupled energy donor (TO) and the proximity of an unexcited acceptor (TR). In case of the TO/TR pair, excitonic interactions can be observed mainly by the difference in TR extinction. Obviously, the DNA double helical architecture and the distance between the fluorophores control the angle between the transition dipole moments and thereby influence the energy transfer efficiency. This effect was similarly, but more systematically, found with extended cytosine fluorophores by Wilhelmsson et al.[39] and pyrene/perylene fluorophores by Asanuma et al.[40]

■ APPLICATIONS OF "DNA/RNA TRAFFIC LIGHTS"

Conventional fluorescent tools for nucleic acid imaging such as molecular beacons (MBs) typically monitor fluorescence intensity changes.[41,42] However, imaging that relies on a single wavelength in cellular media always carries the risk of wrong readout due to undesired nonspecific opening of the MBs. Thus, many efforts have put into the design low-noise stem-less PNA MBs,[43] quencher-free MBs,[44] or MBs based on excimer fluorescence readout.[45−47] New in-stem-labeled MBs with clear fluorescence color readout were developed with **10** (TO) and **12** (TR) as an energy transfer pair (Figure 4). When the hairpin **DNA9** hybridizes with the complementary target, a gradual color change from green (530 nm) to red (670 nm) can be observed.[48] To compare with conventional, termini-labeled MBs, we used the enhancement factor f that represents the fluorescence ratio I_{530}/I_{670} of the duplex relative to that of the hairpin form.[49] **DNA9** gave an enhancement factor f of 34, whereas a conventional MB that was terminally labeled with fluorescein and rhodamine[48] revealed a factor f of 3.9, a value that is almost one magnitude lower than that of **DNA9**.

In the past years, a number of aptamer-based sensors (the so-called "aptasensors") were described with respect to different bioanalytical applications.[50−52] Our wavelength-shifting aptasensor **DNA10a/b**[38] is based on a 27-mer sequence originally reported by Patel et al.[53] and cut between nucleotides 14 and 15 to form two single strands (Figure 5). To ensure good target binding properties, **10** (TO) and **12** (TR) were placed near the left terminus embedded in a short extension sequence that builds the required DNA architecture. During stepwise addition of adenosine (A), the red TR signal rises due to

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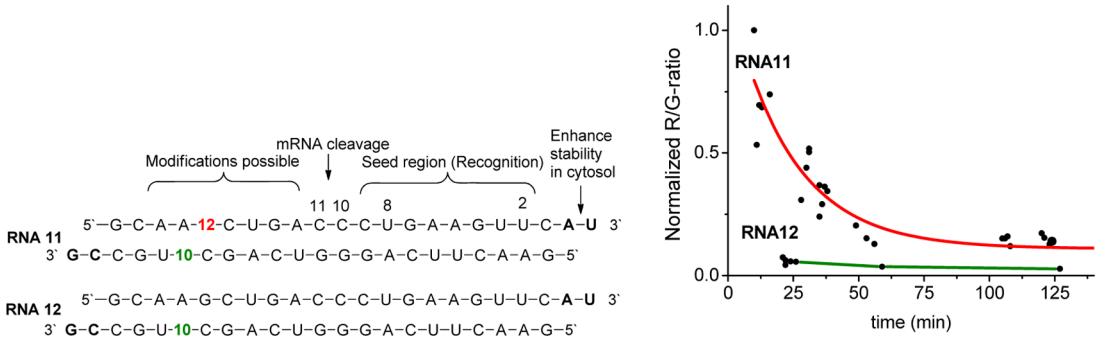


Figure 6. Sequences of **RNA11** and **RNA12** (left). R/G ratios measured in CHO-K1 cells (right).

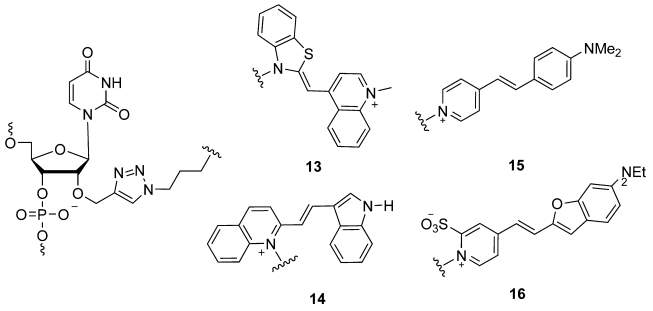
target-mediated duplex formation. The green emission in the presence of targets with high structural similarity, such as 2-aminopurine (2-AP) and deoxyguanosine (dG), revealed a high selectivity of this aptasensor.[38] Recently, we have shown that our DNA "traffic lights" can also visualize two consecutive DNA strand displacements due to a distinct fluorescence color change from green to red and back to green.[54] These results highlight the potential of our concept as a bioanalytic tool to study more complex DNA nanostructures as well as macromolecular switches in the future.

The more challenging application for the TO/TR pairs is molecular imaging. The potential of small interfering RNA (siRNA) not only as a tool to study gene functions in eukaryotic cells[55−57] but also in terms of therapeutics suffers from poor stability in biological media and insufficient delivery to target cells. To overcome these drawbacks, it is crucial to be able to track siRNA integrity during the delivery process. However, only very few approaches with a dual emission readout are described in the literature.[58−60] Conventional labeling at the 3′- and 5′-termini suffers from an inefficient energy transfer between the chromophores and a very poor dynamic range from 0 to 10% for the discrimination of processed siRNA. We used the TO/TR-modified RNA to create a novel biosensor with bright fluorescence and applicable contrast ratios (R/G = 6:1 in cell lysate).[61] The labeling region within the siRNA sequence of **RNA11** and **RNA12** was carefully chosen to preserve the gene silencing function toward the knockdown of enhanced green fluorescence protein (EGFP) in CHO-K1 cells (Figure 6). The TO/TR combination destabilizes RNA duplexes typically by only 3 °C (ΔT_m). An exceptional broad and sensitive range from 0 to 50% processed siRNA was observable. Real time experiments allowed monitoring cellular uptake and integrity in living cells by the R/G ratio. Thereby, our RNA "traffic lights" show higher sensitivity compared to several single and multicolor fluorescent mRNA sensors developed on RNA base surrogates recently.[62−66]

EXTENSION OF THE CONCEPT: 2′-"CLICK" FLUOROPHORES AND NIR

So far, TO, TR, and other dyes were incorporated into DNA by linking them to (*S*)-3-amino-1,2-propanediol.[37] However, each artificial DNA building block requires a time-consuming synthesis. On the other hand, the Huisgen−Sharpless−Meldal cycloaddition "click"-type chemistry[67−69] is well-established for oligonucleotide modification[70−72] and allows evaluating dyes very rapidly as covalent DNA and RNA labels. We focused on the postsynthetic "click"-type modification of 2′-propargylated uridine and showed that this type of modification does not affect the thermal duplex stability and that the dye-modified uridine exhibits preferential stability with adenine as a counter base.[73] The combination of TO as energy donor that was incorporated via such postsynthetic 2′-modification **13** with **12** (TR) as "conventional" DNA base substitute yielded dual emitting DNA probes with good fluorescence readout properties.[74] Moreover, the styryl dye CyIQ (cyanine indole quinoline) **14** exhibits not only bright fluorescence but also excellent photostability and hence represents an important alternative to the rather photolabile TO.[74,75]

Molecular imaging by means of so-called far-red, near-infrared (NIR) fluorophores is becoming more important based on their high sensitivity, excellent temporal and spatial resolution, and their potential for multichannel imaging. Hence, our postsynthetic labeling approach was extended to fluorophores with large apparent Stokes' shifts (>100 nm for MegaStokes dyes). The combination of the 2′-modifications **15** and **16** in two complementary strands enhanced the apparent Stokes' shifts up to 230 nm. The FRET-type process shifts the emission to the NIR range. On the other hand, upconversion nanoparticles (UCNPs) are capable of converting low-energy NIR radiation into visible light by a two-photon process. The silica shells of these UCNPs bear azide groups. Via terminal 2′-ethynyl groups, DNA can be conjugated, which makes these particle soluble in water.[76] Confocal microscopy by our lab[76] and similarly by Lu et al.[77] revealed the high potential of these bright UCNP−DNA conjugates for live cell imaging in the NIR range where they act as "nano-sized" lamps.



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Notes

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