Published in final edited form as:

Mol Biosyst. 2013 October ; 9(10): 2447–2453. doi:10.1039/c3mb70108e.

The three-dimensional context of a double helix determines fluorescence of the internucleoside-tethered pair of fluorophores

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Abstract

We report a general phenomenon of the formation of either a fluorescent, or of an entirely quenched oligodeoxynucleotide (ODN) duplex system by hybridizing pairs of complementary ODNs with identical chemical composition. The ODNs carried internucleoside tether-linked cyanines, where the cyanines were chosen to form a Förster's resonance energy transfer (FRET) donor/acceptor pair. The fluorescent and quenched ODN duplex systems differed only in that the cyanines linked to the respective ODNs were linked either closer to the 5'-, or closer to the 3'-ends of the molecule. In either case however, the dyes were separated by an identical number (7 or 8) of base pairs. Characterization by molecular modeling and energy minimization using a conformational search algorithm in a molecular operating environment (MOE) revealed that linking of the dyes closer to the 5'-ends resulted in their reciprocal orientation across the major groove which allowed a closely interacting dye pair to be formed. This overlap between the donor and acceptor dye molecules resulted in changes of absorbance spectra consistent with the formation of H-aggregates. Conversely, dyes linked closer to 3'-ends exhibited emissive FRET and formed a pair of dyes that interacted with the DNA helix only weakly. Induced CD spectra analysis suggested that interaction with the double helix was weaker than in the case of the closely interacting cyanine dye pair. Linking the dyes such that the base pair separation was 10 or 0 favored energy transfer with subsequent acceptor emission. Our results suggest that when interpreting FRET measurements from nucleic acids, the use of a "spectroscopic ruler" principle which takes into account the 3D helical context of the double helix will allow more accurate interpretation of fluorescence emission.

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[†]Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x

The authors declare that they have no conflicts of interest.

Introduction

The analysis of emissive or non-emissive FRET effects in fluorophore- or fluorophore/quencher-labeled ODNs ¹⁻³ is commonly used for detecting interactions between complementary nucleic acid sequences in vitro and in living systems. The fidelity of FRET as a readout for sequence-specific interactions has been further improved by using a dual beacon system ⁴. More recently, monitoring of FRET efficiency changes was proposed for imaging of protein-oligonucleotide binding ⁵⁻⁸. We previously reported several NF- κ B sensing DNA probes consisting of fluorescent or non-fluorescent hairpins and 3'- and 5'-phosphorothioate stabilized oligodeoxynucleotide (ODN) duplexes ^{8, 9}. We hypothesized that if two fluorophores are localized within the transcription factor (e.g. NF- κ B) binding site they can potentially form a FRET dye pair and that the resultant amino acid residues of transcription factors interacting in close proximity to the dye pair would interfere with FRET in this pair of fluorophores ^{5, 8, 10}. It is now well established that transcription factors induce a variety of DNA conformational changes, from unwinding and bending in the case of TATA-binding proteins ^{11, 12} to more subtle changes in the DNA double helix curvature as observed in the case of NF- κ B and STAT3, which involve all base pairs of the binding site ^{13, 14}. By recording the changes in FRET, one would be potentially able to track the DNA-protein interactions without the need to separate the ODN duplex and the protein from the mixture of non-binding proteins and a non-interacting ODN sensor.

We report here several initial findings proving that after fluorophores are linked to complementary strands of an ODN duplex, the mode of fluorophores interactions depends on: 1) inter-dye distance along the duplex (i.e. base pair separation); 2) dye structure, and; 3) reciprocal orientation along the duplex sequence that translates into the positioning of the dyes across either the major groove, or alternatively, the minor groove of the duplex. While the first two factors can be easily anticipated, the third factor is non-trivial, is the direct consequence of the double helical conformation of ODNs and, unlike model duplex systems that included terminally tethered fluorophores ¹⁵ has not been investigated before.

Results and Discussion

The importance of the spatial separation distance of two fluorescent dyes ¹⁶ was recognized as a “molecular ruler” main principle in investigating the interactions between the complementary pairs of oligonucleotides using FRET ¹. The contribution of non-emissive energy transfer (contact fluorescence quenching) was assumed to be low, and conversely, the efficacy of emissive FRET due to the fluorescence of the acceptor was assumed to be high if the fluorophores covalently linked to ODN duplex termini were spatially separated by an optimal number of base pairs according to Förster's description of FRET ¹⁷. This assumption has been subsequently carefully tested by using complementary ODNs of various lengths that were labeled at the 5'-end ¹⁸. The observed periodicity effect of FRET efficacy, which is related to stacking of the fluorophores on the ends of the helix with resultant orientation of transition dipole moments accounted for three-dimensional properties of a single helix in a given ODN duplex ^{18, 19}. Furthermore, the linking of sulfonated cyanines via long flexible linkers could not prevent the end-stacking ²⁰.

The experimental setting applied in this study is different from that of ^{18, 19} – instead of linking the fluorophores to the termini we covalently linked them at the internucleoside positions that were defined by the ODN sequence design. By using this strategy that does not favor fluorophore-base pair stacking we discovered that the “molecular ruler” principle is not always applicable, i.e. the base pair separation that usually results in the changes of linear inter-dye distance and non-linear (helix-defined) orientation of transition dipole moments.

We chose polymethine cyanine dyes as a sensing donor/acceptor pair for several reasons: 1) the spectral properties of these dyes, i.e. absorbance and emission in the near-infrared (NIR) result in less background signal due to the low autofluorescence of the majority of biological samples in the NIR range; 2) the fluorescence fluctuation is largely reduced when two cyanine dyes are paired together; 3) the chosen cyanine dyes include dimethylindole heterocycles that have low intrinsic DNA binding affinity (either by intercalation or groove binding) due to sterical bulk introduced by geminal methyl groups^{21, 22}; 4) the dyes are large enough to increase the probability of the protein mediated interference with the dye pair interaction, i.e. of the proteins comprising active NF-κB (e.g. p50/p65 heterodimer) and the pair of dyes covalently linked to the oligonucleotides. To ensure better solubility of the DNA duplex probe and to prevent the potential binding of the dyes to the surface of the DNA duplex we chose soluble sulfonated cyanine dyes such as Cy5.5, Cy7 and 800CW fluorophores. The quenching levels of cyanine fluorescence by nucleotides on the opposing complementary ODN is not high or different: i.e. dA quenching efficiency is 15%, dC - 15%, dG - 17% and dT - 18%²³. To have an option of changing the position of dye linking to the oligonucleotide relative to the nearby purine or pyrimidine and to freely change the distance between the points of linking of the dyes along the DNA sequence by design, we used a strategy of internucleoside hydrophilic aminoethyl-di(ethylene glycol) linkers. These CH₂CH₂OCH₂CH₂OCH₂CH₂NH linkers are 9-atom long and they can be easily introduced in the oligonucleotide sequence during the ODN synthesis using novel nucleoside synthons (see Supplemental Fig. 1S). The linker's amino group was used for the linking of fluorophores which extended the dye's own linkers thereby providing additional separation from the ODN duplex. All above factors could enable free diffusion of the dyes in the sterically allowed space²⁴. Using hydroxysuccinimide esters of cyanines we achieved high yields of linking to hydrophilic aminoethyl-di(ethylene glycol) residues²⁵ and cyanine-ODN conjugates were purified using ion-pairing HPLC. A set of about 40 oligonucleotides with various donor-acceptor pairs was synthesized to study the spectral effects of the inter-dye distance and reciprocal dye orientation along the duplex sequence. The ODNs in this study contained consensus NF-κB and STAT3 binding sequences as well as control sequences that lacked the consensus sequence.

The synthesized oligonucleotides formed duplexes upon hybridization and as a result, the donor and acceptor fluorophores that were linked to complementary ODNs were separated by 0-13 base pairs. We have studied spectroscopic properties of the obtained duplexes in view of the observation made previously²³ suggesting that strong changes of absorbance spectra of fluorophores in the visible range will result when the energy transfer between the fluorophores is mediated via a contact mode of quenching but not FRET. The studied ODN duplexes can be divided into three groups (see Supplementary Tables S1-S3) based on comparing the spectra of ODN duplexes carrying one cyanine and the duplexes to which a donor/acceptor pair of cyanine dyes was linked. We identified duplexes with linked dyes that are engaged in the contact mode of quenching (Table S1), in emissive FRET (Table S2) and lastly, duplexes in which the cyanine dye interactions result in an intermediate mode of fluorescence (Table S3). A superimposition of the two individual absorbance spectra of donor and acceptor fluorophores was observed only in the case of emissive FRET. If the donor and acceptor fluorophores were separated by at least 10 nucleotide base pairs (b.p.), these fluorophores were capable of efficient FRET as determined by unchanged absorbance spectrum and by measuring the relative changes of donor and acceptor fluorescence intensities (duplex 1, Table 1). However, if the separation distance was decreased to 7-8 b.p. and each one of the two dyes was closer to the 5'-end of the corresponding ODN than to the 3'-end, the duplexes were no longer fluorescent (Table 1, duplexes 2,3 and Table S1). Instead, fluorescence intensities of the donor and acceptor were dramatically decreased independently of the ODN nucleotide sequence. The same quenching effect was observed when the donor- and acceptor-linked ODNs were switched around (compare duplexes 2 and

3, Table 1). Unlike the absorbance spectra resulting from the FRET duplex, the spectra of “quenched” duplexes did not resemble the superimposed spectra of the two individual dyes. The observed FRET efficacy in this case was close to zero and cyanine fluorescence was quenched. In particular, in the case of the dye pair Cy5.5/800CW, such quenching was very efficient, and associated with profound changes in absorbance spectra of the dyes. A new absorbance peak with a maximum of 645 nm was present suggesting a potential static close quenching due to formation of a fluorophore dimer.

In contrast, the duplexes having the same separation distance of 7-8 nucleotide b.p. between the dyes as above, but having the opposite reciprocal orientation along the ODN duplex (Table 1, duplex 4 and Table S2), showed an increase of acceptor fluorescence intensity that closely resembled the increase resulting from FRET between the dyes separated by 10 base pairs. No absorbance peak shifts in visible or near infrared ranges of absorbance spectrum were observed in such duplexes.

To eliminate the potential influence of ODN sequence variations and to verify the observed correlation between the orientation of dyes and spectral properties, we synthesized an ODN with the linker positioned approximately in the middle of the ODN sequence and hybridized it with complementary ODNs carrying internucleoside linkers positioned either 8 b.p. downstream, or 8 b.p. upstream. In these duplexes with identical sequences in directionally opposite locations of the acceptor dye in antiparallel strands we observed the same spectroscopic effects, i.e. duplex A was non-fluorescent with a strong change in absorbance spectrum (Fig. 1, trace a), while duplex B showed no shifts in absorbance of cyanines and efficient FRET between Cy5.5 and 800CW dyes (Fig. 1, trace b). The fluorescence spectral data obtained using the dyes linked to duplexes A and B is shown in Fig. 2. We observed a clear-cut effect associated with emissive FRET in the case of Cy5.5 donor dye that showed the decrease of fluorescence intensity of the peak in the case of duplex B (emissive FRET) and a further decrease of intensity of the peak in duplex A (non-emissive FRET, due to interactions of the donor and acceptor dyes and quenching). The observed effects were paralleled by a decrease of Cy5.5 fluorescence lifetime (FL) from the average 1.24 ns to a statistically significant shorter values in emissive, and non-emissive duplexes (0.94 and 0.84 ns, respectively), Fig. 2A. As expected, the fluorescence spectra corresponding to the acceptor dye 800CW excited at characteristic 778 nm demonstrated a sharp decrease of fluorescence intensity only in the case of a non-emissive dye pair within duplex A with the corresponding decrease in FL from the average of 0.75 ns to 0.64 ns (Fig. 2B). These results suggest that though the pair of Cy5.5-800CW dyes in duplex A was located close to the less kinetically stable 3'-5' end of the duplex (resulting in a higher rotational freedom of each dye and less stable inter-dye interactions), the fluorescence spectra still reflected the prevalence of inter-dye interactions in the non-emissive FRET orientation. Furthermore, the importance of the three-dimensional double helix conformation can be illustrated by paradoxically efficient FRET in the case where the dyes are positioned across from each other in the duplex (zero base pair separation) (see duplexes Table 1, duplex 5). If both the donor and acceptor fluorophores were positioned on the complementary ODN strands across from each other with zero b.p. separation along the sequence, we usually observed emissive FRET and no change in the absorbance spectra (Table 1, duplex 5).

To provide a possible explanation of the observed effects we performed simulations in a molecular operating environment (MOE) to obtain realistic models of covalently linked cyanine donor/acceptor dye pairs. We utilized minimizations of the potential energy of the two-dye system via conformational searches that produce a discrete sampling of the space confined to local minima of the potential energy function. We performed LowModeMD searches that are concentrated exclusively on realistic low energy states and transitions between these states. These searches assume that the relevant molecular conformations of

the dyes are connected by low-frequency vibrational mode transitions. After 1000 iterations that included energy minimization steps (100 iterations per each conformational search iteration) we obtained 3-D models with values of the potential energy of the conformations that were below 4000 units compared to the initial hydrated cyanines (n=6 layers of water molecules in the hydration layers). Our strategy was to add hydration to cyanine dyes linked to the ODN duplex after pulling the dyes together to a distance that was closer than realistic for the energy state of the system (i.e. resultant non-minimized potential energy) and then performing LowModeMD in MMFF forcefield refinement of the system to the nearest local energy minimum. The same forcefield was used to define the partial charges of the atoms. The obtained results were in agreement with the actual fluorescent spectroscopy done in duplexes differing in the reciprocal orientation of the dye linking along the ODNs (Fig. 2). Duplex 2 (Table 1) had the Cy5.5/800CW dye pair interacting closely with strong overlap (Fig. 3A,B) suggesting the formation of an H-aggregate as implied by the absorbance spectrum. In the case of Cy5.5/Cy7 dyes, the obtained model (Fig. 3C) suggested a much smaller overlap with the near-perpendicular reciprocal orientations of dyes suggesting lower probability of close inter-dye interactions. This dye orientation also suggests near-perpendicular orientation of transition dipole moments of the cyanine donor and acceptor at the equilibrium that results in low values of orientation κ^2 factor ²⁶ corresponding to near-zero FRET efficiency ²⁷ (Fig. 3C). The dependence of κ^2 from the distance between cyanine donor/acceptor pair of fluorophores linked to DNA duplexes has been demonstrated previously in an experiment ¹⁵ proving that the orientation of transition dipole moments changes as the distance between the dyes changes in various donor-acceptor configurations affecting κ^2 values. Since the directions of transition dipole moments of cyanines are known ²⁷, MOE simulations of dye orientations performed with conformational search provide excellent prediction of emitting or non-emitting FRET scenarios.

Finally, in all cases where the dyes were facing each other across the minor groove of the ODN duplex, the minimizations with conformational searches indicated that the dyes were separated at a distance favoring efficient FRET (Fig. 3D). Therefore, the MOE simulations suggested that dye separation by the minor groove does not support the formation of a quenched dimer. It should be noted that we studied oligonucleotide duplexes with different primary structures (containing consensus NF- κ B, STAT3, control sequences) as well as various locations of the linkers after all four nucleotides in the oligonucleotide chain (dT, dA, dG or dC, see Tables S1-S3). The obtained results showed that the chemistry of nearby base pairs surrounding the linked dyes had negligible effect on FRET efficacy indicating that the dyes were separated from the double helix. To investigate, whether the latter assumption was correct, we performed circular dichroism (CD) spectra acquisitions of the duplexes in the near-infrared range (650-750 nm) ²⁸. All fluorophores studied in this work were achiral and no CD signal should have been observed in the absence of interaction between the dyes with chiral DNA helix that induces circular dichroism in the near-infrared range of spectrum. As expected, we observed weak but very distinct induced CD chiroptical signatures for Cy5.5-800CW pair of fluorophores in emissive orientation vs. close contact (quenched) orientation (Fig. 4, traces a and b) with a much weaker positive Cotton effects at 680 nm and an absent positive band at 670 nm in the case of the close contacting dyes. The CD spectrum for a single Cy5.5-labeled duplex was similar to that of emissive FRET pair (Fig 4, trace c) and contained a dual-band positive signature. It should be noted that each one of the dyes in these ODNs were surrounded by an identical adjacent GC pair in 5'- and 3'- positions and the observed Cotton effects were similar to previously reported for other cyanine monomers interacting with dG-dC dimers ²⁸. These results support the notion that in the case of a major-groove oriented pair of cyanines (quenched pair), the interaction of cyanines with the chirally active DNA is much less probable.

Importantly, the chemistry of dyes (as some polysulfonated cyanine dyes are resistant to static quenching) and close proximity of the dyes to either the 3'- or 5'- end of the ODN duplex (e.g. duplexes 4 and 8, Table S1) both had clear-cut effects on FRET. The latter could be due to a lower kinetic stability of the ODN duplex termini base pairing than that which occurs in the center of the duplex. Both factors will require a more detailed investigation in the future.

Conclusions

In conclusion, we demonstrated that if the donor and acceptor cyanine fluorophores are linked to different complementary ODNs at internucleoside positions, the FRET effect in the resultant duplex is not only influenced by base pair separation, but is also a function of reciprocal position of the dyes alongside the duplex. We also demonstrated that the understanding of the ability of a given dye pair to interact in the context of double helix 3D geometry enables one to design either predominantly quenched, or non-quenched FRET probes by using identical ODN sequences and the same dyes. This observation is important considering that the use of the internucleoside linker technology has ample potential in ODN-based sensor design. It has a great potential for facile introduction of multiple functionalities into the duplex probes in addition to the traditional modification of oligonucleotide termini.

Experimental

Oligodeoxyribonucleotides and dye conjugation

Four nucleoside phosphoramidite synthons (Figure 1S) developed for introducing internucleoside linkers in ODNs were synthesized following a previously published approach²⁵. The 5'-O-(4, 4'-dimethoxytrityl)-terminated ODNs were cleaved from support and deprotected using ammonia, precipitated, and purified on C18-HPLC column. The conjugation of N-hydroxysuccinimide (NHS) esters of Cy5.5, Cy7 (GE Healthcare, Piscataway NJ) or IRDye 800CW (Li-COR, Lincoln NE) to ODNs bearing internucleoside amino linkers was performed using a method previously described in ⁵. Cyanine dye Cy5.5 NHS ester was used for modification of internucleoside hydrophilic amino linker to obtain a "donor" ODN, whereas 800CW and Cy7 NHS esters were used to synthesize the "acceptor" ODNs. The dye-conjugated ODNs were passed through Bio-Spin P6 columns (Bio-Rad) followed by a purification using a gradient of acetonitrile in 0.1 M TEAA, pH 7.0 on C18-HPLC columns. The purified dye-conjugated ODNs were precipitated from ethanol/sodium acetate and characterized by using electrospray mass-spectrometry, e.g. 5'-**AGCCTG***(Cy5.5)**GAAAGTCCCACATCG**-3' m/z calc 7525.3, found 7527; 5'-**CGATG***(800CW)**TGGGACTTTCCAGGCT**-3' m/z calc 7633.2, found 7634 (*-denotes the amino linker, phosphorothioate bonded nucleosides are shown in boldface). The absorbance and fluorescence spectra measurements were performed on Cary 50 and Cary Eclipse fluorescence spectrophotometers (Varian), respectively. ODN duplexes were prepared by combining equimolar amounts of the corresponding ODNs dissolved in 25 mM Hepes, 1 mM MgCl₂, 50 mM NaCl, pH 7.4, heating at 95°C for 5 min and cooling at room temperature. The formation of ODN duplexes was confirmed by performing melting point determinations using UV spectrophotometer. CD spectra of oligonucleotides were acquired on CS/2 Chirscan (Applied Photophysics) at 2 μM ODN duplex in 10 mM sodium phosphate, 50 mM NaCl, 0.1 mM EDTA, pH 7.0.

Molecular modeling was performed using Molecular Operating Environment v 2.0 software (MOE2010, Chemical Computing Group Inc., Montreal CA) that was run on 2×2.4GHz Intel 4-Core Xeon MacPro (Apple Inc). To generate energetically reasonable 3D atomic configurations with geometric constraints and fixed ODN chain atoms, we performed

conformational searches using LowModeMD Search, i.e. a series of molecular dynamics simulations using velocities with little kinetic energy on the high-frequency vibrational modes²⁹. We freed the mobility of cyanine dyes and fixed the potentials of the duplex oligonucleotides in simulated solvent (water, n=6 layer) and counter ions (NaCl). One thousand conformations of hydrated cyanine dyes were tested with potential energy minimization iterations in Cartesian coordinates per each molecular dynamic simulation. The algorithm was terminated if the total number of iterations exceeded the Iteration limit parameter set at 100 or RMS gradient falling below the 0.05 test value.

Fluorescence lifetime measurements

A femtosecond Ti:Sapphire laser (Mai Tai, Spectra Physics) operating at 750 nm was used as an excitaiton source¹⁰. The time resolved emission was detected using a CCD camera (LaVision GmbH, Germany) coupled to a gated-image-intensifier (LaVision GmbH). Excitation of Cy5.5 dye was performed by filtering (650nm-40nm bandwidth filter, Chroma) the broadband output (500nm-700nm) obtained from a secondary, super-continuum (SC) source system based on photonic crystal fiber technology (NL-PM-750, Thorlabs). The fluorescence was detected using either a 716-40 bandpass filter (for Cy5.5) or 800 nm longpass filter (for 800CW). The measured fluorecence decay curves were analyzed using multi-exponential fitting algorithms based on non-linear least squares to obtain the lifetimes. If a single lifetime is present in the measured spot, the slope of the decay in the log scale directly provides the lifetime. The nonlinear fits were performed, using the MATLAB (Mathworks) function, “fminsearch,” which employs an unconstrained nonlinear optimization using the Nelder–Mead simplex approach. Three separate acquisitions form spatially separated regions in the samples were performed.

Statistical analysis

All experiments were performed in triplicate. Values were expressed as the mean $\pm$ the standard deviation (SD) and compared using double-tailed unpaired *t* test with Welch's correction (Prism 4.0, GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the National Cancer Institute IMAT grant R33CA134385 and R01AG026240 to A.B. and R01EB015325 to A.T.N.K. The authors are grateful to Dr. Jeff Bacon (Department of Chemistry, Boston University) for his help with CD spectra acquisitions.

References

1. Cardullo RA, Agrawal S, Flores C, Zamecnik PC, Wolf DE. Proc Natl Acad Sci U S A. 1988; 85:8790–8794. [PubMed: 3194390]
2. Tyagi S, Kramer FR. Nat Biotechnol. 1996; 14:303–308. [PubMed: 9630890]
3. Sokol DL, Zhang X, Lu P, Gewirtz AM. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:11538–11543. [PubMed: 9751701]
4. Santangelo PJ, Nix B, Tsourkas A, Bao G. Nucleic Acids Res. 2004; 32:e57. [PubMed: 15084672]
5. Zhang S, Metelev V, Tabatadze D, Zamecnik PC, Bogdanov A Jr. Proc Natl Acad Sci U S A. 2008; 105:4156–4161. [PubMed: 18337505]
6. Zhang S, Metelev V, Tabatadze D, Zamecnik P, Bogdanov A Jr. Oligonucleotides. 2008; 18:235–243. [PubMed: 18699740]

7. Zhao W, Schafer S, Choi J, Yamanaka YJ, Lombardi ML, Bose S, Carlson AL, Phillips JA, Teo W, Droujinine IA, Cui CH, Jain RK, Lammerding J, Love JC, Lin CP, Sarkar D, Karnik R, Karp JM. *Nat Nanotechnol.* 2011; 6:524–531. [PubMed: 21765401]
8. Metelev V, Zhang S, Tabatadze D, Bogdanov A. *Bioconjug Chem.* 2011; 22:759–765. [PubMed: 21417216]
9. Metelev V, Weissleder R, Bogdanov A Jr. *Bioconjugate Chemistry.* 2004; 15:1481–1487. [PubMed: 15546218]
10. Bogdanov A Jr, Metelev V, Zhang S, Kumar AT. *Mol Biosyst.* 2012; 8:2166–2173. [PubMed: 22710322]
11. Burley SK. *Philos Trans R Soc Lond B Biol Sci.* 1996; 351:483–489. [PubMed: 8735270]
12. Nikolov DB, Burley SK. *Nat Struct Biol.* 1994; 1:621–637. [PubMed: 7634102]
13. Phelps CB, Sengchanthalangsy LL, Malek S, Ghosh G. *J Biol Chem.* 2000; 275:24392–24399. [PubMed: 10825175]
14. Ghosh G, van Duyne G, Ghosh S, Sigler PB. *Nature.* 1995; 373:303–310. [PubMed: 7530332]
15. Ouellet J, Schorr S, Iqbal A, Wilson TJ, Lilley DM. *Biophys J.* 2011; 101:1148–1154. [PubMed: 21889452]
16. Stryer L, Haugland RP. *Proc Natl Acad Sci U S A.* 1967; 58:719–726. [PubMed: 5233469]
17. Lakowicz, J. *Principles of fluorescence spectroscopy.* 2nd. Plenum; New York, NY: 1999.
18. Iqbal A, Arslan S, Okumus B, Wilson TJ, Giraud G, Norman DG, Ha T, Lilley DM. *Proc Natl Acad Sci U S A.* 2008; 105:11176–11181. [PubMed: 18676615]
19. Iqbal A, Wang L, Thompson KC, Lilley DM, Norman DG. *Biochemistry.* 2008; 47:7857–7862. [PubMed: 18597488]
20. Urnavicius L, McPhee SA, Lilley DM, Norman DG. *Biophys J.* 2012; 102:561–568. [PubMed: 22325279]
21. Armitage B. *Topics Curr Chem.* 2005; 253:55–76.
22. Di Fiori N, Meller A. *Biophys J.* 2010; 98:2265–2272. [PubMed: 20483335]
23. Marras S, Kramer F, Tyagi S. *Nucleic Acids Res.* 2002; 30:122–130.
24. Sindbert S, Kalinin S, Nguyen H, Kienzler A, Clima L, Bannwarth W, Appel B, Muller S, Seidel CA. *J Am Chem Soc.* 2011; 133:2463–2480. [PubMed: 21291253]
25. Tabatadze D, Zamecnik P, Yanachkov I, Wright G, Pierson K, Zhang S, Bogdanov A Jr, Metelev V. *Nucleosides Nucleotides Nucl Acids.* 2008; 27:157–172.
26. Knox RS. *J Biomed Opt.* 2012; 17:011003. [PubMed: 22352637]
27. Munoz-Losa A, Curutchet C, Krueger BP, Hartsell LR, Mennucci B. *Biophys J.* 2009; 96:4779–4788. [PubMed: 19527638]
28. Choi JK, D'Urso A, Trauernicht M, Shabbir-Hussain M, Holmes AE, Balaz M. *Int J Mol Sci.* 2011; 12:8052–8062. [PubMed: 22174649]
29. Labute P. *J Chem Inf Model.* 2010; 50:792–800. [PubMed: 20429574]

Fig. 1. Absorbance spectra dependence on the orientation of cyanine dyes along the ODN duplex: a) a blue-shift of Cy5.5 absorbance peak in duplex A suggesting the formation of H-aggregate; b) - a superimposition of spectra **c** and **d** with no spectral shifts in duplex B. Spectrum **c** corresponds to the absorbance of ODN duplex linked Cy5.5, and spectrum **d** corresponds to a single 800CW dye labelled duplex. The positions of the cyanine dyes are colour-coded and the structures of Cy5.5 (donor) and 800CW (acceptor) are shown below.

Fig. 2. Fluorescent properties of Cy5.5 donor and IRDye 800CW acceptor dyes linked to ODN duplexes A and B (sequence shown in Fig 1). **A**-fluorescence of NIR dyes ($\lambda_{\text{exc}} = 675\text{nm}$) red- single Cy5.5 duplex A ; blue- Cy5.5/800CW-duplex B (emissive FRET); purple – Cy5.5/800CW-duplex A; dashed lines – single dye labelled IRDye 800CW-labeled duplexes A and B; **B** – fluorescence of IRDye 800CW - duplex A (single dye, dashed purple); Cy5.5/800CW-duplexB (blue); IRDye 800CW-duplex B (dashed blue) and Cy5.5/800CW-duplex A, purple. Fluorescence lifetimes of Cy5.5 dye (**A**) and IRDye 800CW (**B**) are shown above the corresponding spectra (in ns, presented as mean $\pm$ SD).

Fig. 3. ODN duplex-linked cyanine dye interactions obtained using MOE. A - the results of cyanines energy minimization in Cy5.5-800CW duplex (major groove orientation with the cyanine dyes separated by 8 b.p.); B - 90° view of (A) for visualization of the extent of dye overlap; C- Cy7 and 800CW dyes overlap; D- a lack of Cy5.5 and 800CW overlap over the minor groove. Van der Waals surfaces (white dotted) and hydration sphere (n=6 layer, blue) are shown in (A).

Fig. 4. Induced CD spectra acquired in the near-infrared range of wavelengths showing similar CD signatures of Cy5.5 in a radiative FRET duplex 4 (Table 1) and a single-dye Cy5.5 duplex 4 (trace c). Non-fluorescent duplex 2 (trace b) showed a distinct Cotton effect at 670 nm.

Table 1

Fluorescent properties of ODN duplexes carrying a pair of donor/acceptor cyanine fluorophores.

N	Duplex a)	Inter-linker distance in b.p., nucleotides	Absorbance spectra changes b)	Cy5.5 donor fluorescence intensity change, % c)	800CW acceptor fluorescence intensity change, % d)	Dye orientation
1	5'AGCCTG C TCGAGTCACATCG3' 3'TCGGAACGTCAGT C GTAGCS'	10 T,T	-	-72.1	+ 107.8	-
2	5'AGCCTG C AAAGTCC-CACATCG3' 3'TCGGAC-CTTTCAGG C GTAGCS'	8 G,G		-87.3±0.9	-72.8±1.7	Across major groove
3	5'AGCCTG C AAAGTCC-CACATCG3' 3'TCGGAC-CTTTCAGG C GTAGCS'	8 G,G		-83.4±3.4	-69.8±1.5	Across major groove
4	5'AGCCTG C AAAGTCC C CACATCG3' 3'TCGGAC C CTTTCAGG-GGTAGCS'	8 C,C	-	-66.1±5.0	+ 119.0±4.0	Across minor groove
5	5'AGCCTGGAA C TTTCCACATCG3' 3'TCGGAACCT C AAAGGGGTAGCS'	0 A,A	-	-88.8	+ 113.9	Directly opposing each other

a) **C**= CH₂CH₂OCH₂CH₂OCH₂CH₂NH-Cy5.5, **C**= CH₂CH₂OCH₂CH₂OCH₂CH₂NH-800CW;

b) : new peak at 645 nm, with the concomitant decrease of absorbance at 675 nm;

c) Fluorescence intensity change at 700 nm (excited at 675 nm) (%);

d) Fluorescence intensity change at 800 nm (excited at 675 nm) (%). The results are shown as means or mean±SD (n=2-3). Phosphorothioate bonded nucleosides are indicated in boldface.