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Sensing of transcription factor binding via cyanine dye pair fluorescence lifetime changes

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Abstract

We designed and synthesized sensors for imaging transcription factor-DNA interactions using a complementary pair of 21-base pair long oligonucleotides (ODN) carrying two internucleoside phosphate-linked cyanine fluorophores that can either engage in Förster's resonance energy transfer (FRET) with fluorescence emission or assemble into a ground state quenched dimer with short fluorescence lifetimes (FL). Cyanine fluorophores were linked to ODNs within the NF- κ B binding site. These sensors were tested in the presence of recombinant p50 and p65 NF- κ B proteins or constitutively NF- κ B activating HeLa cell lysates. By using a coherent light excitation source we followed changes in fluorescence lifetime of the donor (Cy5.5) at the donor's excitation and emission light wavelengths, as well as the acceptor (800CW or Cy7 cyanine fluorophores) in FRET mode. We observed increases of the donor lifetime in both emitting (0.08–0.15 ns) and non-emitting quenched (0.21 ns) sensors in response to protein binding. The measurements of lifetimes in FRET mode in quenched pair-carrying ODN duplex sensors showed significant differences in FL of the acceptor cyanine fluorophore between NF- κ B-containing and NF- κ B-free samples but not in control sensors with ODN sequences that have decreased binding affinity to NF- κ B. We anticipate that the observed effects will be instrumental for developing sensors enabling non-invasive imaging in cells that undergo activation of NF- κ B.

Introduction

Recent progress in chemical modification of oligonucleotides (ODN) with fluorophores¹ has enabled precise positioning of fluorophores along the ODN sequence between any number of nucleosides without interfering with base pairing^{2–4}. Using this strategy, we have assembled duplexes carrying either closely interacting, or non-interacting and completely separated, fluorescent dyes located at various distances from each other along the duplex. It is known that ground state quenched complex formation between two fluorophores⁵ results in very short lifetimes of the excited state^{6, 7}. Consequently, a transition of fluorophore from non-fluorescent, ground state complex with another dye (the molecular exciton⁸) to emitting state should allow Förster's resonance energy transfer (FRET) (i.e. a transfer of excited state energy to another fluorophore) and should result in a measureable increase of fluorescence lifetime. The quenching efficiency and, consequently, the lifetime, depends strongly on the

separation between the two dyes, i.e. the effective distance between the non-perpendicular molecular dipolar moments at thermal equilibrium. Most of the fluorophores covalently linked to macromolecules in close proximity to each other engage in non-covalent interaction with the formation of ground-state quenched dimers (also known as H-aggregates) or multimeric aggregates^{9, 10}. For example, after linking to 3' and 5' termini of a hairpin-forming oligonucleotide we previously observed blue-shifted H-aggregate formation in the case of both Cy3 and Cy5.5¹¹. We anticipated that even subtle conformational changes could potentially be amplified if detected by FRET sensing of dye pair separation altered by duplex-interacting polypeptides or by binding of a dye to the interacting protein instead of binding to another dye. To test this assumption we synthesized novel ODNs bearing covalently linked near-infrared fluorophore pairs positioned at various distances from each other along the ODN sequence.

We hypothesized that the fluorescence lifetime measurements could be used for “sensing” and monitoring the binding of proteins to the motifs encoded in ODN duplex sequence (referred below as duplex sensor). It is well known that the interactions of DNA-binding polypeptide chains include multi-point association of amino acid residues with the oligonucleotide duplex resulting in a variety of conformational changes in the vicinity of the binding site^{12, 13}. Depending on the DNA binding protein specificity (e.g. minor groove-binding zinc finger domains or fast acting major groove binding transcription factors) these changes can range from strand separation to a relatively minor duplex bending. Major groove binding transcription factors (e.g. NF- κ B and STAT proteins) bind to DNA elements with high affinity¹⁴ and play important roles in cytokine-mediated signal transduction involved in pathogenesis of major diseases. Heterodimeric nuclear transcription factor kappa B (p50-p65) recognizes B elements with a consensus DNA sequence of 5'-GGGRNYYYCC-3' (R for purine, Y for pyrimidine, and N for any nucleotide). Most STAT family members bind to DNA target sites as dimers¹⁵. There is no universal agreement regarding the STAT3 consensus sequence, however, it appears that 5'-CCTTCNGGAATT-3' describes it most closely¹⁶. There is structural and functional similarity between the STAT and Rel/NF- κ B family of transcription factors, i.e. obligatory dimerization although there is no amino acid sequence homology between the members of these protein families. Both STAT and Rel family members form contacts with double helices using loops protruding from a β -barrel domain. Four loops per STAT monomer vs. ten NF- κ B loops (5 loops per monomer) are involved in DNA binding and there are less direct base contacts in the case of STAT3. As a result, the overall difference in dissociation constants of NF- κ B and STAT3 complexes with B-DNA is several orders of magnitude with the dissociation constant for NF- κ B being in the high picomolar range, whereas STAT3 constant is in the nanomolar range¹⁵. However, in both cases the affinity of major groove binding dimerized transcription factors is high enough for pursuing the direct detection using duplex sensors.

Consequently, the goal of the current study was to investigate interactions of a model transcription factor (NF κ B) with near-infrared quenched DNA duplex sensors by measuring changes in NIR sensor fluorescence lifetime using time domain approach (FL-TD). This approach can be combined with multiexponential fluorescence decay analysis for resolving discrete fluorescence lifetimes (FL) that can be present in a complex mixture of fluorescent dye-linked sensors^{17, 18}, fluorescent dye-linked sensors^{17, 18}.

Results and Discussion

Recent years have witnessed rapid progress in molecular imaging of living systems including the development of specific imaging probes and cross-sectional optical imaging (reviewed in¹⁹⁻²¹). The development of imaging sensors designed for imaging of molecular

processes at the intracellular level usually requires a “knock-in” of special genetic reporters^{22, 23}. The transformation of traditional biochemical assays that do not require such reporters into *in vivo* applicable methods still represents a serious challenge. For example, biochemical detection of nucleic acid-protein binding can be performed in complex mixtures but requires separating the components for positive identification of the formation of the protein-nucleic acid complex²⁴. With the exception of surface plasmon resonance^{25, 26}, the identification is conditional to successful labeling of nucleic acids or labeling of antibodies that interact with the nucleic-acid bound proteins. The first labeling strategy is suitable for the screening of the unknown nucleic-acid binding proteins, the second one requires *a priori* knowledge of the binding complex composition and depends on the availability of specific antibodies for positive identification of these proteins. The detection of protein/nucleic acid interactions under native conditions and/or using homogenous assays would require sensors that change their properties upon binding of the protein. These changes are generally limited to subtle conformational transitions. Therefore, the detection of binding based on protein-induced changes in nucleic-acid conformation is a complex task requiring carefully designed sensors. Among all potential strategies, the development of fluorescent labelled duplex sensors appears most promising. There are several known reports utilizing aptamers²⁷ or triplex-forming oligonucleotides²⁸ that are capable of undergoing transitions usually resulting in either a decrease, or an increase of FRET between the pair of fluorescent dyes linked to the sensor. Linking of fluorophores to ODNs for the purpose of synthesizing molecular beacons is usually limited to the modifications of ODN 3'- and 5'- ends⁵, or covalent linking of the dyes to the nucleic acid bases, which in turn, could potentially interfere with base pairing. We circumvented this potential limitation by using internucleoside linkers that allowed conjugating amine-reactive dyes between any two nucleotide bases without interfering with the base pairing. We synthesized complementary oligonucleotides with hydrophilic internucleoside amino linkers that were introduced during the synthesis using two phosphoramidite synthons¹ (see Fig. 1a). The synthesis resulted in deprotected ODNs carrying a free amino group positioned between dG and dC and any other nucleoside that underwent further covalent modification. The modification yielded ODN duplexes carrying either donor (Cy5.5) or acceptor (800CW or Cy7 Fig. 1b) cyanine dyes. The synthesized ODNs formed duplexes with complementary ODNs carrying either acceptor or donor dyes linked at precise positions shown in Fig. 1c. The duplex with a “specific” NF- κ B binding site contained a binding sequence derived from Ig κ B -box or from IFN β - κ B binding site, with both sites exhibiting identical DNA duplex-binding affinity²⁹. The control duplex sensors (D4 and D9, see Supplementary Table 1S) contained “non-specific” sequences that did not include NF- κ B consensus sequence. During sensor optimization we found that by using two internucleoside amino linkers within complementary ODNs we could obtain various duplexes with identical chemical composition and either different, or identical base pair separation lengths between the donor and acceptor fluorophores (Table 1). These chemically identical or nearly identical duplexes (e.g. D1, D2, D3 Table 1) could have completely different optical properties. If the donor and acceptor were separated by at least 10 nucleotide base pairs, the pair of donor/acceptor cyanine dyes was capable of efficient FRET as determined by measuring the relative changes of donor and acceptor fluorescence intensities (Table 1, duplex D1). However, if the separation distance was decreased to 7-8 base pairs (bp), and each one of the two dyes was closer to the 5'-end of the corresponding ODN than to the 3'-end (as shown in Table 1) the duplexes were no longer fluorescent (Table 1, duplex D2). Instead, fluorescence intensities of the donor and acceptor linked to D2 were dramatically decreased (Fig. 2a). Similar fluorescence quenching effect was observed when the donor- and acceptor dye linked ODNs were switched around (duplex D2A, Table 1). Unlike the absorbance spectra of a fluorescent duplex D3 (Fig. 2d), the spectra of quenched duplexes did not resemble the superimposed spectra of the two individual dye-linked ODNs (see Fig 2b). In contrast, a new absorbance peak with a maximum of 645 nm was present (Fig. 2b), suggesting the

potential static close quenching due to formation of a fluorophore dimer. Fluorescence intensities of both donor and acceptor dyes in such duplexes was decreased regardless of whether they were excited in the regular or FRET modes (Fig. 2a). At the same separation distance of 7-8 bp, but in the opposite reciprocal orientation along the duplex (Table 1, D3), we observed the acceptor fluorescence in emissive FRET mode (Fig. 2c) similar to that of the sensor with 10 bp separation of fluorophores (Table 1, D1).

The models of duplexes with two covalently linked cyanine dyes obtained using Molecular Operating Environment (MOE) suggested that due to the double helical conformation of the fluorophore-labeled duplex, a pair of covalently linked fluorophores separated by 7-8 bp on antiparallel DNA strands will be positioned across from each other in the major groove of the duplex only if each one of the fluorophores is linked closer to the 5'-end of ODN than the to the 3'-end (Fig. 3). In this configuration the fluorophores can form dimers in close proximity to each other in a non-parallel orientation (Fig. 3a). The analysis of the available X-ray crystallography data for NF- κ B/DNA complexes revealed that amino acid residues within DNA-binding polypeptide chains of both p50 and p65 NF- κ B proteins are localized within the major groove of duplex and can potentially perturb the formation of the fluorophore dimers (Fig. 3b). We further performed dual wavelength imaging of EMSA gels to verify that both single dye- and double fluorophore-labelled duplexes are capable of binding NF- κ B proteins (Fig. 4a). Upon the addition of a mixture of recombinant NF- κ B proteins p50 and p65 a characteristic shift was observed indicating the formation of a complex with the duplex sensors (lanes 1-4). Similar shifts were observed if HeLa nuclear lysates were used instead of p50/p65 mixture. Due to fluorescence quenching, the formation of the complex was mostly obscured (lanes 5-8) and had to be verified by using fluorescence emitting duplexes (lanes 9-12). HeLa nuclear lysates were clearly an adequate model protein mixture for comparing fluorescent properties of complexes and free duplexes. There was also evidence of more efficient binding of HeLa proteins to the specific duplex containing Ig κ B box sequence than to the control duplex. However, the overall ODN sequence-independent level of protein binding in those lysates was very high (compare lanes 10 and 12) suggesting cautious interpretation of the lysate experiments.

The above results indirectly suggested that the binding of transcription factor proteins to fluorescent duplex sensors can be followed by measuring changes in fluorescent properties of the cyanine dye pairs. This was accomplished initially by tracking fluorescence intensity increase after the addition of recombinant p50 and p65 NF- κ B proteins to duplex sensors in solution at 1:6 molar ratios (ODN duplex/protein, mol/mol). Initially EMSA was used for testing of the binding of p50/p65 recombinant proteins to both fluorescent and quenched duplex sensors. We investigated two quenched duplex sensors carrying the Cy5.5/800CW pair at two different locations along the duplex (D2 and D10, see Supplemental information) and a total of three sensors with different structures resulting in radiative FRET sensors (D6, D7, D8, Fig.1c). We excited Cy5.5 fluorescence at 675 nm and measured: 1) relative change of the donor fluorescence at 700 nm and; 2) relative acceptor fluorescence change at 800 nm. Both fluorescent and quenched duplex sensors showed Cy5.5 donor and 800CW acceptor fluorescence intensity increase after the addition of p50/p65 NF- κ B protein mixture. The overall changes in fluorescence intensity were nearly instantaneous and stable over a period of 40-60 min (the total time of observation). The overall relative increase of 800CW fluorescence intensity was greater in quenched duplex sensors (Fig. 4b) if compared to fluorescence emitting duplexes. The relative 800CW fluorescence intensity change in FRET mode (Fig. 4b) was greater than the dequenching of the donor dye, Fig. 4c). In both cases quenched duplexes showed higher ratios of fluorescence intensities measured prior to and after the addition of NF- κ B proteins than fluorescence emitting duplexes (Fig. 4b,c). The latter showed only marginally higher increases of fluorescence intensities if compared to D2 carrying only one fluorophore instead of a dye pair. The lack of measurable changes

of single fluorophore fluorescence were observed in both FRET (Fig. 4b) and Cy5.5 relative increase of Cy5.5 fluorescence signal (i.e. the donor fluorescence (Fig. 4c) modes.

The results of our experiments suggested that the observed differences in fluorescence intensities should be detectable using a time-resolved fluorescence lifetime (FL-TD) imaging setup. It should be noted that there are multiple advantages of FL-TD strategy over other fluorescent assays: (1) unlike continuous waveform (CW) excitation of fluorescence, the FL-TD approach is more robust and quantitative and is not affected by the sample concentration and excitation intensity, which are in many cases difficult to control; (2) FL-TD is also generally independent on the excitation wavelength; (3) FL-TD is not affected by excitation leakage into the fluorescence filter, whereas in CW excitation this can be a major source of light contamination that is difficult to eliminate (the presence of excitation leakage is readily identified in FL-TD as a sharp peak in the initial rise of temporal decay profile (see Fig. 5) whereas the long time-decay is unaffected by the initial rise and can be used for the lifetime fitting; (4) the lifetime assays can potentially be performed in multiple formats: on a chip or in solution by simple mixing of the sensor with the sample in a plate.

Since FL is exquisitely sensitive to the changes of microenvironment, we tested whether the sensors based on close positioning of donor/acceptor pair of fluorophores would “sense” the protein binding events. Using a time-domain setup³⁰ we were able to measure FL in solutions containing between 50 and 100 nM of Cy5.5 and/or Cy7 and 800CW (2.5-5 pmol fluorophore/sample). Initially we measured FL of the fluorophores that were used for linking to ODNs. Both fluorophores, as expected, showed some FL lengthening after linking them to the duplexes due to the decrease of rotational freedom and the increase of rotational correlation time³¹ (Supplementary Table 2S). Furthermore, the FL increased to the baseline average of 1.19 ns in the case of Cy5.5 after hybridizing Cy5.5-labeled ODN to a complementary ODN. This property was not shared by acceptor fluorophores. However, their FL values also increased slightly after the conjugation to ODNs. We further determined the sensitivity of FL-TD to the presence of “dequenched” duplex sensors in mixtures that contained sensor populations with long and short FL. Towards this goal we used model mixtures of fluorescence emitting FRET (Cy5.5/Cy7) and emitting non-FRET (Cy5.5 only) duplex sensors (D3, Fig. 1c). In these mixtures, FL of Cy5.5 was always shorter in the case of FRET (D3-Cy5.5/Cy7, Fig. 5a). Biexponential data fit of fluorescence decay curves enabled fractional analysis of the mixtures containing various amounts of long-lifetime components (D3-Cy5.5) in mixtures with short lifetime components (D3-Cy5.5/Cy7, Fig. 5b). The FL-TD approach showed a sensitivity limit of approximately 10% of non-FRET duplex in 50 μ l samples of mixtures with a total concentration of 100 nM of duplex sensor. Under the above conditions, the accurate two-component lifetime unmixing was possible at 25% duplex-linked fluorophore in the absence of FRET, i.e. D3-Cy5.5.

The initial FL-TD experiments with recombinant NF- κ B proteins showed that the addition of 3-fold molar excess of p50 and p65 per mole of D3 duplex sensor carrying Cy5.5 dye and FRET acceptor dye resulted in a measurable increase of Cy5.5 dye FL (Fig. 5 c,d). Fluorescence lifetime of the donor fluorophore Cy5.5 increased by 0.08 ns in the case of Cy7, and by 0.15 ns in the case of 800CW acceptors, respectively. The 35 p50/p65 binding resulted in higher FL changes if we used non-fluorescent quenched sensors (e.g. duplex sensor D2, Table 2). The difference between the baseline FL and the FL of the donor Cy5.5 dye measured after adding p50/p65 to D2 increased to 0.21 ns (0.19 ns in the case of Cy5.5/Cy7 pair) compared to FL values measured using emitting FRET sensors (e.g. D3). The addition of the excess of NF- κ B positive HeLa nuclear extract resulted in FL differences exceeding 0.4 ns in duplex sensors carrying either Cy5.5/800CW or Cy5.5/Cy7 pairs (Table 2). The analysis of electrophoretic migration shift assay results showed that unlike recombinant NF- κ B proteins, HeLa cell nuclear extract caused a 100% shifting of the ODN

duplex band under the identical electrophoresis conditions (Fig. 4a). The binding of the proteins comprising HeLa nuclear extract to duplex sensors was very efficient but non-specific since we observed a shift in migration of control sequence D9 that contained no NF- κ B binding sequence. FL measurements in FRET mode (i.e. at the excitation of 650 nm and emission at >800 nm) showed higher specificity of p50/p65 interaction with the NF- κ B binding sensor D2 than the control sensor D4 (Table 2). The average non-specific change of FL (0.03 ns) was 4-times lower than specific (0.12 ns) change of FL in the presence of p50/p65 protein mixture. The observed differences in average FL values were further visualized by scanning the surface of the samples and representing the FL data in pseudo-colour maps (Fig. 6).

Conclusions

Therefore, the observation that donor fluorescence intensity change in non-radiative, quenched duplex sensors were greater than in radiative FRET sensors led us to a transcription factor binding study that provided measurements of FL changes detectable using both Cy5.5 fluorescence emission and FRET modes (Table 2). The significant changes in FL values were observed upon the binding of the duplexes suggesting that the close interaction between the donor and acceptor dyes was perturbed and these changes were substantially greater in the case of quenched “across-the-major-groove” orientation of cyanine fluorophores. One of the potential limitations of sensors based on cyanine pair interaction effect is in short lifetimes of these dyes³². Recent advances in new chemistry of cyanine fluorophores³³ are potentially capable of providing alternative dyes with long fluorescence lifetimes. We anticipate that further development of chemically stabilized sensors for direct imaging of transcription factor interactions in living systems using readout changes in fluorescence lifetimes (FL-TD) will be useful for increasing sensitivity of response to the binding of the components involved in transcriptional activation of gene expression.

Experimental

Synthesis and chemical modification

Oligodeoxyribonucleotides were synthesized using semiautomated mode on Expedite 8909 DNA/RNA synthesizer (Applied Biosystems Inc.) using Expedite monomers, and 1 μ M CPG columns (Glen Research). The reagents for ODN synthesis and purification were obtained from Glen Research (Sterling VA). Nucleoside phosphoramidite synthons: dG-phosphoramidite and dC-phosphoramidite (Fig. 1a) were synthesized for introducing hydrophilic aminoethyl(ethylene glycol) internucleoside linkers that separate fluorophores from DNA duplex. These phosphoramidites were synthesized using a synthetic approach described in¹. Cyanine near-infrared dye Cy5.5 hydroxysuccinimide (NHS) ester and Cy7 NHS ester were purchased from GE Healthcare (Piscataway NJ); IRDye 800CW NHS ester was obtained from Li-COR (Lincoln NE). The conjugation of Cy5.5 (donor) or IRDye 800CW (acceptor) fluorophores to the internucleoside amino linkers was performed by adding 10-fold molar excess of the activated ester in DMSO to HPLC-purified ODN in 0.1 M sodium bicarbonate, pH 8. After incubating for 2 h modified ODNs were separated using Bio-Spin P6 microcolumns (Bio-Rad) and precipitated with ethanol. The obtained ODNs were purified on C18-HPLC columns (Discovery C18, 5 μ m, Supelco) using a gradient of acetonitrile in 0.1 M TEAA, pH 7.0, precipitated from ethanol/sodium acetate and characterized by using electrospray mass- spectrometry.

Spectral measurements

All UV spectral measurements were performed at room temperature with a Cary 50 spectrophotometer (Agilent Technologies). All fluorescence spectral measurements were carried out at room temperature in quartz microcuvettes using a Cary Eclipse fluorescence spectrophotometer (Agilent).

Preparation of ODN duplexes, protein complexes and electrophoretic mobility shift assays (EMSAs)

ODN duplexes were prepared by mixing equimolar amounts of the corresponding ODNs dissolved in 25 mM Hepes, 1 mM MgCl₂, 50 mM NaCl, pH 7.4. The solutions were heated to 95°C for 5 min and cooled at room temperature. The duplex formation was confirmed by recording duplex melting curves and measuring changes of Cy5.5 fluorescence intensity increase with the increase of temperature in the cuvette controlled by an external Peltier heater. EMSAs were performed using a mixture containing 20 nM ODN duplexes incubated 30 min at RT in a 0.01 ml equimolar mixture of NF-κB p50/p65 solution (final concentration – 5 μg each protein/ml) diluted at 1:1 (vol/vol) with 10mM Tris, 100 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1 mg/ml tRNA and 0.5 mM DTT, pH 7.5. Complexes for fluorescence lifetime measurements were prepared as above at the concentration of 50 nM of ODN duplex and at the 1:6 (duplex:NF-κB heterodimer) molar ratio. HeLa cell nuclear protein lysate (Promega Inc.) was added at ODN duplex to achieve the final concentration of 500 μg/ml. EMSA samples were run on 15% TBE Ready Gels (Bio-Rad Laboratories, Hercules CA) using 0.5×TBE buffer. The gels were imaged and digitized using an Odyssey Infrared Imaging system (Li-COR Biosciences, Lincoln NE, USA).

Molecular modeling was performed with Molecular Operating Environment v 2.0 software (MOE, Chemical Computing Group Inc., Montreal CA). The general strategy included exporting ODN sequences in MOE, manually “linking” NIR fluorophores at desired internucleoside positions and assembling duplex models using energy minimization (force field calculation) followed by use of atom coordinates from available NF-κB-DNA complex X-ray crystallography PDB data files (PDB access number 1LE9, SwissProt database)²⁹. First, the positions of all hydrogen atoms were added, partial charges of atoms were calculated, AMBER94 force field was applied for all-atom molecular dynamics of ODN double helix and the potentials of ODNs comprising the duplex were fixed. The models were further optimized using MMFF force field simulations, with the atomic charges specified by the same force field. In our work we used interactive free energy minimizations via systematic conformational database searches in n=4-6 layer of interacting H₂O/Na⁺ solvent.

Fluorescence lifetime measurements

Cyanine dye fluorescence was excited by femtosecond Ti:Sapphire laser (Mai Tai, Spectra Physics) at 750 nm (Cy7 and 800 CW). The emitted time resolved fluorescence was registered using a CCD camera (LaVision GmbH, Germany) coupled to a gated-image-intensifier (LaVision GmbH) that provides a 300 picosecond time resolution. Excitation of Cy5.5 dye was performed by filtering (650 nm-40nm bandwidth filter, Chroma) the broadband output (500 nm-700 nm) obtained from a secondary, super-continuum (SC) source system based on photonic crystal fiber (NL-PM-750, Thorlabs) technology³⁴. The fluorescence was detected using either a 716-40 bandpass filter (for Cy5.5) or 800 nm longpass filter (800CW and FRET mode detection) attached to a camera lens coupled to the CCD camera. The measured fluorescence decay curves were analyzed using multi-exponential fitting algorithms based on non-linear least squares to obtain the lifetimes. If a single lifetime was present in the measured spot, the slope of the decay in the log scale

directly provided 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 from spatially separated regions in the samples were usually performed and averaged. Fractional analysis (Fig. 5a,b) was performed by expressing the total fluorescence signal as a sum of two “basis functions” that are essentially the full temporal response curves corresponding to the short lifetime quenched duplex ($B_F(t)$ Fig. 5a, 100% FRET) and to the longer lifetime non-FRET single dye Cy5.5 component ($B_N(t)$, Fig. 5a, Donor only):

$$I(t) = A_0 + A_F B_F(t) + A_N B_N(t) \quad (1)$$

where A_F and A_N are the corresponding amplitudes and A_0 is a constant to account for background leakage. The experimental decay data was subjected to linear-least squares fit using equation (1) to determine the amplitudes. The fractional contribution of each of FRET and non-FRET components, calculated as: $C_F = A_F/(A_0 + A_F + A_N)$ and $C_N = A_N/(A_0 + A_F + A_N)$, are shown in Fig. 5b along with the known fraction of the components in the mixture.

To obtain the FL maps (Fig. 6) we performed scanning of the whole area of the samples placed in 0.2 ml colourless transparent PCR tubes and then the data pixels were represented in pseudocolour according to their lifetimes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig.1.

a) oligodeoxynucleotide (ODN) modifiers: dC- and dG-amidite synthons used to covalently link near-infrared donor and acceptor cyanine fluorophores via the hydrophilic tri(ethylene glycol) amino linkers after ODN deprotection; b) donor and acceptor cyanine fluorophores used for ODN modification; c) ODN duplex sequences showing the positions of amino linkers within the sequences indicated as ⊗.

Fig.2.

Spectra of duplex sensors: a) superimposed fluorescence spectra of D2-Cy5.5 (black) D2-800CW (green); D2-Cy5.5/800CW (purple) demonstrating the close quenching of cyanines excited at 675 nm. Red traced spectrum shows fluorescence of 800CW excited at 774 nm in D2-Cy5.5/800CW; b) superimposed absorbance spectra of D2 sensor carrying one dye or a dye pair: c) superimposed fluorescence spectra of emitting D3 sensor; D3-Cy5.5 (black) D3-800CW (green); D3-Cy5.5/800CW (purple) demonstrating efficient emissive FRET if excited at 675 nm; d) -superimposed absorbance spectra of D3 sensor carrying one dye or a dye pair.

Fig. 3. Representative molecular models built in MOE as described in Materials and Methods: a) - closely interacting dyes in the non-parallel aggregate of Cy5.5 and 800CW dyes within the major groove of ODN duplex resulting in the lack of fluorescence emission as evidenced from the results shown in Fig. 2a. b)- X-ray crystallography data suggesting that DNA-interacting polypeptides of p50 and p65 subunits are interacting with major groove in close proximity from the space occupied by the donor/acceptor pair shown in (a).

Fig. 4.

Fluorescent analysis of protein binding to the duplex sensors: a) electrophoretic mobility shift assay in the (+) presence or (–) absence of p50/p65 or HeLa cell nuclear lysate and the following sensors: D3-Cy5.5 (lanes 1,2); D3-Cy5.5/800CW (lanes 3,4), D2-Cy5.5/800CW (lanes 5,6), D4-Cy5.5/800CW (lanes 7,8), D3-Cy5.5/800CW (lanes 9,10) and control D9-Cy5.5/800CW (lanes 11,12). Shifted band of the sensor-protein complex is shown with an arrowhead; b,c) - changes of fluorescence intensity upon the addition of p50 and p65 NF-κB protein mixture: b) relative change in FRET (excitation – 675 nm/emission 800 nm); c) relative change of donor fluorescence intensity (excitation – 675 nm/emission 700 nm). Duplex sensors used in each of these experiments are indicated below the bars.

Fig. 5. Sensitivity of FL-TD measurements. a) Cy5.5 fluorescence decay curves showing a bi-exponential behaviour due to varying proportions of D3-Cy5.5 and D3-Cy5.5/Cy7; b) the comparison of the true fractional content (in %) of a single Cy5.5 dye duplex sensor (D3-Cy5.5) and the content determined by measuring FL using multi-exponential decay curve analysis; c,d)- fluorescence decay curve of D3 duplex sensors carrying pairs of Cy5.5/800CW (c) and Cy5.5/Cy7 (d) incubated at the concentration of 75 nM in the absence (blue traces) or in the presence (red traces) of human recombinant p50/p65 NF-κB proteins. The FL (ns) values in c) and d) were determined by using monoexponential data fits.

Fig. 6.

Pseudo-colour maps showing FL distribution in experimental and control ODN sensor samples in the absence (–) or in the presence (+) of p50/p65 mixture measured in FRET mode. The sample FL scanning results obtained using the experimental NF- κ B - specific sensor with binding sequence (D2) labelled either with Cy5.5/800CW or Cy5.5/Cy7 dye pairs is shown as two top rows, the control D4 results are shown as two bottom rows. Pixel size – 0.5×0.5 mm. Pseudo-colour scale and the corresponding FL values in nanoseconds are shown on the right.

Table 1

The dependence of fluorescence on separation and reciprocal orientation of the donor/acceptor pair in representative ODN duplex sensors.

	ODNs forming a duplex ^{d)}	b.p.distance, nucleotides	Absorbance Spectra changes ^{b)}	Donor fluorescence intensity decrease, % ^{c)}	Acceptor fluorescence intensity change, % ^{d)}	Acceptor fluorescence intensity change, % ^{e)}
D1	5' AGCCT GGAAAGTCC-ACATCG 3' 3' TCGGAC-CCTTTCAGG GTGAGC 5'	10 T,T	-	70.6	+103.6	-6.2
D2	5' AGCCTG GGAAAGTCC-CACATCG 3' 3' TCGGAC-CCTTTCAGG GTGAGC 5'	8 G,G	+	87.3±1.0	-72.8±1.7	-84.2±2.2
D2A	5' AGCCTG GGAAAGTCC-CACATCG 3' 3' TCGGAC-CCTTTCAGG GTGAGC 5'	8 G,G	+	83.4±3.4	-69.8±1.5	-84.1±0.6
D3	5' AGCCTG-GAATTTC CACATCG 3' 3' TCGGAC CTAAAGG-GTGAGC 5'	8 C,C	-	63.4±4.0	+115.8±0.2	+0.6±0.1
D4	5' CGTATG AGTGACTG-CAGAGCT 3' 3' GCATAC-TCACTGAC GTCTCGA 5'	8 G,G	+	93.1±0.5	-81.4±0.1	-86.9±1.2

a) internucleoside positions of donor and acceptor cyanines; blue – Cy5.5 (donor), green- 800CW, acceptor;

b) + Indicates the presence of the of 640 nm absorbance peak;

c) Fluorescence intensity change at 700 nm (λ_{ex} =675nm) (%);

d) Fluorescence intensity change at 800 nm (λ_{ex} =675 nm) (%);

e) Fluorescence intensity change at 800 nm (λ_{ex} =778 nm) (%);

c,d,e) - The results are shown as mean±SD (n=2-3). Phosphorothioates are shown in bold.

Table 2

Fluorescence lifetimes measured at 650/716 band pass (donor dye Cy5.5) and 650/800 long pass (acceptor dye, 800CW and Cy7) in duplex sensors in the absence and in the presence of DNA binding proteins. The results of 2-3 independent measurements shown as mean $\pm$ SD.

Duplex sensor	Fluorophores <i>a)</i>		FL donor, ns	FL donor, with p50/p65 ^{c)} , ns	FL donor, with HeLa nuclear extract ^{c)} , ns	FL FRET, ns	FL FRET p50/p65, ns
	donor	acceptor					
Ig κ B derived sequence (D2) 5' AGCCTG $\otimes$ GAAAGTCCCACATCG 5' 3' TCGGACCTTCAGG $\otimes$ GTGTAGC 3'	Cy5.5		1.24 $\pm$ 0.04	-	-	-	-
	Cy5.5	800	0.94 $\pm$ 0.05	1.15 $\pm$ 0.04	1.23 $\pm$ 0.05	0.68 $\pm$ 0.02	0.76 $\pm$ 0.02
	Cy5.5	Cy7	0.80 $\pm$ 0.03	1.11 $\pm$ 0.04	1.36 $\pm$ 0.03	0.75 $\pm$ 0.03	0.87 $\pm$ 0.06
Control sequence (D4) 5' CGTATG $\otimes$ AGTGACTGCAGAGCT 3' 3' GCATACTCACTGAC $\otimes$ GTCTCGA 5'	Cy5.5	800	1.20 $\pm$ 0.02	NM <i>b)</i>	1.54 $\pm$ 0.3	0.77 $\pm$ 0.03	0.75 $\pm$ 0.03
	Cy5.5	Cy7	1.10 $\pm$ 0.03	NM	1.46 $\pm$ 0.02	0.70 $\pm$ 0.02	0.73 $\pm$ 0.02

a) duplex (and each dye) concentration – 50 nM;

b) NM - not measurable;

c) protein concentration – p50/p65 - 5 μ g/ml; HeLa nuclear extract concentration – 500 μ g/ml.