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Plate capture assay of fluorescent oligonucleotide duplex reporter-transcription factor complexes

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Abstract

We previously developed prototype oligodeoxyribonucleotide (ODN) duplex fluorescence energy transfer (FRET) reporters for optical sensing of NF- κ B transcription factor. We report here a plate-binding assay designed for optimizing the above reporters. Nitrilotriacetate-bearing plates were prepared by using either sequential 1) aminosilylation; 2) carboxylation; 3) coupling of N α ,N α -bis(carboxymethyl)-L-lysine or, alternatively, by replacing steps 1 and 2 by treating the glass with 3-(triethoxysilyl)propylsuccinic anhydride. FRET reporters were obtained by covalent linking of Cy5.5 (fluorescence donor) and IRdye800CW (fluorescence acceptor) to complementary ODN strands encoding a high affinity p50 binding site. Recombinant 6xHis tagged NF- κ B p50 was used for immobilizing the protein on glass plates via linked NTA-Ni(II) groups. Imaging and quantification of the fluorescence intensity in the wells was performed in two channels (700 and 800 nm) using a near-infrared scanning device with microscopic resolution. The fluorescence intensity of the ODN duplex reporter was detectable in the plates at the concentration of 5 pM. NF- κ B p50-ODN reporter interaction was studied by measuring the ratio of 700 nm (donor) to 800 nm (acceptor) fluorescence intensities. Using the plate assay we were able to measure p50-mediated interference with FRET at low density of plate binding.

Introduction

We previously developed probes for optical sensing of NF- κ B p50 protein-oligodeoxyribonucleotide (ODN) duplex interactions (1,2). The sensors were based on the effect of fluorescence resonance energy transfer between fluorescence donor (Cy5.5 carbocyanine dye) and acceptor (e.g. IRdye800CW), which were covalently linked to complementary strands encoding a high-affinity transcription factor binding site. We determined that if the donor and acceptor dyes were separated by 8 base pairs the radiative FRET efficiency exceeded 75%. Since the efficiency of FRET between a pair of near-infrared dyes is sensitive to protein binding to ODN duplex-encoded sequence, the reporters can be potentially used for direct sensing protein–DNA interactions (1,2). We initially measured the changes of donor and acceptor fluorescence by using a single-cuvette spectrofluorometer. This testing approach provided high spectral accuracy and precision in determining normalized fluorescence intensity ratios. However, measurements performed in the presence of non-stoichiometric ratios of oligonucleotide probe/transcription factor could also be affected by the excess of free ODN duplex reporter. In addition, the setup lacked

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multiplexing capabilities and had a low throughput. By using a microtiter assay format one can potentially achieve high throughputs and better accuracy since protein-probe complex can be isolated from the non-bound fluorescence probes by using binding of transcription factor to the plate surface. For isolating such reporter complexes it is desirable to immobilize them on the microplates using non-covalent, affinity-based strategies (3). There are several alternative strategies for achieving the above goal. The alternatives include the use of specific antibodies against transcription factor epitopes (4,5), as well as against fusion peptide tags introduced in the sequence of recombinant transcription factor proteins (6). We chose to study ODN duplex reporter-p50 protein complex separation conditions using NTA-Ni(II)-mediated affinity binding of the p50's fusion hexahistidine residue (6xHis) tag (7) since commercially available purified transcription factors are usually available as affinity-tagged proteins. We anticipated a potentially high density of 6xHis-tag binding sites that can be covalently linked to the glass surface, in contrast to a less dense coating with antibodies that are prone to activity loss during the storage. Glass has low autofluorescence in red and near-infrared spectral ranges and can be easily modified with silylation agents affording further chemical modification. We hypothesized that high efficacy of ODN duplex reporter-p50 protein complex retrieval from the solution could potentially translate into more accurate fluorescence readouts.

Therefore, we compared glass microplate modification protocols and tested the obtained plates for immobilizing NF- κ B p50 and for detecting the interactions between NF- κ B p50 and ODN duplex.

Materials and methods

Materials

3-(triethoxysilyl)propylsuccinic anhydride (TPSA) was purchased from Gelest Inc. (Morrisville PA). 3-aminopropyltriethoxysilane (APTS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), ethylenediamine, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and glycine were purchased from Sigma-Aldrich (St. Louis MO). Tris (2-carboxyethyl)phosphine (TCEP) was obtained from Pierce (Rockford IL). α , α -bis(carboxymethyl)-L-lysine hydrate (BCML) was purchased from Fluka (Switzerland). Triethylammonium acetate solution (2 M, TEAA), pH 7.0 was purchased from Glen Research (Sterling VA); Cy5.5 maleimide was from GE Healthcare (Piscataway NJ); IRDye 800CW NHS ester was from Li-COR (Lincoln NE). 96 well glass-bottom microplates were purchased from Thermo Fisher Scientific (Hudson NH).

Oligodeoxyribonucleotide (ODN) synthesis and modification

The synthesis, purification and characterization of the ODNs used in this study were performed as in (8). The capped 3'-dithiolinker was introduced by using commercially available CPG derivatives (Glen Research). The conjugation of the donor fluorochrome (Cy5.5 mono maleimide) was achieved after reducing hydroxyethyl dithiopropyl linker on the 3'-end of the oligonucleotide 5'-AGCTATGAGGGACTTTCCG-Y-3' (ODN1) with DTT. The conjugation of N-hydroxysuccinimide ester of IRDye 800CW acceptor fluorochrome was performed by using an internucleoside amino linker positioned between thymidine and cytosine in complementary 5'-CGGAAAGT*CCCTCATAGCT-3' (ODN2), designated by an asterisk. The latter was introduced by using a thymidine phosphoramidite synthon (5'-O-DMTr-thymidine-3'-O-(2-(2-(2-trifluoroacetamidoethoxy)ethoxy)ethyl)-N,N-diisopropylphosphoramidite) with a protected primary amino group linked via a triethylene glycol linker to phosphorus. The separation of donor and acceptor fluorochromes by 8 base pairs resulted in measured radiative FRET efficiency exceeding 75%.

Spectral measurements—All UV spectral measurements were performed at room temperature unless otherwise stated by using Cary 50 spectrophotometer (Varian). All fluorescence spectral measurements were carried out at room temperature unless otherwise noted in quartz microcuvettes using Cary Eclipse fluorescence spectrophotometer (Varian).

Preparation of ODN duplexes—Oligodeoxynucleotide (ODN) duplexes were prepared by mixing equimolar amounts of the corresponding ODNs dissolved in 25mM Hepes, 1mM MgCl₂, 50mM NaCl, pH7.4. The duplex mixtures were heated between 90–95°C for 5 min to dissociate any intrastrand secondary structures, and allowed to cool at room temperature to attain equilibrium. Each duplex formation was confirmed by measuring UV and fluorescence spectra. Two duplexes were used in this study: 1) FRET duplex: Cy5.5-ODN1/800CW-ODN2; 2) 800 nm fluorescent control duplex: ODN1/800CW-ODN2.

Plate surface treatment—For glass surface activation the following sequence of washes was used: 1) 1 M NaOH, 10 min; 2) water, 5 min; 3) 1 M HCl, 1 h; 3) water, 5 min; 4) H₂SO₄/H₂O, (1/1, v/v), 5 min. The plate was then washed in water followed by anhydrous ethanol and dried in vacuum oven at RT for 1–2h.

Two-step method of NTA linking to the glass surface (Fig. 1A)—5% 3-(triethoxysilyl) propylsuccinic anhydride (TPSA) in anhydrous DMSO was added to the plate and reacted for 1h followed by washing with DMSO three times; 0.025 ml of 0.2 M Na_α,Na-bis(carboxymethyl)-L-lysine (BCML) in 0.2M Na₂CO₃ (pH 9) and 0.075 ml DMSO were added to each well and incubated for 3h, followed by washings with water.

Mutli-step method of NTA linking (Fig. 1B)—Amino sylilation was performed by adding APTS/DMSO (5/95, v/v) mixture to the plate and incubating for 1 h (9,10). Sylilated plate was then washed three times with DMSO. Plate carboxylation was performed by mixing 0.03 ml 0.1 M succinic anhydride in DMSO with 0.27 ml, 0.2M NaHCO₃ in the wells of the plate, incubated at room temperature for 1–2h and washed with water three times. Fifty μL of water was added to each well and the plate was put on ice. To each well a mixture of 50mM EDC/20mM sulfo-NHS in water was added, followed by incubating for 5min. Then 0.05 ml of 0.1 M BCML/0.2M NaHCO₃ solution was added. The mixture was incubated with mixing at room temperature for 2h. The plate was washed three times with water.

Amino group titration—For determining amino groups on the glass surface 0.1 ml of 0.1 M sodium tetraborate pH 9.0, 0.05 ml 0.1% Ipegal and 0.1 ml 10mM TNBS were added to the wells. The plate was incubated 1 hour at RT and the absorbance was measured on SpectraMax M5 microplate reader (Molecular Devices). To test carboxylate-modified plates, ethylenediamine was used for reacting with EDC-activated carboxyl groups in the presence of sulfo-NHS. TNBS/tetraborate/igepal solution was then added to each well. Unmodified plate wells were used as blank controls, and amino group terminated plate wells were used as positive controls. Carboxyl group terminated plate well was used as a negative control.

Ni(II) chelation—0.1 ml of 0.1 mM NiCl₂, 10mM sodium acetate (pH 5.0) was added to each well and the plate was incubated at room temperature for 1h. The plate was washed with 0.05% Tween-20 and then 50mM Tris HCl, 500mM NaCl, pH 7.5.

NF-κB p50 direct labeling by fluorochromes and plate binding assay—NF-κB p50 (2μg in 0.05 ml of 0.1M NaHCO₃, 0.1% Tween-20, pH8.5) was incubated in the presence of 5-fold molar excess of IRDye800CW-NHS at room temperature for 1h. The mixture was separated on Micro Bio-Spin P-30 columns (Bio-Rad) equilibrated with the

plate binding buffer (PPB: 10 mM Tris, 100 mM KCl, 2 mM MgCl₂, 0.1 mg/ml tRNA, 10% glycerol, 5 mM TCEP, pH 7.5). The labeled NF- κ B p50–800CW protein conjugate was added to the plate in the binding buffer and incubated at room temperature for 1h. The negative control sample was prepared as above but in the absence of NF- κ B p50. The plate was washed with washing buffer (WB: 10mM Tris HCl, 500mM NaCl, 0.05% Tween-20, pH7.5) three times followed by fluorescence readout as described below.

NF- κ B p50 binding assay—ODN duplex-p50 complex solution in 0.05 ml PPB was prepared by adding 0.4 fmol ODN duplex and 1350ng NF- κ B p50. Incubated at room temperature for 30min before loaded to the plate. The plate was incubated at room temperature for 1h followed by three washes with WB.

mAb-coated plate—According to the manufacturer's instructions, affinity-purified rabbit polyclonal antibody (NF κ B p105/p50, Abcam Inc., Cambridge, MA) was adsorbed on a surface of polystyrene 96-well plate at 1 μ g/well in 0.01 M carbonate buffer, the plate was washed with PBS-T (0.05% Tween 20) and blocked with BSA. Complexes of ODN and p50 were incubated in the plate for 1h at room temperature and fluorescence was determined by using Odyssey system as described below.

Electrophoretic mobility shift assays (EMSAs)—EMSAs were performed using a reaction mixture containing 18 nM ODN duplexes incubated 30 min RT in a volume of 0.01 ml NF- κ B p50 solution in PPB. Samples were loaded and run on 10% TBE Ready Gels (Bio-Rad Laboratories, Hercules CA) using 0.5 $\times$ TBE buffer. The gels were imaged and digitized using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE).

Fluorescence measurements using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE). The instrument utilizes simultaneous solid diode excitation of fluorescence at 685 and 785 nm and measures emitted light by using dichroic mirror transmitting above 810 nm ("800 channel" fluorescence band) and reflecting below 750 nm ("700 channel" fluorescence band). Fluorescence signals were processed to filter scattered and stray light. The obtained images (16 bit TIFF) were analyzed, colorized, and fused using ImageJ 1.38 (NIH).

The standard for measurements in 800 channel was prepared by loading the plate with different concentrations of 800 channel control duplex. FRET duplex was also measured to calculate FRET efficiency.

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).

Results and discussion

Preparation and testing of NTA glass plates

We initially compared two strategies of carboxyl group linking to the surface of the glass plates: 1) silylation with 3-(triethoxysilyl) propylsuccinic anhydride and reacting the obtained anhydride group with α , α -bis(carboxymethyl)-L-lysine (Fig. 1A); 2) a multistep synthesis requiring aminosilylation using 3-aminopropyltriethoxysilane (APTS), reacting the glass-linked amino groups with succinic anhydride and, finally, activating the coupled carboxyls by using water-soluble carbodiimide before adding BCML (Fig. 1B). To determine the amounts of amino groups and carboxyl groups on the surface of the plates we used TNBS as amino group test reagent and measured absorbance of the mixture at 420nm (11).

We used ethylene diamine coupling to either anhydride (first two-step method, Fig. 1A) or to EDC/Sulfo-NHS activated carboxyls (Fig. 2B) for comparing the efficacy of plate surface modification. By reacting anhydride residues with diamine and treating the conjugated amino groups with TNBS we showed that at least 60nmol of reactive carboxyl groups were linked to the surface of each well of a 96-well plate as a result of silylation with TPSA. In contrast, the multi-step approach resulted in 2-times less amino groups linked to each well. The multi-step conjugation strategy has potential advantages allowing conjugation via longer linker between the glass and chelating group and the absence of additional carboxyl groups in the linker moiety that can potentially weakly bind Ni^{2+} cations. However, the advantage of the first method was in simplicity due to a two-step activation and the higher number of reactive groups linked to the glass. Since the first strategy resulted in 2-times more reactive carboxyl groups/well we used the two-step glass modification approach in the further experiments.

To obtain glass-immobilized quadridentate NTA-Ni(II) chelate and to reduce non-specific binding the plate was first blocked with BSA followed by nickel chelation with glass-conjugated NTA groups. To test whether the obtained NTA-Ni(II) plate has 6xHis-tag binding affinity we labeled NF- κ B p50 directly with IRDye800CW fluorochrome and compared binding of labeled protein with the free dye (Fig. 2A). No free IRDye800CW binding to the plate was detected whereas the 800CW-labeled NF- κ B p50 showed binding that was directly proportional to the added protein concentration. As shown in Fig. 2A in the range below 50 fmol (less than 1 nM p50 added) 90%-95% 800CW-p50 was bound to the NTA-Ni plate.

NTA-Ni(II) plate binding assay for detecting ODN reporter-p50 interactions

We tested two types of p50-binding ODN duplexes: a reporter duplex Cy5.5-ODN1/800CW-ODN2 carrying a pair of FRET fluorochromes (Cy5.5, donor and 800CW, acceptor) on complementary strands, as well as non-FRET duplex ODN1/800CW-ODN2 as a standard. According to the results of EMSA, both duplex probes (i.e. reporter and control) were efficiently binding p50 (not shown). ODN duplex ODN1/800CW-ODN2 was initially chosen for testing the binding of ODN duplex probes in the NTA-Ni(II) plate binding assay since fluorescent signal at 800 nm has a very high signal/background ratio (Fig. 2B). ODN1/800CW-ODN2 duplex was first incubated with human recombinant Hisx6-tagged p50 at various duplex/p50 molar ratios and then added to the wells of NTA-Ni(II) plates. The measured bound fluorescence values showed that approximately 1–2% of added duplex-p50 complex was bound to the NTA-Ni plate (Fig. 2C, squares). Alternatively, ODN duplex-p50 complex (1:12 molar ratio) was captured on the plates using affinity-purified antibody. In contrast to NTA-Ni(II) strategy only 0.34% of added duplexes were bound to the plate surface reflecting random immobilization of antibody molecules on the plate and, consequently, a potentially lower density of p50 binding centers (Fig. 2C, triangles).

Fluorescence measurements in glass plates demonstrated high sensitivity to the presence of ODN reporter. The limit of FRET ODN duplex reporter detection in solution was 5 pM when fluorescence was measured at 800 nm. To perform testing of ODN duplex probes in the developed NTA-Ni(II) plate binding assay, ODN duplexes (2.4 pmol, 8 nM) were first incubated with human recombinant p50 at the molar ratio of 1:12 at room temperature for 30 min. To test the specificity of p50-ODN duplex recovery from the solution we added 0–8 nM ODN duplex to the NTA-Ni(II) plate, either in the presence, or in the absence of p50 protein. After a 1h-incubation followed by washings we measured the fluorescence intensity associated with the plate using a two-channel imaging system (Fig. 3A). For quantification of both free and bound duplex the fluorescence intensity was measured in the 800 nm channel was excited at 785nm, i.e. the fluorescence intensities of non-FRET duplex ODN1/800CW-ODN2 and FRET duplex Cy5.5-ODN1/800CW-ODN2 measured at 800 nm

were treated as unaffected by FRET and plotted in the same scale (Fig. 3B). Fluorescence intensities of both Cy5.5 and 800CW fluorochromes measured in the wells after adding FRET ODN duplex-p50 complex showed duplex concentration-dependent increase (Fig. 3A,B), whereas free FRET ODN duplex showed no concentration-dependent association with plate surface (see Fig. 3A, upper row). To determine, whether FRET efficiency in ODN duplex reporter complex was changing as a result of p50 binding we performed measurements of fluorescence at 700 nm (donor fluorochrome) to 800 nm (acceptor fluorochrome) and calculated the resultant emission light intensity ratios. Measurements of fluorescence intensity at 800 nm enabled calculating the duplex amount bound to the plate. By comparing ratiometric measurements performed in the case of free Cy5.5-ODN1/800CW-ODN2 duplex and Cy5.5-ODN1/800CW-ODN2-p50 complex bound to NTA-Ni(II) plate we showed that in the low range of bound duplex amounts (less than 20 fmol/well) FRET ODN duplex reporter- p50 complex had a higher Cy5.5/800CW ratio values than the free duplex (Fig. 3C). The above result indicates that p50 partially interferes with intra-duplex FRET effect normally present in p50-free duplex reporter. However, at higher amounts of the duplex bound to the well (>60 fmol/well) we observed a loss of measurable ratiometric difference between duplex reporter-p50 complex bound to the plate or free duplex reporter alone (Fig. 3C). This effect can be explained by Cy5.5 fluorochrome partial self quenching at high local concentrations next to the plate surface (non-emitting FRET).

Therefore, we performed optimization steps for preparing NTA-Ni(II) linked glass plate and used the obtained plates for separating free ODN duplexes from a complex formed between the Hisx6-tagged NF- κ B p50 protein and fluorescent ODN duplex. This approach enabled reliable ratiometric measurements of FRET effects in ODN reporters bound to the plate surface. The alternative approaches would require coating of the plate with anti-transcription factor antibodies or linking the duplexes to the surface as described in (5,12). The antibody coated plate showed a lower efficacy than NTA-Ni(II) strategy. The use of covalent conjugation of fluorochrome-linked duplexes to the plate with the subsequent analysis of fluorescence changes after incubating with recombinant NF- κ B proteins can be used as a potential alternative. The advantage is in that the use of His-tagged proteins, which have a lower DNA-binding affinity (13) is not required. However, the latter strategy requires the use of additional linking of reporter ODNs to the glass, for example via 3'- or 5'-dithio linkers (14) creating potential sterical hindrances for transcriptional factor binding. The assay proposed in our work was specifically designed to capture pre-formed transcription factor-duplex complexes and allowed selective measurement of FRET in the fraction of protein-ODN duplex complexes. The developed method will be used for optimizing duplex ODN reporter design for potential detection of functional NF- κ B components *in vitro* and *in vivo*.

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A

B

Figure 1.

Scheme of NTA-Ni(II) glass plate surface activation. **A**- a two-step method including glass surface activation using silylation with 3-(triethoxysilyl) propylsuccinic anhydride (TPSA) followed by coupling of N_{α}, N_{α} -bis(carboxymethyl)-L-lysine hydrate (BCML); **B**- a multi-step synthesis requiring aminosilylation using 3-aminopropyltriethoxysilane (APTS) with subsequent acylation with succinic anhydride and coupling of BCML in the presence of water soluble carbodiimide and sulfosuccinimide.

Figure 2.

Testing NTA-Ni(II) plate in binding assays. **A-** p50 binding to NTA-Ni(II) plate. NF- κ B p50 labeled with IRDye800CW (squares), IRDye800CW dye alone (triangles); **B-** ODN1/800CW-ODN2 binding to NTA-Ni(II) plate either in the presence of p50 (squares) or in the absence of p50 (diamonds). **C-** efficacy of p50- duplex binding to the NTA-Ni(II) plate (squares) at various p50:duplex molar ratios or to anti-p50 antibody coated plate (triangle) at p50:duplex molar ratio of 12:1.

Figure 3.

NTA-Ni(II) plate binding of two-fluorochrome labeled FRET reporter duplexes. **A**- Dual channel fluorescence imaging of NTA-Ni(II) plate. The upper row: binding of FRET duplex Cy5.5-ODN1/800CW-ODN2 to the plate surface in the absence of p50, the lower row binding of the FRET duplex to the plate after a complex formation with p50. Lanes 1–8 duplex amounts: lanes 1,5 - control, 0 fmol; lanes 2, 6 – 2 fmol; lanes 3, 7 – 8 fmol; lanes 4, 8 - 65 fmol. Lanes 9–12 show an overlay of images acquired at 700 nm (red) and 800 nm (green); **B**- ODN duplex reporters Cy5.5-ODN1/800CW-ODN2 binding to the NTA-Ni(II) plate in the absence or in the presence of p50 protein. Triangles (solid line) –fluorescence intensity at 800 nm, squares (hatched line) –fluorescence intensity at 700 nm. Results presented as mean $\pm$ SD (n=3); **C**- Ratiometric measurements (700 channel/800 channel fluorescence intensities) performed using NTA-Ni(II) plate bound Cy5.5-ODN1/800CW-ODN2 ODN duplex reporters in the absence (open bars, free duplex in solution) or in the presence of p50 protein (closed bars, p50-duplex complex bound to the plate).