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Comparison of several linear fluorophore-and quencher conjugated oligomer duplexes for stability, fluorescence quenching and kinetics in vitro and in vivo in mice: Fluorescent DNA duplexes for imaging applications

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Abstract

A useful property of optical imaging is the potential to modulate the detectable signal to improve target/nontarget ratios. When administered as a dimer of a fluorophore- and a quencher-conjugated duplex arranged to inhibit fluorescence but designed to dissociate only in the presence of its target, the fluorescence signal should in principle appear only in the target. This laboratory has demonstrated the feasibility of this approach by using a duplex consisting of a linear oligomer conjugated with Cy5.5 (emitter) hybridized to another linear oligomer conjugated with Iowa Black (quencher) in a pretargeting optical study.

Methods—Eight duplexes consisting of combinations of 18 mer linear phosphodiester (PO) and phosphorothioate (PS) DNAs and phosphorodiamidate morpholinos (MORFs) conjugated with Cy5.5 (emitter) and Iowa Black (quencher) were variously screened for in vitro duplex stability. The MORF/PO duplex was selected for further study based on evidence of stability in 37 °C serum. Simultaneously, the kinetics of quenching was investigated in vitro and in vivo in mice. Thereafter, mice were implanted in one thigh with MORF/PO Cy 5.5 microspheres and the complementary PS Iowa Black administered IV to measure the extent and kinetics of duplex formation in the target.

Results—While all duplexes were stable in buffer, only the MORF/PO duplexes and possibly all PS containing duplexes were stable in 37°C serum for at least 4 h. The kinetics of quenching were found to be rapid in vitro, with a 80–90% decrease in Cy5.5 fluorescence immediately followed formation of a PS/PS homoduplex and in vivo, with a 27 to 38% decrease in target thigh/nontarget ratio within 1 h following administration of the complementary PS Iowa Black complementary DNA but not the random control DNA to mice implanted with MORF/PO Cy5.5 microspheres.

Conclusions—This investigation has provided additional evidence that Cy5.5 may be efficiently and rapidly quenched by Iowa Black when both are conjugated to complementary oligomers and that the resulting inhibition of fluorescence is sufficiently persistent for imaging.

Keywords

optical imaging; linear oligomers; fluorescence quenching

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INTRODUCTION

While nuclear imaging offers many advantages including high sensitivity of detection, quantitation accuracy and the availability of numerous contrast agents, the physics involved does not permit varying the nuclear emissions. By contrast, a property of optical imaging unique among most molecular imaging modalities is the potential to modulate the detectable signal in the target. By bringing into close proximity a fluorophore and its quencher, it is possible to inhibit fluorescence by contact fluorescence inhibition (1). This laboratory is investigating antisense and pretargeting of tumor and other applications of radiolabeled oligomers (2,3,4). In the course of these investigations, it became apparent that the hybridization properties of oligomers may have applications in optical imaging as well. When properly designed, it should be possible with fluorophore-conjugated linear duplexes to limit fluorescence in a living subject except at the target site expressing a complementary oligomer capable of dissociating the duplex. Molecular beacons consisting of DNAs in the form of hairpins with two fluorophores or a fluorophore and a quencher on opposite ends are already in use in a similar manner for in vitro assays and for vivo studies as well (5~11). Possible applications include antisense targeting in which the target mRNA serves as the complementary oligomer and pretargeting in which the complementary oligomer is localized in the target by a preliminary administration. This laboratory has recently reported on a proof of concept study of optical pretargeting showing that a linear fluorophore/quencher-conjugated oligomer duplex may be designed to dissociate in its target with the release of fluorescence inhibition (12). The further development of optical imaging using linear duplexes will require additional investigations of imaging sensitivity, duplex stability and the kinetics of fluorescent inhibition.

We report herein on the screening of duplexes consisting of phosphodiester (PO) and phosphorothioate (PS) DNAs and/or phosphoramidate morpholinos (MORFs) conjugated with Cy5.5 as emitter and Iowa Black as quencher to evaluate duplex stability in vitro and in vivo and to measure the pharmacokinetic and optical imaging properties of a stable duplex in a mouse microsphere model. The results of these measurements will be required to select duplexes for future investigations.

EXPERIMENTAL PROCEDURES

All oligomer were either uniform PO, PS or MORF throughout their length. All DNAs, fluorophore/quencher conjugated or native, were purchased HPLC purified (Integrated DNA Technologies, Inc. Coralville, IA) as were the MORFs, biotin conjugated or native (GeneTools, Philomath, OR). The DNAs were purchased with the fluorophore/quenchers attached directly while the biotin was attached to the MORF via a 6 carbon linker. The oligomer base sequences of this investigation are shown in Table 1. For convenience, the 18 mer sequence was selected as the standard sequence of ongoing pretargeting studies in this laboratory (13). The calculated melting temperatures of PO homoduplexes with the 18 base standard sequence and with the new sequence of this investigation are 43.8 °C. The melting temperatures of the PS and MORF duplexes of this investigation are also expected to exceed 37 °C whether native or with fluorophore/quencher attached (14,15). Complementary sequences are designated herein as cDNA or c'DNA and random sequences are designated as rDNAs. The base sequence of both the 36 mer PO and PS DNAs consisted of two complementary regions, an 18 mer sequence complementary to the above standard sequence (i.e. cDNA1) and an additional 18 mer new sequence (i.e. c'DNA2) complementary to that of a new 18 mer PS DNA2 sequence. Therefore, in this article, both the 36 mer PO and PS DNAs are represented as cc'DNAs to illustrate this structure. The new sequence was selected to avoid hairpin formation and to bring the fluorophore/quencher into the proper configuration for quenching after hybridization (Scitools OligoAnalyzer 3.0, Integrated DNA Technologies). The control PS rDNA1 and PS rDNA2

were also 18 mer but with randomized sequences. The 1.0 μm streptavidin coated polystyrene microspheres had a 3.5 $\mu\text{g/mL}$ biotin binding capacity (Polysciences, Inc. Warrington, PA).

All centrifuge tubes, pipette tips and PBS buffers were autoclaved before use. The Cy5.5 fluorophore was selected because its emission maximum is at 694 nm and therefore in the near infrared where light absorbance in tissue reaches a minimum. The Cy5.5 fluorescence can therefore be detected in vivo at subnanomolar concentrations and at depths sufficient for experimental studies in small animals and possibly in patients (16~19). The Iowa Black RQ™ dye was selected because it has a broad absorbance spectrum ranging from 500 to 700 nm with peak absorbance at 656 nm and therefore that overlaps the emission spectrum of Cy5.5 such that the emissions of Cy5.5-DNA may be efficiently inhibited (quenched). The Cy5.5 fluorescence was detected using a 615–665 nm excitation filter combined with a 695–770 nm emission bandpass filter on a IVIS 100 optical camera (Xenogen, Alameda, CA). Regions of interest were set and data were analyzed with Living Image software (Xenogen). To reduce autofluorescence, the animals were hairless male SKH-1 (Charles River Breeding Labs, Wilmington, Mass.) and were fed on a chlorophyll-free diet (AIN-93G Purified Diet, Harlan Teklad, Madison, WI).

Duplex stability studies by PAGE

All oligomers for the duplex stability studies were native rather than fluorophore/quencher conjugated and all were prepared at 10 μM concentration. The PAGE Ready Gel 15% TBE gel was purchased (Bio-Rad, Richmond, CA) and run in 1XTBE (Tris-borate buffer) at 87 v for 1 h then stained with ethidium bromide (0.5 mg/mL in 1X Tris-borate buffer).

The following four duplexes were prepared: PO DNA1/PO cc'DNA; MORF1/PO cc'DNA; MORF1/PS cc'DNA; and PO DNA1/PS cc'DNA. All duplexes were first formed by heating at 90 °C for 10 min before cooling to room temperature. Samples were then incubated in PBS at 37 °C for 2, 4 or 9 h before analysis. For analysis, 1 μL was added to each well. Samples were also incubated in fresh mouse serum by adding 10 μL of each sample to 30 μL of serum followed by incubation at 37°C for 2 or 4 h before analysis. For analysis, 4 μL was added to each well except for serum alone in which 3 μL was added.

Confirmation of fluorescent hybridization quenching in vitro

The 18 mer PS Cy5.5 cDNA1 was first added in triplicate to Costar® 96 well Black polystyrene flat bottom assay plate (Corning Incorporated, Corning, NY) at concentrations of 1, 2 or 5 μM in room temperature 50 μL PBS ($[\text{Na}^+] = 150 \text{ mM}$, pH 7.4) or 75% fresh mouse serum followed immediately by the addition of the 18 mer PS Iowa Black DNA1 to the wells at different cDNA1 : DNA1 molar ratios. Fluorescence intensity was measured over 1.5 h thereafter on the IVIS camera. As control, the identical study was repeated with the random control PS Cy5.5 rDNA1 in place of the study PS Cy5.5 cDNA1. The kinetics of quenching was also measured in 75% fresh mouse serum in wells containing 5 μM PS Cy5.5 cDNA1 to which the PS Iowa Black DNA1 was added at a 1 : 1 molar ratio.

Before performing imaging studies on animal implanted with microspheres, the ability of PS Iowa Black DNA2 to quench the fluorescence of PO Cy5.5 cc'DNA immobilized on microspheres was established. Streptavidin coated microspheres (100 μL) were added to a solution of 0.8 nmol of the duplex formed previously between biotin-MORF1 and PO cc'DNA and incubated at room temperature for 30 min. Thereafter the microspheres were separated by centrifugation and washed in PBS/BSA binding buffer three times.

Approximately 20 μL of microspheres (Cy5.5 concentration 2 μM) were added to each well of the flat bottom assay plate and suspended in 50 μL of PBS/BSA binding buffer. Thereafter,

the PS Iowa Black DNA2 was added at different cc'DNA : DNA2 molar ratios followed by agitation to suspend the microspheres. The fluorescence intensity was measured at room temperature 20 min and 60 min later on the IVIS camera and the results analyzed by Living Image software as before.

Animal Studies

All animal studies were performed with the approval of the UMMS Institutional Animal Care and Use Committee. For in vivo studies, the identical illumination settings (lamp voltage, filters) were used for all images, and fluorescence emission was normalized to photons per second per centimeter squared per steradian (p/s/cm²/sr). All data were processed by Living Image 2.50 software (Xenogen). Prior to performing the imaging studies on microsphere implanted animals, the stoichiometry and kinetics of fluorescent hybridization quenching was studied in vivo. In groups of 3 to 4, male SKH-1 mice were injected via a tail vein with either 0.3 nmol (2 µg) or 0.8 nmol (5 µg) of the PS Cy5.5 cDNA1 in 0.1 mL and thereafter each received via a tail vein either 0.3 nmol, 0.8 nmol or 2.4 nmol of PS Iowa Black DNA1. The molar ratios of Iowa Black DNA1 to Cy5.5 cDNA1 were either 1 : 1 or 3 : 1. The animals were then anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) and imaged on the IVIS camera both in the ventral and dorsal views at various times postinjection while under continuous sedation. All images were acquired with 1 sec exposure (f/stop= 2). A large region of interest was set about the whole animal for quantitation.

Approximately 30 µL of the microspheres prepared as described above and carrying 0.06 nmol of Cy5.5 were implanted intramuscularly in one thigh of each SKH-1 mice. After 15 min, each animal received either 0.06 nmol (N=2), 0.6 nmol (N=2) or 6.0 nmol (N=4) of the PS Iowa Black DNA2 by IV administration and was imaged in both the dorsal and ventral view immediately thereafter. The fluorescence intensity in a region of interest about both thighs was recorded and target to nontarget ratios were compared before and after the administration of PS Iowa Black DNA2 with any decrease defined as the quenching efficiency. As a control, one implanted animal received 6.0 nmoles of the random Iowa Black rDNA2.

Although the amount of microspheres implanted was identical, the depth of intramuscular implantation varied from animal to animal and resulted in differences in the fluorescence intensity. To correct for absorption and scatter, the fluorescence intensity of all mice but one with implanted PO Cy5.5 cc'DNA microspheres were measured before the PS Iowa Black DNA2 was administrated. The remaining mouse left as a further control did not receiving PS Iowa Black DNA2.

RESULTS

Duplex stability studies by PAGE

Figure 1 presents a photograph of the 2 h gel showing a molecular weight ladder in lane 1, the results of incubating at 37 °C in PBS in lanes 2 to 5, the results of incubating in serum in lanes 6 to 9 and serum alone in lane 10. The gels obtained in the 4 h incubations in PBS and serum and the 9 h incubation in PBS show the identical trends (data not presented).

As shown, all four duplexes with either the PS or PO cc'DNAs are stable in 37 °C PBS (lanes 2 to 5). In each case, one intense band at about 50 base pairs appears for both homoduplexes and the MORF1/PS cc'DNA heteroduplex. The two weaker bands in the case of the MORF1/PO cc'DNA heteroduplex (lane 4) appeared consistently in all gels.

However, also as shown in the figure, duplex stability in serum compared to PBS is obviously very different. Of the four duplexes, only the MORF1/PO cc'DNA duplex showed evidence of stability at the earliest time point at 2 h (lane 8). This band was weaker although still readily

apparent at 4 h. The 9 h gels were equivocal as regards duplex stability (data not presented). The weak bands at low molecular weight in the case of the PO DNA1/PO cc'DNA (lane 9) are probably the result of the nuclease instability of this DNA. Finally, a band is apparent at the loading point in all serum samples, including that of serum alone. However, this band is much more intense in the case of the PS cc'DNA duplexes (lanes 6 and 7) presumably because of the protein binding affinity of this DNA. This analysis therefore cannot exclude the possibility that the duplexes with PS cc'DNA were also stable in serum but remained at the loading point.

An additional four 18 mer duplexes were also investigated in buffer and in serum MORF1/PO cDNA1; MORF1/PS cDNA1; PO DNA1/PO cDNA1 and PO DNA1/PS cDNA1 (data not presented). The results show once again that at 2 and 4 h of incubation, the MORF1/PO cDNA is stable in serum.

It is possible to conclude from these duplex stability studies that the PO DNA homoduplex with overhang is unstable as expected due to the recognized instability to nucleases of a single chain PO DNAs (20,21). However nuclease attack on PO DNA, including the single chain overhang, appears to be less effective when hybridized to MORF.

The bead studies described below require streptavidin microspheres to which Cy5.5 conjugated oligomer are bound. Since neither a PO or PS DNA derivitized with both Cy5.5 and a biotin group on opposite ends were commercially available, the results of the above PAGE study provided confidence that microspheres prepared by the addition of biotinylated MORF1 first to the beads followed by addition of PO cc'DNA conjugated Cy5.5 would be stable.

Confirmation of fluorescent hybridization quenching in vitro

The histograms of Figure 2 show that the fluorescence in PBS buffer of the PS Cy5.5 cDNA1 was reduced by more than 80% following addition of the PS Iowa Black DNA1 even at the lowest 1 : 1 cDNA1 : DNA1 molar ratio and more than 90% at cDNA1 : DNA1 molar ratios 1 : 2 or higher. This quenching of Cy5.5 fluorescence was not apparent when the Iowa Black was added to the control nonspecific PS Cy5.5 rDNA. The figure presents results (N=3) obtained at 30 min and at the 5 μ M concentration of PS Cy5.5 cDNA1.

The kinetics of quenching in wells (N=3) with 5 μ M PS Cy5.5 cDNA1 following addition of PS Iowa Black DNA1 at three cDNA1 : DNA1 molar ratios from 1 : 1 to 1 : 3 showed that under the conditions of this in vitro study, quenching occurred extremely rapidly, reaching more than 80% within 5 min even at cDNA1 : DNA1 molar ratio of 1 : 1 and greater than 90% at higher molar ratios. The quenching was persistent thereafter at least until the end of observation at 90 min. The quenching was found to be equally rapid in serum as well as in buffer (data not presented). Compared to the fluorescence of wells without the Iowa Black, the fluorescence intensity in wells containing 5 μ M PS Cy5.5 cDNA1 in 75% serum fell by 73% immediately upon addition of the PS Iowa Black DNA1 at 1 : 1 molar ratio at room temperature and rose only slightly to 77% and 83% at 8 and 57 min respectively. However, the quenching efficiency was somewhat reduced in serum over the 80% achieved in buffer under essentially identical experimental conditions.

Figure 3 presents histograms showing that Cy5.5 on the 3' end of the 36 mer PO cc' DNA even when tethered via MORF1 to streptavidin coated microspheres loses 82% of its fluorescence by the addition of Iowa Black DNA2 in PBS/BSA buffer within 20 min at room temperature even at a 1 : 1 molar ratio with little change at higher molar ratios or at 60 min (N=3).

Animal Studies

Camera sensitivity was sufficient to provide an acceptable fluorescent image within 10 min following the administration of 0.3 nmol of PS Cy5.5 cDNA1 alone. However following

administration of PS Iowa Black DNA1, image quality was markedly superior when 0.8 nmol of PS Cy5.5 cDNA1 was initially administered. Accordingly, the 0.8 nmol dosage of Cy5.5 was selected for subsequent studies.

Figure 4 presents the images obtained in the study of the stoichiometry and kinetics of fluorescent hybridization quenching in vivo and shows both the ventral (left panel) and dorsal (right panel) whole body fluorescent images in animals receiving from left to right: nothing, PS Cy5.5 cDNA1 alone (0.8 nmoles), and PS Cy5.5 cDNA1 followed by PS Iowa Black DNA1 at a 1 and 3 fold molar excess 10 min later and imaged 30 min thereafter. The figure shows obvious quenching in both the ventral and dorsal views of PS Cy5.5 cDNA1 fluorescence following administration of PS Iowa Black DNA1. As expected, the quenching is more pronounced following administration of PS Iowa Black DNA1 at a three fold molar excess. Fluorescence intensity in these hairless animals fed on a chlorophyll-free diet appears to be concentrated in the kidneys as evident in the dorsal view and in the GI tract and possibly the thyroid as evident in the ventral view. The fluorescence from these and other tissues tended to change over time (data not presented).

The quenching efficiency was determined by subtracting from unity the ratio obtained by dividing the whole body fluorescence intensity of the mice receiving both PS Cy5.5 cDNA1 and PS Iowa Black DNA1 by the fluorescence intensity of the mice receive PS Cy5.5 cDNA1 alone. As shown in Figure 5, when measured in this manner, the quenching efficiency at 20 min post administration ($N = 3$ in all cases) at a 3 fold molar excess of PS Iowa Black DNA1 was $25.0 \pm 4.8\%$ (dorsal view) and $34.1 \pm 5.6\%$ (ventral view) with little change at 60 min ($26.6 \pm 9.6\%$ and $35.9 \pm 6.7\%$ respectively).

Thereafter, the streptavidin microspheres carrying 0.06 nmol of biotinylated MORF1/PO Cy5.5 cc'DNA were implanted intramuscularly in one thigh of SKH-1 mice and, after 15 min, each animal received either 0.06 nmol ($N=2$), 0.6 nmol ($N=2$) or 6.0 nmol ($N=4$) of the PS Iowa Black DNA2 by IV administration. One mouse received 6.0 nmol of the random PS Iowa Black rDNA2 as control. The animals were imaged in both the dorsal and ventral view immediately thereafter. The fluorescence intensity of the implanted thigh compared to that of the contralateral thigh was measured and the decrease in this ratio after administration of the Iowa Black was defined as the percent quenching efficiency as before. Figure 6 presents the results obtained over 90 min in mice receiving 6.0 nmoles of the PS Iowa Black DNA2 at a 100 fold molar excess over the PO Cy5.5 ($N=4$). Lower dosage of Iowa Black DNA2 (10 and 1 fold molar excess) gave an unremarkable quenching effect.

Mice implanted with streptavidin beads binding the 36 mer PO Cy5.5 cc'DNA showed a decrease in target/nontarget ratio of $27\% \pm 12\%$ (dorsal view) and $38\% \pm 6\%$ (ventral view) within 1 h of administration of the 18 mer PS Iowa Black DNA2 but not the Iowa Black rDNA2 random control. When administered at a 100 fold molar excess, the target/nontarget ratio was increased 20 % (dorsal view) and 14 % (ventral view) at 40 min postadministration of the random Iowa Black rDNA2 control. Therefore, despite the barriers to in vivo hybridization including rapid clearance and poor access, the quenching of the tethered Cy5.5 fluorescence was remarkably effective following intravenous administration of the Iowa Black DNA2.

DISCUSSION

To the best of our knowledge, pretargeting and similar applications of optical imaging have not been previously considered especially with linear oligomers. This laboratory recently completed a optical pretargeting study in which microspheres with a 25 mer cMORF were implanted in mice that subsequently received a duplex consisting of a 25 mer Cy5.5-MORF hybridized with a 18 mer BHQ3-PS DNA (12). The results of that study confirm that

fluorescent conjugated duplexes may be designed to dissociate rapidly in the presence of the target. We report herein on an investigation of the *vivo* stability of novel duplexes, the sensitivity of detection, the kinetics of hybridization and the pharmacokinetics of *in vivo* quenching. While these measurements are not directly related to pretargeting and similar applications of optical imaging, the results described herein will be required in the further development of optical imaging with linear duplexes.

Of the eight duplexes between PO DNA, PS DNA and MORF, gel electrophoresis showed stability only for the MORF/PO DNA and possibly the MORF/PS DNA duplexes following incubations in 37°C serum for 2 h (figure 1) and 4 h (data not presented). To measure the kinetics of quenching, the fluorescence intensity of a Cy5.5 conjugated PS DNA was measured *in vitro* following addition of various molar ratios of Iowa Black conjugated complementary PS cDNA. The quenching was initially found to be essentially completed within 30 min (figure 2) while a more focused study of kinetics indicated that quenching was completed within several minutes. By contrast no quenching was observed if the study Cy5.5 cDNA was replaced with the control Cy5.5 rDNA. Quenching *in vivo* in mice was also found to be rapid and essentially completed within 20 min (figure 5).

The most plausible mechanism to explain the fluorescence inhibition of a Cy5.5 conjugated PS DNA following addition of the Iowa Black complementary but not random control DNA is contact fluorescence inhibition following duplex formation. That the quenching and therefore hybridization is rapid is encouraging since rapid kinetics will be required for the *in vivo* applications contemplated. Rapid hybridization was previously observed in this laboratory by surface plasmon resonance in which a 25 mer Cy5.5 MORF duplexed with an 18 mer BHQ3 DNA was allowed to flow over an immobilized 18 mer PS DNA complementary to the MORF (12). Dissociation of the duplex with capture of the MORF was completed in less than 1 min.

Figure 4 presents whole body optical images of mice showing fluorescence inhibition at 30 min after the IV administration of Cy5.5-cDNA1 to mice administered IV Iowa Black-DNA1 10 min earlier. The results presented in Figure 5 show that at 20 min postadministration of Iowa Black DNA1, the quenching efficiency was 25 to 34% depending upon the view, with little change thereafter.

Fluorescence was apparent at all dosages in hairless the SKH-1 mice administered IV Cy5.5 cDNA1 alone, however the administration of 0.8 nmol (5 µg) of the Cy5.5 cDNA1 provided the most intense image. We conclude from these studies that the 0.8 nmol of Cy5.5 cDNA1 fluorescence may be detected with sufficient sensitivity and that the IV administration of 2.4 nmol of Iowa Black DNA1 (i.e. a 3 fold molar excess) provided obvious inhibition, superior to an equimolar administration and superior to the administration of 0.3 nmol of Cy5.5 cDNA1. The results confirm that a 0.8 nmol dosage of Cy5.5 cDNA1 provides sufficient fluorescent intensity for subsequent *in vivo* measurements with the camera of this investigation, that fluorescent intensity is stable over at least 1 h, that *in vivo* quenching of fluorescence occurs rapidly consistent with the earliest practical times between administrations and imaging.

Having thus established that rapid fluorescence quenching follows hybridization in solution both *in vitro* and *in vivo*, it was necessary to ensure that immobilization does not adversely influence these properties. Since MORF/PO DNA duplexes are stable in serum, an 18 mer PO immobilized microsphere was constructed consisting of a biotinylated MORF1 added first to the streptavidin coated bead followed by addition of the 36 mer PO Cy5.5 DNA with two 18 mer complementary regions, one against the standard sequence (i.e. cDNA1) and one against a new sequence (i.e. c'DNA2). Thereafter, the PS conjugated Iowa Black DNA2 quencher complementary to the 18 mer PO overhang of the bead duplex was administered and the fluorescence intensity was measured. As shown in figure 3, quenching inhibition in buffer was

completed in 20 min or less and with about 82% inhibition at the lowest 1 : 1 molar ratio despite the immobilization.

Finally, as shown in Figure 6, in mice implanted with the 36 mer PO Cy5.5 cc'DNA microspheres binding showed a decrease in target/nontarget ratio of 27% (dorsal view) and 38% (ventral view) within 1 h of administration of the 18 mer PS Iowa Black DNA2 but not the Iowa Black rDNA2 random control. The quenching efficiency was therefore remarkable despite the rapid clearance from circulation of the PS Iowa Black DNA2 and the poor accessibility of the subcutaneous microspheres from the circulation.

In conclusion, the results of this proof of concept study show that duplexes of a fluorophore- and a quencher-conjugated linear oligomer can be designed to be sufficiently stable for use in vivo, that fluorescence quenching follows duplex hybridization of Cy 5.5 and Iowa Black conjugated oligomers and can be detected in mice at reasonable concentrations, that the hybridization and therefore quenching is rapid whether free or immobilized and that the quenching is persistent. Future studies will apply the results of this investigation of fluorescent quenching to applications such as optical antisense and pretargeting imaging.

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Figure 1.

PAGE gel photograph presenting results of 37 °C stability measurements after 2 h of incubation at 37 °C. Lane 1- Low molecular weight DNA ladder; Lane 2-PBS incubation of PO DNA1/PS cc'DNA; Lane 3-PBS incubation of MORF1/PS cc'DNA; Lane 4-PBS incubation of MORF1/PO cc'DNA; Lane 5-PBS incubation of PO DNA1/PO cc'DNA; Lane 6-serum incubation of PO DNA1/PS cc'DNA; Lane 7-serum incubation of MORF1/PS cc'DNA; Lane 8-serum incubation of MORF1/PO cc'DNA; Lane 9-serum incubation of PO DNA1/PO cc'DNA; Lane 10-serum alone.

Figure 2.

Fluorescence intensity of wells containing the study PS Cy5.5 cDNA1 (open bars) and control PS Cy5.5 rDNA (closed bars) 30 min after the addition of PS Iowa Black DNA1 at different DNA1 : cDNA1 molar ratios from 0:1 to 3:1 in PBS buffer. Error bars represent one s.d. (N = 3).

Figure 3. Fluorescence intensity of wells with MORF1/PO Cy5.5 cc'DNA microspheres at 20 min (open bars) and 60 min (hatched bars) following addition of PS Iowa Black DNA2 at 0 to 15 fold molar excess to PO Cy5.5 cc'DNA in PBS/BSA binding buffer. Error bars represent one s.d, (N = 3).

Figure 4. Whole body ventral (left panel) and dorsal (right panel) fluorescent images of SKH-1 mice. From left to right in both panels: blank control, Cy5.5-cDNA1 alone, 1:1 and 3:1 molar ratios of Iowa Black-DNA1 to Cy5.5-cDNA1 with 10 min between administrations. Images at 30 min.

Figure 5.

Histograms showing the quenching efficiency in SKH-1 mice receiving IV 0.8 nmoles of PS Cy5.5 cDNA1 followed by a three fold molar excess of PS Iowa Black DNA1 10 min later. Results presented for both the dorsal view (open bars) and ventral view (hatched bars) obtained between 20 and 120 min thereafter. Error bars represent one s.d, (N = 3).

Figure 6.

The quenching efficiency 10 to 90 min after IV administration of 6.0 nmoles of PS Iowa Black DNA2 in SKH-1 mice implanted with 0.06 nmoles PO Cy5.5 cc'DNA microspheres. Results presented for both dorsal view (open bars) and ventral view (hatched bars). Error bars represent one s.d., (N=4).

Table 1

The base sequences of the PO and PS DNAs and MORF of this investigation.

Oligomer Type	Base Sequence	Modifications
MORF1, 18mer	5'-TCT-TCT-ACT-TCA-CAA-CTA	Native or 3' biotin conjugated
PO DNA1, 18 mer	5'-TCT-TCT-ACT- TCA-CAA-CTA	Native only
PO cc'DNA 36 mer	5'-TAG-TTG-TGA-AGT-AGA-AGA-GGT-GTA-GGA-GTC-GGT-GTT-3'	Native and 3' Cy5.5 conjugated
PS DNA1 18 mer	5'-TCT-TCT-ACT-TCA-CAA-CTA -	Native and 3' Iowa Black conjugated
PS cDNA1 18 mer	5'-TAG-TTG-TGA-AGT-AGA-AGA	5' Cy5.5 conjugated
PS random rDNA1 18 mer	5'-AGA-ATG-ATG- GTT-ATA-AGG	5' Cy5.5 conjugated
PS random rDNA2 18 mer	5'-ATA-CCA-ACC- GCC-TCC-ACA	5' Iowa Black conjugated
PS DNA2 18 mer	5'-AAC-ACC-GAC- TCC-TAC-ACC	5' Iowa Black conjugated
PS cc'DNA 36 mer	5'-TAG-TTG-TGA-AGT- AGA-AGA-GGT-GTA GGA-GTC-GGT-GTT-3'	Native

Complementary DNAs identified as cDNAs, random DNAs identified as rDNAs, DNAs with two complementary regions identified as cc'DNAs