Supplementary information

Imaging mRNA expression levels in living cells with PNA·DNA binary FRET probes delivered by cationic shell-crosslinked nanoparticles

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Experimental

Dissociation constant of PNA-261 for iNOS mRNA. This procedure follows the two step procedure described in *Bioconjugate Chemistry* 2012, 23, 574-585 involving the binding constant determination of an antisense ODN for the mRNA site in question (the actual site targeted by PNA-Cy5 is underlined), and then determining the binding constant of the PNA for the mRNA target site by a competition assay with the ODN.

Dissociation constant of ODN-261 for iNOS mRNA by a Dynabead-based binding assay. The radiolabeled ODN-261 d(GTTTTCTTCACGTTGTTGTTA) (100 pM) was incubated with biotinylated mRNA (0.01, 0.1, 1, 10, 100 nM) and 1 μL of RNase inhibitor for 4 h at 37 °C in a total volume of 100 μL. Streptavidin coated Dynabeads were added and mixed for 30 min and then separated by a magnet, washed twice and resuspended in 100 μL of hybridization buffer (5 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.1 M NaCl). The bound and free solutions were then assayed by liquid scintillation counting. The dissociation constant was determined by non-linear fitting of the fraction bound versus RNA concentration to equation (1) using the Kaleidagraph program.

$$(1)\ F_B = NSB + \frac{SB * (([L] + K_d + [RNA]) - \sqrt{([L] + K_d + [RNA])^2 - 4 * [RNA]})}{2 * [L]}$$

where FB is the fraction of bound ODN, NSB is the nonspecifically bound fraction, SB is the specifically bound fraction and was set equal to (1-NSB), [L] is total ODN concentration, K_d is dissociation constant of the ODN and [RNA] is total RNA concentration. The dissociation constants from three separate experiments were then averaged (Figure S1).

Dissociation constant of PNA-261 for iNOS mRNA by a competition assay.

Radiolabeled ODN (1000 pM) was incubated with biotinylated mRNA (10 pM) and 1 µL of RNase inhibitor for 4 h at 37 °C, to which unlabeled competitor PNA-261 5'-GTTTTCTTCACGTTGTTG-3' (0, 0.001, 0.01, 0.1, 1, 10 nM) was added. Streptavidin coated Dynabeads were then added and incubated for another 30 min at 37 °C. Following incubation, the reaction mixture was separated with a magnet, washed twice and resuspended in hybridization buffer (5 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.1 M NaCl). The solutions containing bound and free ODN were counted by liquid scintillation. The fraction of bound ODN (B) was then plotted against the PNA concentration ([PNA]), and the IC_{50} value was obtained by fitting the data to equation (2). The K_d for the PNA was then obtained using equation (3). The dissociation constants from three separate competition experiments were then averaged (Figure S2).

$$(2)\ B = B_{min} + \frac{B_{max} - B_{min}}{1 + 10^{\log[PNA] - \log(IC_{50})}}$$

$$(3)\ K_d(PNA) = \frac{IC_{50}}{1 + \frac{[ODN]}{K_d(ODN)}}$$

Table S1. BLAST of PNA-Cy5

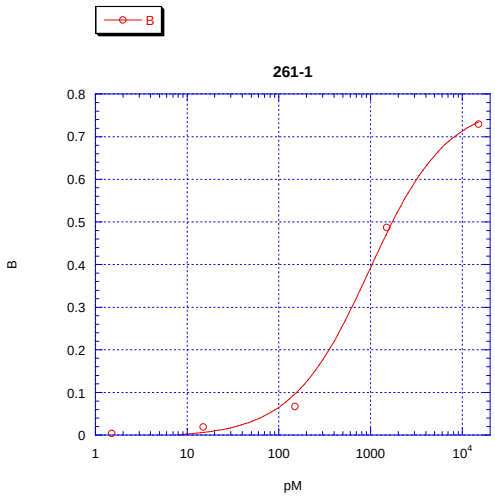
mRNA	alignment	
Mus musculus nitric oxide synthase 2, inducible (Nos2), mRNA	Query 1 TCTTCACGTTGTTGT 15 ||||||||||||||| Sbjct 271 TCTTCACGTTGTTGT 257	15/15
Mus musculus G protein-coupled receptor 107 (Gpr107), mRNA	Query 2 CTTCACGTTGTTG 14 ||||||||||||| Sbjct 739 CTTCACGTTGTTG 727	14/15
Mus musculus phosphoinositide-3-kinase, catalytic, gamma polypeptide (Pik3cg), transcript variant 2, mRNA	Query 1 TCTTCACGTTGTT 13 ||||||||||||| Sbjct 6664 TCTTCACGTTGTT 6652	13/15
Mus musculus glucan (1,4-alpha-), branching enzyme 1 (Gbe1), mRNA	Query 3 TTCACGTTGTTGT 15 ||||||||||||| Sbjct 581 TTCACGTTGTTGT 569	13/15

Table S2. Blast of FAM-PNA

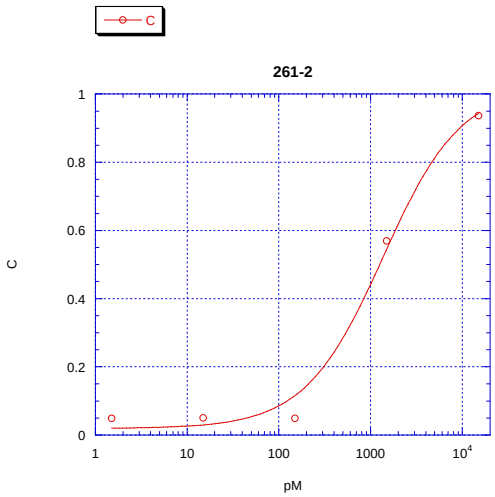
Mus musculus nitric oxide synthase 2, inducible (Nos2), mRNA	Query 1 ATGTCCTTTTCCTCT 15 ||||||||||||||| Sbjct 254 ATGTCCTTTTCCTCT 240	15/15
Mus musculus dynein, axonemal, heavy chain 10 (Dnahc10), mRNA	Query 2 TGTCCTTTTCCTCT 15 |||||||||||||| Sbjct 9119 TGTCCTTTTCCTCT 9106	14/15
Mus musculus cDNA sequence BC005561 (BC005561), mRNA	Query 19 TGTCCTTTTCCTCT 32 |||||||||||||| Sbjct 4837 TGTCCTTTTCCTCT 4824	14/15
Mus musculus pleckstrin homology domain containing, family A member 6 (Plekha6), transcript variant 2, mRNA	Query 1 ATGTCCTTTTCCT 13 ||||||||||||| Sbjct 5675 ATGTCCTTTTCCT 5663	13/15
Mus musculus SPARC related modular calcium binding 1 (Smoc1), transcript variant 1, mRNA	Query 2 TGTCCTTTTCCTC 14 ||||||||||||| Sbjct 2454 TGTCCTTTTCCTC 2442	13/15
Mus musculus sperm antigen with calponin homology and coiled-coil domains 1-like (Specc1l), transcript variant 1, mRNA	Query 2 TGTCCTTTTCCTC 14 ||||||||||||| Sbjct 2457 TGTCCTTTTCCTC 2445	13/15
Mus musculus lysine (K)-specific demethylase 5C (Kdm5c), mRNA	Query 2 TGTCCTTTTCCTC 14 ||||||||||||| Sbjct 893 TGTCCTTTTCCTC 881	13/15
Mus musculus even skipped homeotic gene 1 homolog (Evx1), mRNA	Query 2 TGTCCTTTTCCTC 14 ||||||||||||| Sbjct 2711 TGTCCTTTTCCTC 2699	13/15
Mus musculus laminin, alpha 5 (Lama5), mRNA	Query 3 GTCCTTTTCCTCT 15 ||||||||||||| Sbjct 11300 GTCCTTTTCCTCT 11288	13/15
Mus musculus nucleoporin 160 (Nup160), mRNA	Query 1 ATGTCCTTTTCCT 13 ||||||||||||| Sbjct 4647 ATGTCCTTTTCCT 4635	13/15
Mus musculus sarcolemma associated protein (Slmap), mRNA	Query 2 TGTCCTTTTCCTC 14 ||||||||||||| Sbjct 1905 TGTCCTTTTCCTC 1893	13/15

Table S3. Complete target site

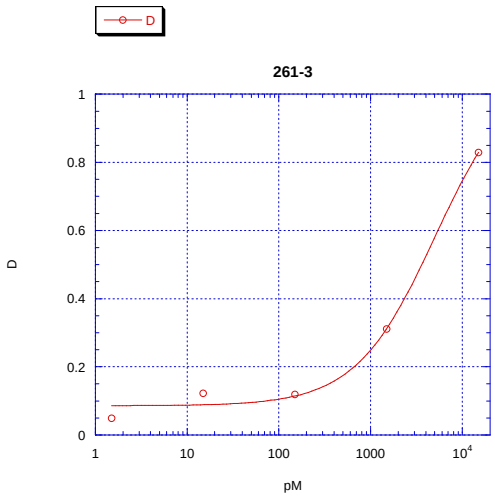
Mus musculus nitric oxide synthase 2, inducible (Nos2), mRNA	Query 1 TCTTCACGTTGTTGTNNATGTCCTTTTCCTCT 32 |||||||||||||||||||||||||| Sbjct 271 TCTTCACGTTGTTGTTAATGTCCTTTTCCTCT 240	
No mRNA matches > 15/30		



y = m1+m2*((100+m3+m0)-sqrt(...		
	Value	Error
m1	-0.0051874	0.016979
m2	0.78539	0.033969
m3	921.05	176.55
Chisq	0.0012996	NA
R	0.9985	NA



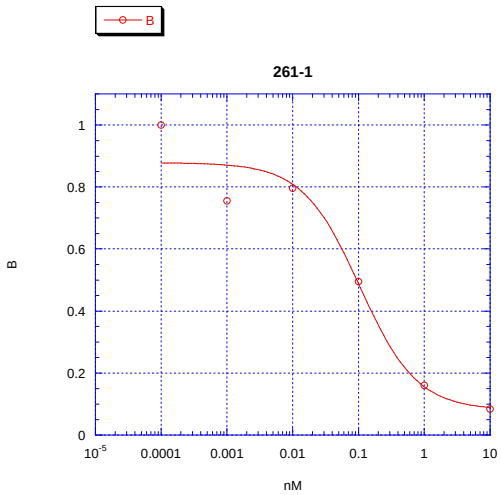
y = m1+m2*((100+m3+m0)-sqrt(...		
	Value	Error
m1	0.019338	0.036365
m2	1.0058	0.08
m3	1317.7	429.8
Chisq	0.0063076	NA
R	0.99522	NA



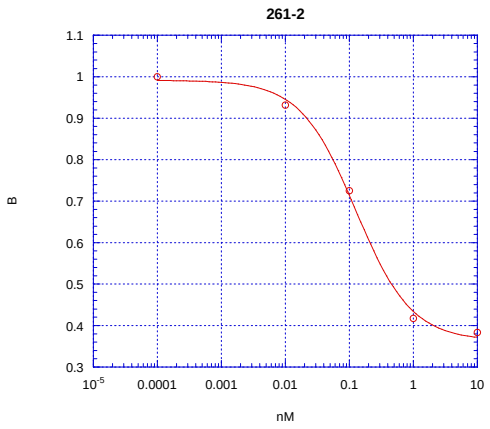
y = m1+m2*((100+m3+m0)-sqrt(...		
	Value	Error
m1	0.086023	0.021737
m2	0.9901	0.10252
m3	4972.5	1753.9
Chisq	0.0025004	NA
R	0.99691	NA

Average K_d(261) = 2.4 ± 0.6 nM

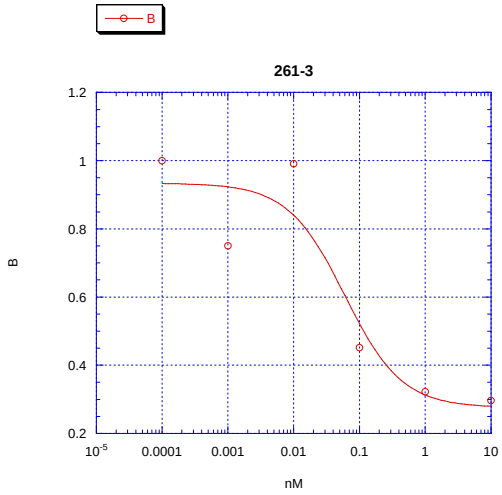
Figure S1. Antisense ODN-261 binding curves. Plots fraction iNOS mRNA bound vs free antisense ODN as a function of iNOS mRNA concentration and experimental fits to the equation described in the experimental section.



y = m1+(m2-m1)/(1+10^(log(m0...		
	Value	Error
m1	0.080567	0.082029
m2	0.8784	0.062555
m3	0.10483	0.061577
Chisq	0.028544	NA
R	0.9787	NA



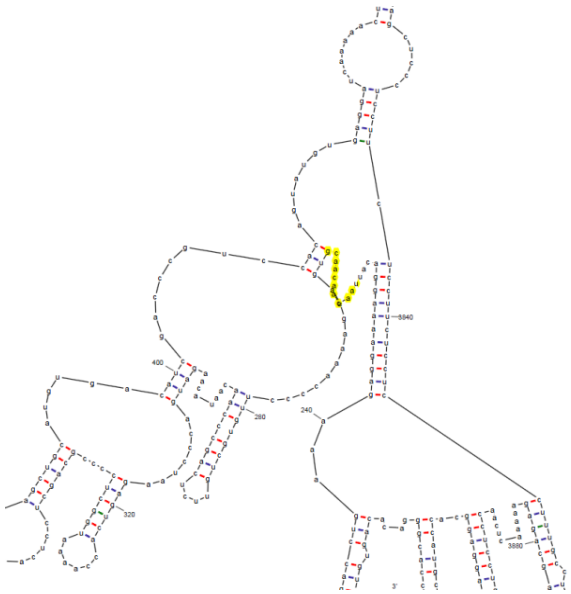
y = m1+(m2-m1)/(1+10^(log(m0...		
	Value	Error
m1	0.36357	0.017591
m2	0.99138	0.016258
m3	0.12693	0.020839
Chisq	0.00082477	NA
R	0.99873	NA



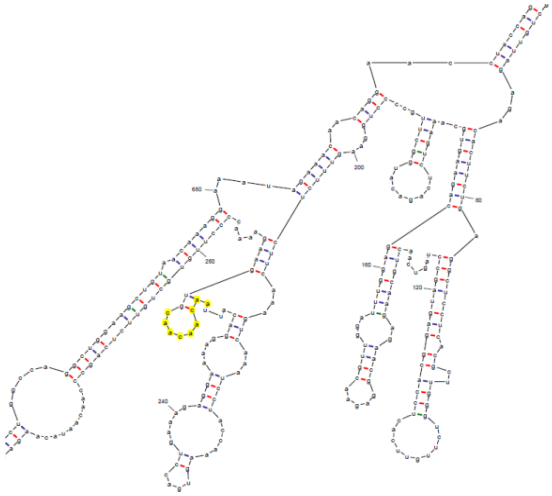
y = m1+(m2-m1)/(1+10^(log(m0...		
	Value	Error
m1	0.27585	0.11411
m2	0.93396	0.097599
m3	0.06001	0.064963
Chisq	0.062652	NA
R	0.93768	NA

Average IC_{50}(261)= 97 ± 30 pM

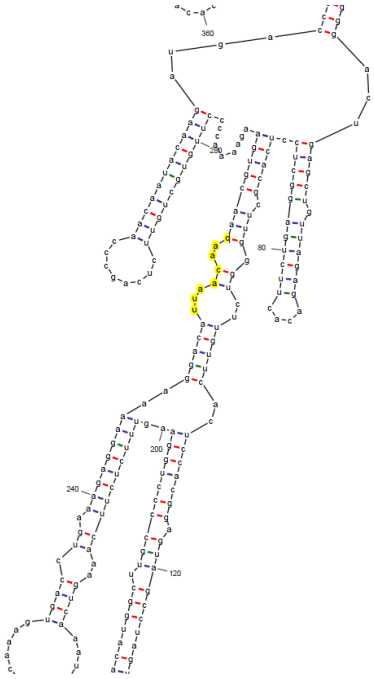
Figure S2. PNA-261 competition experiment. Plots fraction iNOS mRNA bound vs free antisense ODN as a function of PNA concentration in the competition experiment and experimental fits to the equation described in the experimental section.



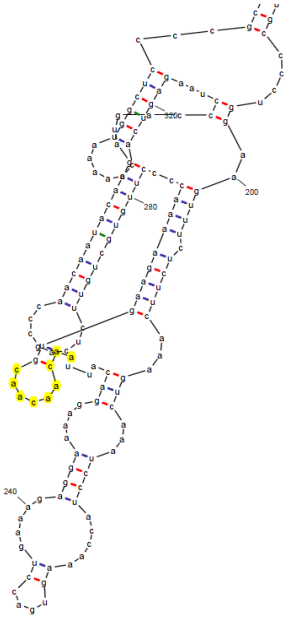
#2 *dG = -1326.30*



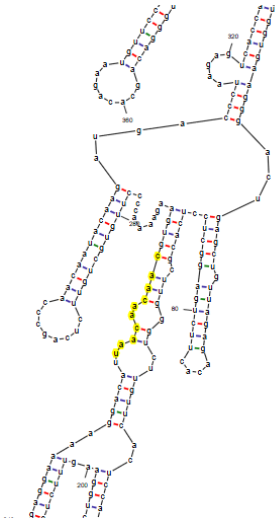
#5 *dG = -1323.56*



#18 *dG = -1322.61*



#6 *dG = -1322.14*



#1 *dG = -1319.22*

Figure S3. The five lowest energy structures predicted for the iNOS mRNA sequence by MFOLD. The target site identified by the RT-ROL method is highlighted in yellow.

Figure S4. Complete folded structure of the lowest energy -1326.30 kcal/mol iNOS mRNA structure. The arrow points to the site experimentally determined to be accessible to an antisense PNA (in yellow in Figure S3). It is highly unlikely that this site would have been selected over any of the other possible accessible sites.

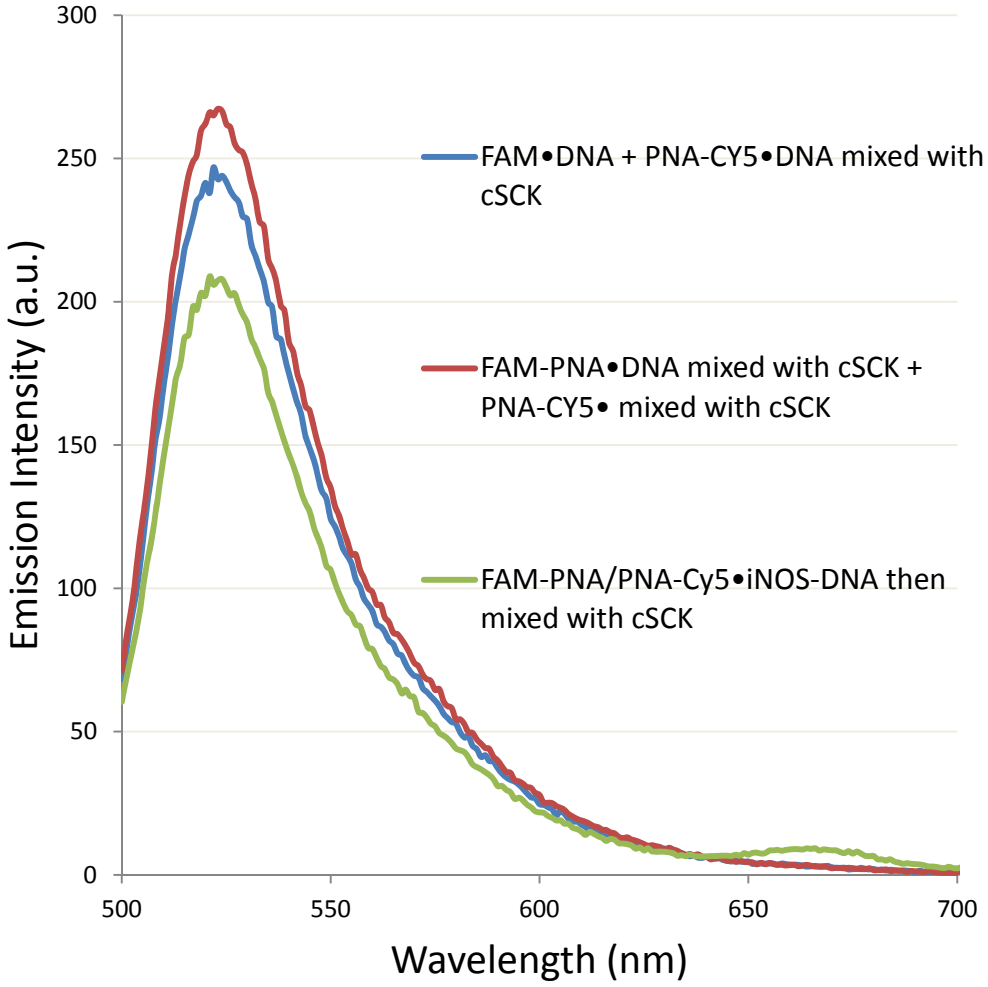


Figure S5. Effect of cSCK binding on FRET between PNA-Cy5•DNA and FAM-PNA•DNA. Probe concentration: 0.2 μM for PNA•DNA and iNOS-DNA. Solution: Opti-MEM at an N/P ratio of 10. The donor and acceptor probes were at 0.2 uM each and preannealed with complementary DNA prior to a) mixing together with cSCK, or b) mixing separately with cSCK and then combining, or c) mixing first with iNOS-DNA and then mixing with the cSCK.