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Synthesis and purification of modified ONs. **Y**-modified ONs were synthesized, purified and controlled for identify and purity, as previously described.[S1] Briefly, ON were synthesized via machine-assisted solid-phase DNA synthesis (0.2 μmol scale; 500 Å succinyl linked LCAA-CPG support) using extended coupling times (4,5-dicyanoimidazole as activator, 15 min, ~98% coupling yield) during incorporation of the corresponding phosphoramidite of monomer **Y** (or its A^{Bz}/C^{Bz}-analogues,[S2] which were used for the synthesis of Invaders used in the nd-FISH study). Cleavage from solid support and removal of protecting groups was accomplished using 32% aq. ammonia (55 °C, 12 h). ONs were purified via ion-pair reverse phase HPLC (XTerra MS C18 column) using a 0.05 mM triethylammonium acetate buffer - 25% water/acetonitrile (v/v) gradient, followed by detritylation (80% aq. AcOH) and precipitation from acetone (-18 °C for 12-16h).

Cy3-labeled **Y**-modified ONs were synthesized and purified as described above with the following modifications: the corresponding Cy3-phospohoramidite (Glen Research) was coupled using 4,5-dicyanoimidazole as an activator (2 min, anhydrous CH_3CN), which was followed by on-column removal of the MMTr group, cleavage from solid support and removal of protecting groups (32% aq. ammonia, 55° C, 3h), and purification by RP-HPLC.

The identity of all synthesized ONs was established through MALDI-MS/MS analysis recorded in positive ion mode on a quadrupole time-of-flight tandem mass spectrometer equipped with a MALDI source using anthranilic acid as a matrix and ammonium citrate as a co-matrix (Table S1), while purity (>80%) was verified by ion-pair reverse phase HPLC running in analytical mode.

Protocol - thermal denaturation studies. ON concentrations were estimated using the following extinction coefficients ($L \times mmol^{-1} \times cm^{-1}$) at 260 nm: dA (15.2), dC (7.05), dG (12.0), T (8.40), pyrene (22.4) and Cy3 (4.93).[S3] ONs (1.0 μM each strand) were thoroughly mixed in the thermal denaturation buffer (T_m buffer, 100 mM NaCl, 0.1 mM EDTA, pH 7.0 adjusted with 10 mM NaH_2PO_4 and 5 mM Na_2HPO_4), denatured by heating, and cooled to the starting temperature of the experiment. Thermal denaturation curves (A_{260} vs T) were recorded using a UV/VIS spectrometer equipped with a Peltier temperature programmer (Cary 100) and quartz optical cells with path-lengths of 10 mm. The temperature was varied from at least 15 °C below to 15 °C above the thermal denaturation temperature using a ramp of 0.5 °C min^{-1}. Thermal denaturation temperatures (T_m's) were determined as the maximum of the first derivative of thermal denaturation curves using the software provided with the UV/VIS spectrometer. Reported T_m's were determined as an average from at least two separate experiments within ±1.0 °C.

Protocol - determination of thermodynamic parameters. Thermodynamic parameters for duplex formation were determined through baseline fitting of denaturation curves using software provided with the UV/VIS spectrometer. Bimolecular reactions, two-state melting behavior, and a heat capacity change of $\Delta C_p = 0$ upon hybridization were assumed.[S4] A minimum of two experimental denaturation curves were each analyzed at least three times by two different operators to minimize errors arising from baseline choice. Averages and standard deviations are listed.

Protocol - recognition of DNA hairpins in cell-free assay. This assay was chosen *in lieu* of footprinting experiments to avoid the use of ^{32}P-labeled targets. Unmodified **DH1-DH7** were obtained from commercial sources and used without further purification. **DH1-DH7** were 3'-DIG-labeled using the 2nd generation DIG Gel Shift Kit (Roche Applied Bioscience) as recommended by the manufacturer. DIG-labeled ONs obtained in this manner, were diluted and used directly in the recognition experiments. An aliquot of probe (to give the probe excess stated in figure legends) was taken from a 1 μM solution in water (annealed at 90 °C for 10 min, cooled to room temperature over 15 min), placed in a vial and evaporated to dryness. To this, was added 2.5 μL 2X HEPES buffer (100 mM HEPES, 200 mM NaCl, 10 mM $MgCl_2$, 20% sucrose, 2.88 mM spermine tetrahydrochloride, pH 7.2) and 2.5 μL of DIG-labeled target from a 68.8 nM stock solution in water (separately annealed as described above). This mixture was incubated for 15h at room temperature, at which point it was diluted with 6x DNA loading dye (Fermentas) and loaded onto a 12% non-denaturing polyacrylamide gel using TBE as a running buffer (89 mM tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was performed using constant voltage (70 V) for 3h at ~4 °C (internally circulating ice-cold water). Gels were blotted onto a positively charged nylon membrane (Roche Applied Bioscience) using constant voltage with external cooling (100V, ~4 °C). The membranes were exposed to anti-digoxigenin-AP F_{ab} fragments as recommend by the manufacturer of the DIG Gel Shift Kit, transferred to a hybridization jacket, and incubated with the substrate (CSPD) in detection buffer for 10 min at 37 °C. The chemiluminescence of the formed product was captured on X-ray film, which was developed using an X-Omatic 1000A X-ray film developer (Kodak). The resulting bands were quantified using a Fluor-S MultiImager (Bio-Rad, Hercules, CA) equipped with Quantity One software. Invasion efficiency was estimated as the intensity ratio between the recognition

complex band and the total lane. An average of three independent experiments is reported along with standard deviations.

Protocol - cell culture and nuclei preparation. Male bovine kidney cells (Madin and Darby Bovine Kidney cells [MDBK], ATCC: CCL-22, Bethesda, MD) were aliquoted into a 25 mL tissue culture flask containing 5 mL DMEM/10% FBS (Invitrogen) and incubated in a 5% CO_2 atmosphere at 37 °C for ~20h. An aliquot of colcemid (65 uL at 10 ug/mL, KaryoMAX, Invitrogen) was added to the cell culture and incubated for additional 20 min. The cells were then trypsinized (0.05% Trypsin/EDTA, Invitrogen) to detach adherent cells. The loosened cells were transferred to a 15 mL conical tube, gently centrifuged, and the supernatant removed. The pellet was resuspended in 75 mM KCl for 20 min at room temperature to lyze the cells. Cells were prefixed for 10 min using Carnoy's fixative (3:1 methanol/acetic acid) and centrifuged (1000 rpm for 10 min). The supernatant was removed and the nuclei fixed for additional 30 min at room temperature using Carnoy's fixative. The suspension was centrifuged as described above and resuspended in Carnoy's fixative. The nuclei were stored at -20 °C until use.

Protocol – nd-FISH experiments. An aliquot (1.5-2.0 uL) of the abovementioned suspension was dropped onto a plastic slide (Sex-Y™, Minitube, Verona, WI) and allowed to dry on a 37 °C warming plate. Unless otherwise mentioned, an aliquot of the labeling buffer [~30 ng of Cy3-labeled probes in 190 uL 1X PCR buffer (10 mM Trizma-HCl, 50mM KCl, pH 8.3; Sigma Aldrich P2317)] was placed on top of the fixed nuclei. The slide was placed in a plastic culture dish, covered with a lid, and moved to a 38.5 °C incubator for 3h, unless otherwise mentioned.

Following incubation, the slide was rinsed for 3 min in warm TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0; 38.5 °C) and left to dry at room temperature. An aliquot (~3 uL) of Gold SlowFade plus DAPI (Invitrogen) was placed directly on the slide and a round coverslip was mounted for fluorescence imaging. A fluorescence microscope (50 W, HBO mercury lamp, Zeiss AxioSkop 40), equipped with Cy3 and DAPI filter sets, was used to visualize the nuclei at 400x magnification. Images of fluorescently labeled nuclei were captured using a Zeiss AxioCam MRc5 camera and processed with appropriate software (AxioVision version 4.8).

Definition if "interstrand zipper arrangement". The following nomenclature describes the relative arrangement between two monomers positioned on opposing strands in a duplex (adapted from reference S5). The number *n* describes the distance measured in number of base pairs and has a positive value if a monomer is shifted toward the 5'-side of its own strand relative to a second reference monomer on the other strand. Conversely, *n* has a negative value if a monomer is shifted toward the 3'-side of its own strand relative to a second reference monomer on the other strand.

Table S1. MALDI-MS of modified oligonucleotides used in this study.[a]

ON	Sequence	Observed m/z $[M+H]^+$	Calculated m/z $[M+H]^+$
Y1	5'-GG**U**ATATATAGGC	4228	4228
Y2	5'-GGTA**U**ATATAGGC	4228	4228
Y3	5'-GGTATATA**U**AGGC	4228	4228
Y4	3'-CCA**U**ATATATCCG	4108	4108
Y5	3'-CCATA**U**ATATCCG	4108	4108
Y6	3'-CCATATATA**U**CCG	4108	4108
Y7	5'-GG**U**A**U**ATATAGGC	4444	4444
Y8	5'-GG**U**ATATA**U**AGGC	4444	4444
Y9	3'-CCA**U**A**U**ATATCCG	4324	4324
Y10	3'-CCA**U**ATATA**U**CCG	4324	4324
Cy3INV-u	5'-[Cy3]-**A**GCCC**U**GTGCC**C**TG	5396	5398
Cy3INV-l	3'-T**C**GGGA**C**ACGGG**A**C-[Cy3]	5509	5510
Cy3INVmm-u	5'-[Cy3]-**A**GC*G*C**U**G*A*G*G*C**C**TG	5486	5486
Cy3INVmm-l	3'-T**C**G*C*GA**C***T*C*C*GG**A**C-[Cy3]	5421	5421
Cy3DNA-u	5'-[Cy3]-AGCCCTGTGCCCTG	4720	4721
Cy3DNA-l	3'-TCGGGACACGGGAC-[Cy3]	4818	4819

[a] **A**, **C** and **U** denote 2'-*O*-(pyren-1-yl)methyladenosine,[S2] 2'-*O*-(pyren-1-yl)methylcytidine[S2] and 2'-*O*-(pyren-1-yl)methyluridine (monomer **Y**)[S1], respectively. "u" and "l" stands for 'upper' and 'lower', respectively.

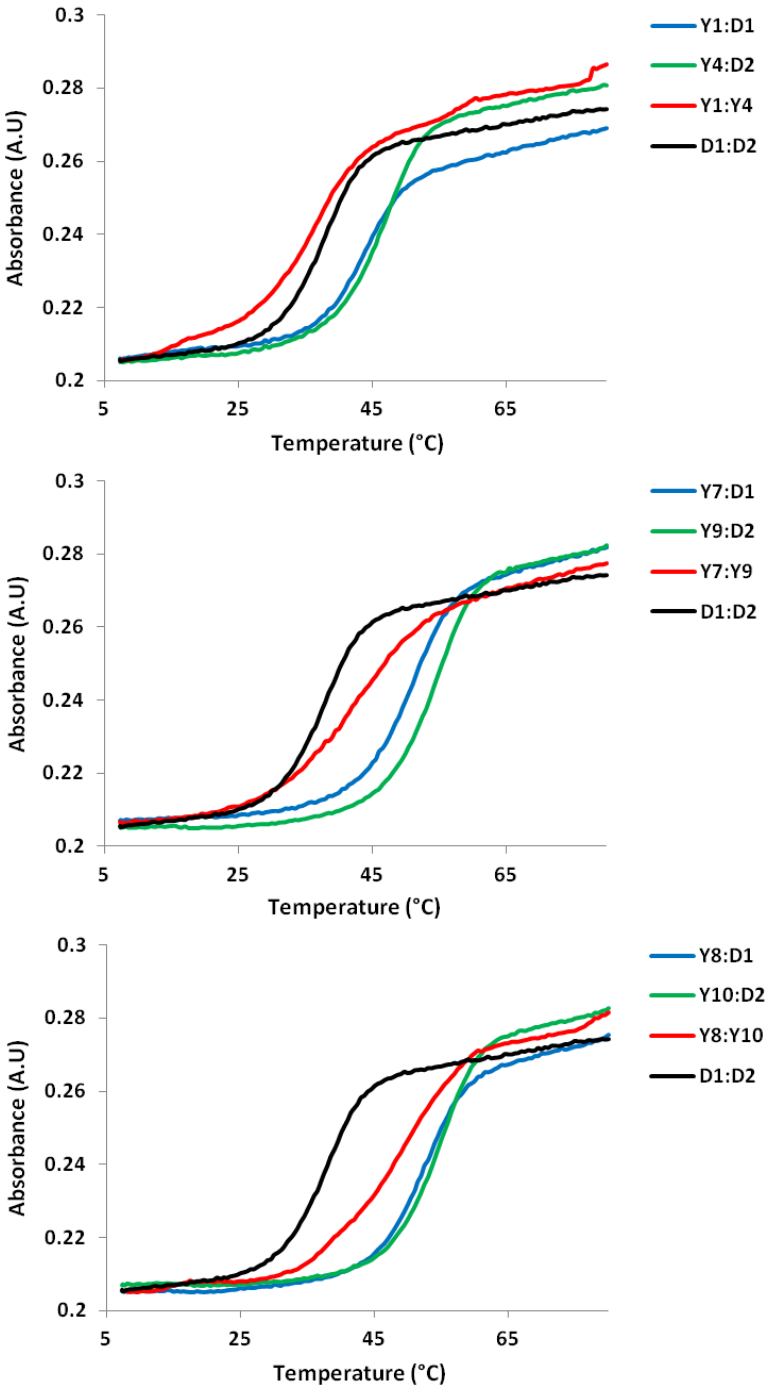

Figure S1. Thermal denaturation curves of representative Invaders (**Y1**:**Y4**, **Y7**:**Y9** and **Y8**:**Y10**), corresponding duplexes between individual probe strands and ssDNA, and unmodified reference duplex **D1**:**D2**. For experimental conditions, see Table 1.

Table S2. Thermodynamic parameters (Gibbs free energy). Change in Gibbs free energy at 293K upon duplex formation (ΔG^{293}) and available free energy upon probe recognition of iso-sequential dsDNA target **D1**:**D2** (ΔG_{rec}^{293}).[a]

Entry	ON	Sequence	$\Delta G^{293}[\Delta\Delta G^{293}]$ (kJ/mol)			ΔG_{rec}^{293} (kJ/mol)
			upper strand vs ssDNA	lower strand vs ssDNA	probe duplex	
1	**Y1** **Y4**	5'-GG**Y**ATATATAGGC 3'-CCA**Y**ATATATCCG	-63±1 [-6]	-65±3 [-8]	-46±1 [+11]	-25
2	**Y2** **Y5**	5'-GGTA**Y**ATATAGGC 3'-CCATA**Y**ATATCCG	-67±3 [-10]	-66±3 [-9]	-49±2 [+8]	-28
3	**Y3** **Y6**	5'-GGTATATA**Y**AGGC 3'-CCATATATA**Y**CCG	-66±2 [-9]	-64±2 [-7]	-45±1 [+12]	-28
4	**Y7** **Y9**	5'-GG**Y**A**Y**ATATAGGC 3'-CCA**Y**A**Y**ATATCCG	-69±3 [-12]	-71±6 [-14]	-50±1 [+7]	-32
5	**Y8** **Y10**	5'-GG**Y**ATATA**Y**AGGC 3'-CCA**Y**ATATA**Y**CCG	-71±4 [-14]	-75±5 [-18]	-56±3 [+1]	-33
6	**Y2** **Y6**	5'-GGTA**Y**ATATAGGC 3'-CCATATATA**Y**CCG	-67±3 [-10]	-64±2 [-7]	-70±2 [-13]	-4
7	**Y3** **Y5**	5'-GGTATATA**Y**AGGC 3'-CCATA**Y**ATATCCG	-66±2 [-9]	-66±3 [-9]	-67±13 [-10]	-8

[a] $\Delta\Delta G^{293}$ is measured relative to ΔG^{293} for **D1**:**D2** = -57 kJ/mol. $\Delta G_{rec}^{293} = \Delta G^{293}$ (upper strand vs ssDNA) + ΔG^{293} (lower strand vs ssDNA) - ΔG^{293} (probe duplex) - ΔG^{293} (dsDNA target). "±" denotes standard deviation.

Table S3. Thermodynamic parameters (enthalpy). Change in enthalpy upon duplex formation (ΔH) and change in enthalpy upon probe recognition of iso-sequential dsDNA target **D1**:**D2** (ΔH_{rec}).[a]

Entry	ON	Sequence	ΔH [$\Delta\Delta H$] (kJ/mol)			ΔH_{rec} (kJ/mol)
			upper strand vs ssDNA	lower strand vs ssDNA	probe duplex	
1	**Y1** **Y4**	5'-GG**Y**ATATATAGGC 3'-CCA**Y**ATATATCCG	-407±7 [-25]	-394±20 [-12]	-267±10 [+115]	-152
2	**Y2** **Y5**	5'-GGTA**Y**ATATAGGC 3'-CCATA**Y**ATATCCG	-415±18 [-33]	-396±21 [-14]	-297±9 [+85]	-131
3	**Y3** **Y6**	5'-GGTATATA**Y**AGGC 3'-CCATATATA**Y**CCG	-407±12 [-25]	-390±12 [-8]	-253±12 [+129]	-162
4	**Y7** **Y9**	5'-GG**Y**A**Y**ATATAGGC 3'-CCA**Y**A**Y**ATATCCG	-385±20 [-3]	-366±53 [+16][b]	-256±7 [+126]	-113
5	**Y8** **Y10**	5'-GG**Y**ATATA**Y**AGGC 3'-CCA**Y**ATATA**Y**CCG	-397±30 [-15]	-404±35 [-22]	-274±27 [+108]	-145
6	**Y2** **Y6**	5'-GGTA**Y**ATATAGGC 3'-CCATATATA**Y**CCG	-415±18 [-33]	-390±12 [-8]	-361±12 [+21]	-62
7	**Y3** **Y5**	5'-GGTATATA**Y**AGGC 3'-CCATA**Y**ATATCCG	-407±12 [-25]	-396±21 [-14]	-300±117 [+82][b]	-121[b]

[a] $\Delta\Delta H$ is measured relative to ΔH for **D1**:**D2** = -382 kJ/mol. ΔH_{rec} = ΔH (upper strand vs ssDNA) + ΔH (lower strand vs ssDNA) - ΔH (probe duplex) - ΔH (dsDNA target). "±" denotes standard deviation.

[b] Please note the large standard deviation.

Table S4. Thermodynamic parameters (entropy). Change in entropy at 293K upon duplex formation ($-T^{293}\Delta S$) and change in entropy upon probe recognition of iso-sequential dsDNA target **D1**:**D2** ($-T^{293}\Delta S_{rec}$).[a]

Entry	ON	Sequence	$-T^{293}\Delta S$ [$\Delta(T^{293}\Delta S)$] (kJ/mol)			$-T^{293}\Delta S_{rec}$ (kJ/mol)
			upper strand vs DNA	lower strand vs DNA	probe duplex	
1	**Y1** **Y4**	5'-GG**Y**ATATATAGGC 3'-CCA**Y**ATATATCCG	344±6 [+19]	329±18 [+4]	221±10 [-104]	127
2	**Y2** **Y5**	5'-GGTA**Y**ATATAGGC 3'-CCATA**Y**ATATCCG	346±14 [+21]	329±18 [+4]	248±8 [-77]	102
3	**Y3** **Y6**	5'-GGTATATA**Y**AGGC 3'-CCATATATA**Y**CCG	341±10 [+16]	327±11 [+2]	208±11 [-117]	135
4	**Y7** **Y9**	5'-GG**Y**A**Y**ATATAGGC 3'-CCA**Y**A**Y**ATATCCG	316±18 [-9]	296±47 [-29]	206±6 [-119]	81
5	**Y8** **Y10**	5'-GG**Y**ATATA**Y**AGGC 3'-CCA**Y**ATATA**Y**CCG	325±26 [0]	329±31 [+4]	218±23 [-107]	111
6	**Y2** **Y6**	5'-GGTA**Y**ATATAGGC 3'-CCATATATA**Y**CCG	346±14 [+21]	327±11 [+2]	291±10 [-34]	57
7	**Y3** **Y5**	5'-GGTATATA**Y**AGGC 3'-CCATA**Y**ATATCCG	341±10 [+16]	329±18 [+4]	233±105 [-92][b]	113[b]

[a] $\Delta(T^{293}\Delta S)$ is measured relative to $-T^{293}\Delta S$ for **D1**:**D2** = 325 kJ/mol. $-T^{293}\Delta S_{rec} = T^{293}\Delta S$ (upper strand vs ssDNA) + $T^{293}\Delta S$ (lower strand vs ssDNA) - $T^{293}\Delta S$ (probe duplex) - $T^{293}\Delta S$ (dsDNA target). "±" denotes standard deviation.

[b] Please note the large standard deviation.

Table S5. Recognition efficiency upon incubation of **DH1** with 200-fold molar excess of Invaders.[a]

Entry	ON	Sequence	Recognition (%)
1	**Y1** **Y4**	5'-GG**Y**ATATATAGGC 3'-CCA**Y**ATATATCCG	46±5
2	**Y2** **Y5**	5'-GGTA**Y**ATATAGGC 3'-CCATA**Y**ATATCCG	40±10
3	**Y3** **Y6**	5'-GGTATATA**Y**AGGC 3'-CCATATATA**Y**CCG	15±7
4	**Y7** **Y9**	5'-GG**Y**A**Y**ATATAGGC 3'-CCA**Y**A**Y**ATATCCG	74±5
5	**Y8** **Y10**	5'-GG**Y**ATATA**Y**AGGC 3'-CCA**Y**ATATA**Y**CCG	43±5
6	**Y2** **Y6**	5'-GGTA**Y**ATATAGGC 3'-CCATATATA**Y**CCG	<10
7	**Y3** **Y5**	5'-GGTATATA**Y**AGGC 3'-CCATA**Y**ATATCCG	<10

[a] Incubation conditions as outlined in Figure 2 in main manuscript. Recognition efficiency was performed in triplicates. "±" denotes standard deviation.

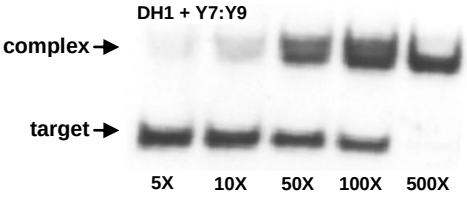

Figure S2. Gel electropherograms illustrating recognition of **DH1** with increasing concentrations of Invader **Y7**:**Y9**. See Figure 2 in main manuscript for incubation conditions. The presence of two bands seen in the recognition regions is attributed to two different conformations of the recognition complex.

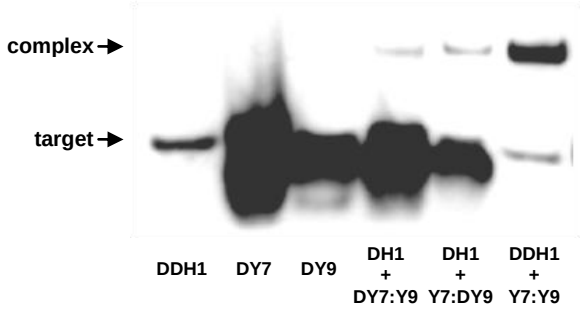

Figure S3. Recognition experiments between dsDNA target **DH1** and 200-fold molar excess of **Y7**:**Y9**, in which either **Y7**, **Y9** or **DH1** are DIG-labeled (i.e., **DY7**, **DY9** or **DDH1**). See Figure 2 for incubation conditions. The intense target signals in lanes 2-5 are caused by excess DIG-labeled species (i.e., **DY7**, **DY9**, **DY7**:**Y9** and **Y7**:**DY9**, respectively).

Table S6. T_m's for probes used in nd-FISH study.[a]

ON	Invader	T_m [ΔT_m] (°C)			
		upper strand vs ssDNA	lower strand vs ssDNA	duplex probe	dsDNA target
Cy3INV	5'-Cy3-**A**GCCC**U**GTGCC**C**TG 3'-T**C**GGGA**C**ACGGG**A**C-Cy3	69.5 [+9.0]	74.0 [+13.5]	66.0 [+5.5]	60.5[b]
Cy3DNA	5'-Cy3-AGCCCTGTGCCCTG 3'-TCGGGACACGGGAC-Cy3	62.5 [+2.0]	64.0 [+3.5]	66.5 [+6.0]	60.5[b]
Cy3INVmm	5'-Cy3-**A**GC*G*C**U**G*A*G*G*C**C**TG 3'-T**C**G*C*GA**C***T*C*C*C*GG**A**C-Cy3	72.5 [+9.0]	78.5 [+15.0]	65.5 [+2.0]	63.5[c]

[a] ΔT_m = change in T_m relative to corresponding unmodified and unlabeled reference duplex; T_m's determined as the first derivative maximum of denaturation curves (A_{260} vs T) recorded in medium salt buffer ([Na^+] = 110 mM, [Cl^-] = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)), using 1.0 µM of each strand. T_m's are averages of at least two measurements within 1.0 °C; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer. **A**, **C** and **U** denote 2'-*O*-(pyren-1-yl)methyladenosine,[S2] 2'-*O*-(pyren-1-yl)methylcytidine[S2] and 2'-*O*-(pyren-1-yl)methyluridine (monomer **Y**),[S1] respectively. **Please note**: the following T_m's are observed for mismatched probe-target duplexes: T_m (**Cy3INVmm upper strand** vs **D4**) = 41.5 °C and T_m (**Cy3INVmm lower strand** vs **D3**) = 33.0 °C, which suggests that **Cy3INVmm** (T_m = 65.5 °C) does not exhibit favorable affinity toward **D3**:**D4** (T_m = 60.5 °C), i.e., the dsDNA target of **Cy3INV**. The shape of the thermal denaturation curves of the mismatched duplexes, did not allow for determination of thermodynamic parameters.

[b] dsDNA target **D3**:**D4** 5'-AGCCCTGTGCCCTG:3'-TCGGGACACGGGAC.

[c] dsDNA target **D5**:**D6** 5'-AGC*G*CTG*A*G*G*CCTG:3'-TCG*C*GAC*TCC*GGAC.

Table S7. Thermodynamic parameters (Gibbs free energy) for probes used in nd-FISH study. Change in Gibbs free energy at 310K upon duplex formation (ΔG^{310}) and available free energy upon probe recognition of iso-sequential dsDNA targets (ΔG^{310}_{rec}).[a]

ON	Sequence	ΔG^{310} [$\Delta\Delta G^{310}$] (kJ/mol)			ΔG^{310}_{rec} (kJ/mol)
		upper strand vs ssDNA	lower strand vs ssDNA	probe duplex	
Cy3INV	5'-Cy3-**A**GCCC**U**GTGCC**C**TG 3'-T**C**GGGA**C**ACGGG**A**C-Cy3	-77±2 [-8]	-83±2 [-14]	-59±3 [+10]	-32
Cy3DNA	5'-Cy3-AGCCCTGTGCCCTG 3'-TCGGGACACGGGAC-Cy3	-68±2 [+1]	-71±2 [-2]	-76±3 [-7]	+6
Cy3INVmm	5'-Cy3-**A**GC*G*C**U**G*A*G*G*C**C**TG 3'-T**C**GCGA**C***T*C*C*GG**A**C-Cy3	-78±4 [-4]	-100±3 [-26]	-61±2 [+13]	-43

[a] $\Delta\Delta G^{310}$ is measured relative to ΔG^{310} for **D3**:**D4 =** -69±2 kJ/mol, except for duplexes involving strands from **Cy3INVmm** (bottom row) in which case $\Delta\Delta G^{310}$ is measured relative to ΔG^{310} for **D5**:**D6 =** -74±2 kJ/mol. ΔG^{310}_{rec} = ΔG^{310} (upper strand vs ssDNA) + ΔG^{310} (lower strand vs ssDNA) – ΔG^{310} (probe duplex) – ΔG^{310} (dsDNA target). "±" denotes standard deviation.

Table S8. Thermodynamic parameters (enthalpy) for probes used in nd-FISH study. Change in enthalpy upon duplex formation (ΔH) and change in enthalpy upon probe recognition of iso-sequential dsDNA targets (ΔH_{rec}).[a]

ON	Sequence	ΔH [$\Delta\Delta H$] (kJ/mol)			ΔH_{rec} (kJ/mol)
		upper strand vs ssDNA	lower strand vs ssDNA	probe duplex	
Cy3INV	5'-Cy3-**A**GCCC**U**GTGCC**C**TG 3'-T**C**GGGA**C**ACGGG**A**C-Cy3	-411±22 [+13]	-435±19 [-11]	-254±21 [+170]	-168
Cy3DNA	5'-Cy3-AGCCCTGTGCCCTG 3'-TCGGGACACGGGAC-Cy3	-395±25 [+29]	-416±35 [+8]	-451±33 [-27]	+64
Cy3INVmm	5'-Cy3-**A**GC*G*C**U**G*A*G*G*C**C**TG 3'-T**C**GCGA**C***T*C*C*GG**A**C-Cy3	-393±46 [+60]	-538±10 [-85]	-257±19 [+196]	-221

[a] $\Delta\Delta H$ is measured relative to ΔH for **D3**:**D4 =** -424±23 kJ/mol, except for duplexes involving strands from **Cy3INVmm** (bottom row) in which case $\Delta\Delta H$ is measured relative to ΔH for **D5**:**D6 =** -453±13 kJ/mol. ΔH_{rec} = ΔH (upper strand vs ssDNA) + ΔH (lower strand vs ssDNA) - ΔH (probe duplex) - ΔH (dsDNA target). "±" denotes standard deviation.

Table S9. Thermodynamic parameters (entropy) for probes used in nd-FISH study. Change in entropy at 310K upon duplex formation ($-T^{310}\Delta S$) and change in entropy upon probe recognition of iso-sequential dsDNA target **D3**:**D4** ($-T^{310}\Delta S_{rec}$).[a]

ON	Sequence	$-T^{310}\Delta S$ [$\Delta(T^{310}\Delta S)$] (kJ/mol)			$-T^{310}\Delta S_{rec}$ (kJ/mol)
		upper strand vs DNA	lower strand vs DNA	probe duplex	
Cy3INV	5'-Cy3-**A**GCCC**U**GTGCC**C**TG 3'-T**C**GGGA**C**ACGGG**A**C-Cy3	334±20 [-21]	352±19 [-3]	195±18 [-160]	+136
Cy3DNA	5'-Cy3-AGCCCTGTGCCCTG 3'-TCGGGACACGGGAC-Cy3	327±23 [-28]	345±32 [-10]	375±30 [+20]	-58
Cy3INVmm	5'-Cy3-**A**GC*G*C**U**G*A*G*G*C**C**TG 3'-T**C**G*C*GA**C**T*C*C*C*GG**A**C-Cy3	315±42 [-64]	438±8 [+59]	196±17 [-183]	+178

[a] $\Delta(T^{310}\Delta S)$ is measured relative to $-T^{310}\Delta S$ for **D3**:**D4** = 355±20 kJ/mol, except for duplexes involving strands from **Cy3INVmm** (bottom row) in which case $\Delta(T^{310}\Delta S)$ is measured relative to $-T^{310}\Delta S$ for **D5**:**D6** = 379±11 kJ/mol. – $T^{310}\Delta S_{rec} = T^{310}\Delta S$ (upper strand vs ssDNA) + $T^{310}\Delta S$ (lower strand vs ssDNA) – $T^{310}\Delta S$ (probe duplex) – $T^{310}\Delta S$ (dsDNA target). "±" denotes standard deviation.

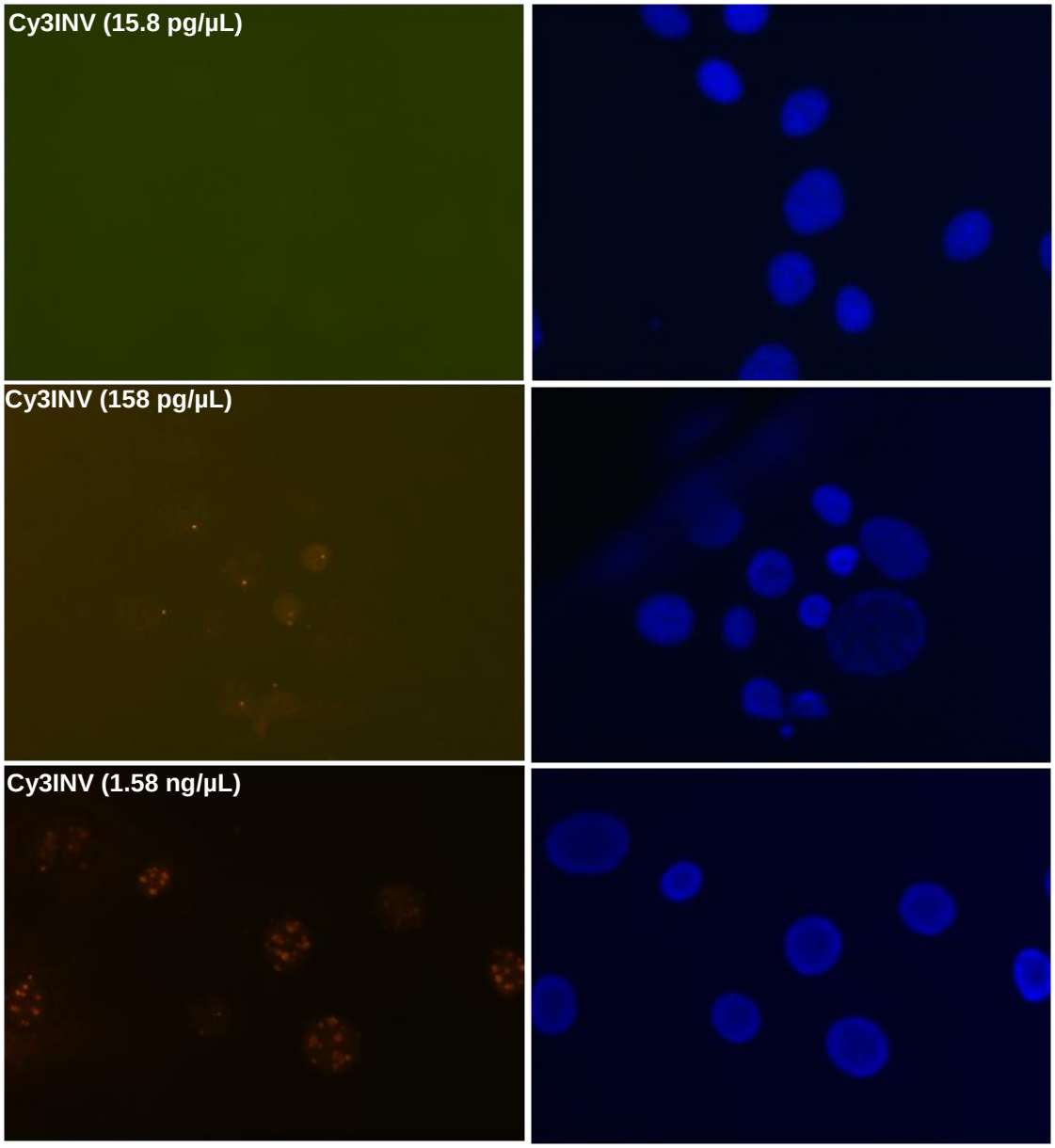

Figure S4. Dose-response nd-FISH studies using **Cy3INV** (180 min incubation). Cy3 and DAPI emission shown in left and right column, respectively. ~30 ng of Cy3INV in 190 uL 1X PCR buffer (i.e., 158 ng/µL) was selected as the optimal dose. As expected, large probe excess results in off-target labeling.

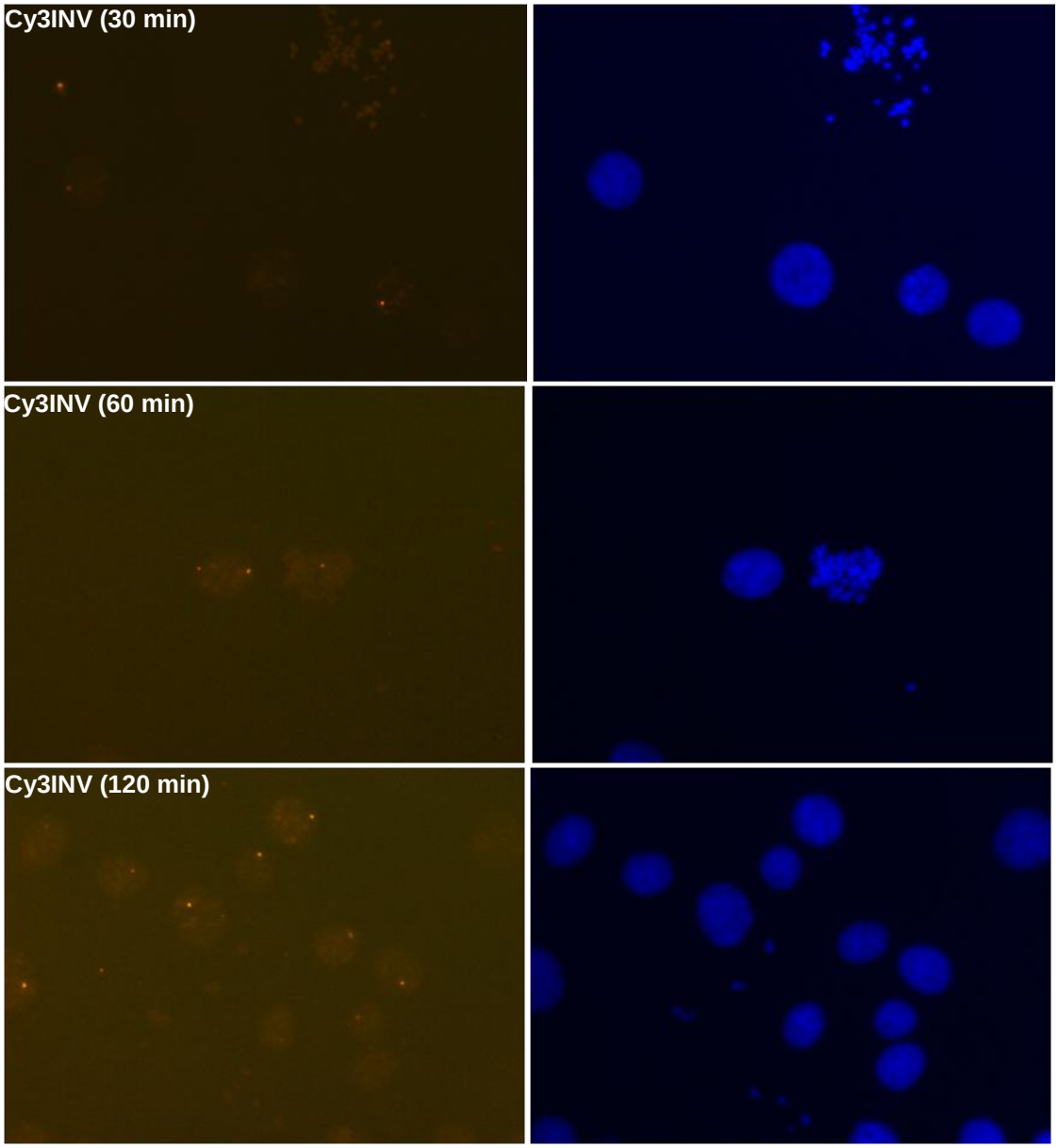

Figure S5. Time-course nd-FISH studies using **Cy3INV** at 158 pg/µL. Cy3 and DAPI emission shown in left and right column, respectively. Signals are observed after 30 min incubation and increase in intensity with longer incubation times.

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