Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2018

Supplemental Information (SI)

Improving NanoCluster Beacon performance by blocking the unlabeled NC probes

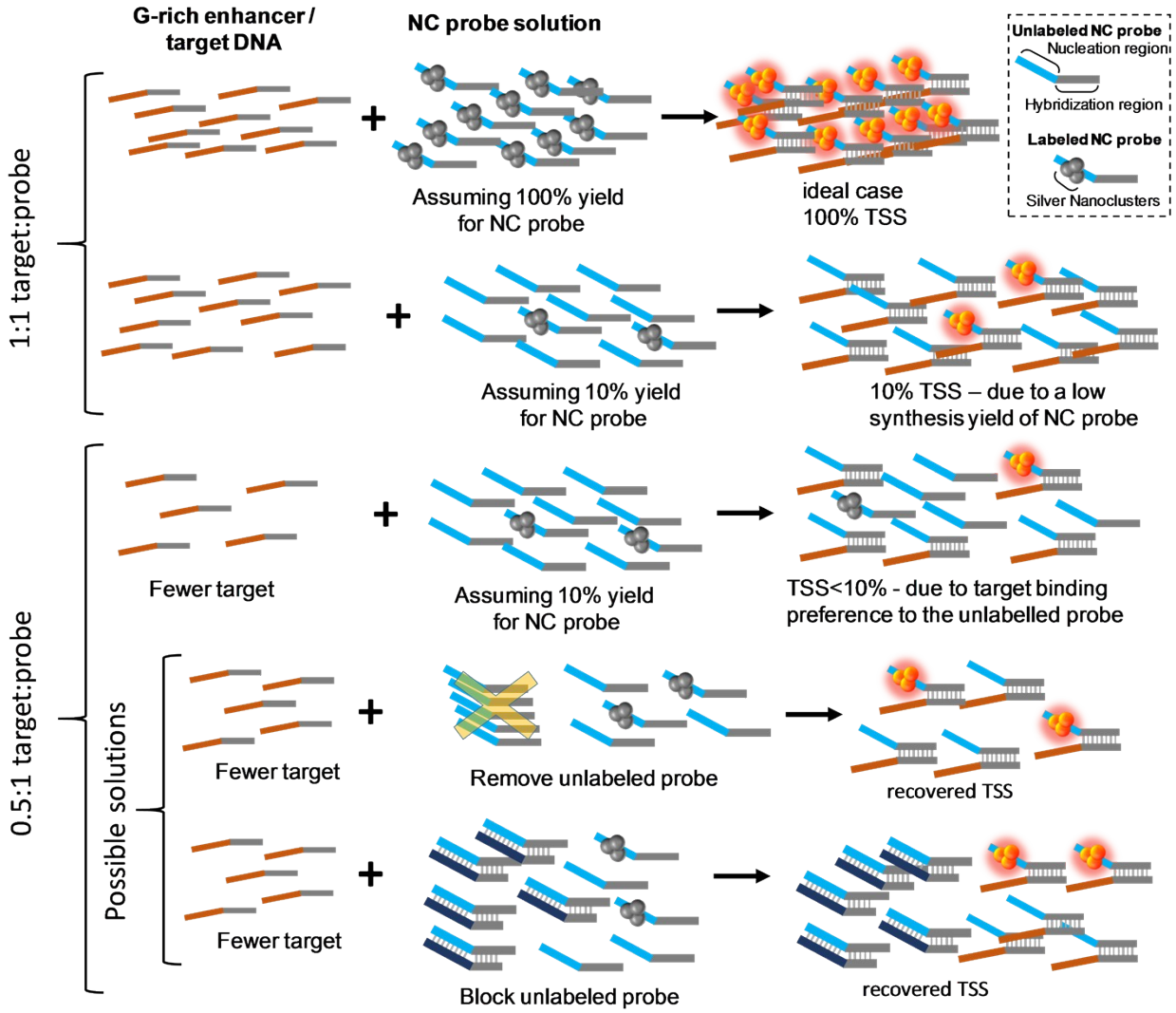
Yu-An Chen[1], Huong T. Vu[1], Yen-Liang Liu[1], Yuan-I Chen[1], Trung Duc Nguyen[1], Yu-An Kuo[1], Soonwoo Hong[1], Yin-An Chen[2], Savannah Carnahan[3], Jeffrey T. Petty[3] and Hsin-Chih Yeh[1,4,*]

[1] Department of Biomedical Engineering, University of Texas at Austin, Austin, Texas, 78712, USA
[2] Department of Chemistry, University of Texas at Austin, Austin, Texas, 78712, USA
[3] Department of Chemistry, Furman University, Greenville, South Carolina, 29613, USA
[4] Texas Materials Institute, University of Texas at Austin, Austin, Texas, 78712, USA

Content

Supplemental Note S1: Definition of target specific fluorescence signal (TSS)

Here we explain the definition of target specific fluorescence signal (TSS). It is the fraction of target strands bound to the functional (labeled) probe and emit fluorescence signal. Ideally, we would want to have 100% target bound to 100% labeled probe (first row). However, we estimated that the TSS can be as low as 10% (second row). In the case of insufficient target strands, the TSS can even be lower (third row). In order to enhance the TSS, we have to either remove or block the unlabeled NCs. The earlier has been adopted before by using size exclusion chromatography to "eliminate" the nonfunctional NC probe (forth row). However, this process can be complicated and sometimes does not work. The latter is the main topic of this paper (fifth row). Here, we design and optimize a blocker strand that can enhance the C33 NCB probe by 14 times at 0.1:1 target probe ratio.



Supplemental Note S2: Materials and sample preparations

Materials. Sodium phosphate dibasic anhydrous (Na_2HPO_4; F.W. 141.96), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$; F.W. 137.99) and sodium borohydride ($NaBH_4$) were purchased from Fisher Scientific, whereas silver nitrate ($AgNO_3$) was acquired from Sigma-Aldrich. All oligonucleotides were purchased from Integrated DNA Technologies and were purified by desalting. De-ionized (DI) water (18 MΩ·cm) was used for all solution preparations.

Preparation of Nanocluster Probes (NC Probes). We adopt the same standard procedure as appeared in Ref. 4, 5 and 10 of the main text. In a typical preparation, a 15 μM (final concentration) NC probe solution was prepared by adding 12.5 μl of 1.2 mM NC probe (Tables S1 and S4) to 940 μl of 20 mM sodium phosphate buffer (pH 6.7). The solution was vortexed for 2 s and centrifuged at 14,000 rpm for 30 s. 45 μl of 4 mM silver nitrate solution was then added and the mixture was again vortexed and centrifuged. The solution was allowed to sit in the dark for 10 min at room temperature. For silver cluster formation, 7 μl of freshly prepared 13.2 mM $NaBH_4$ solution was added, resulting in a pale yellow mixture, which was then stored in the dark overnight. The resulting NC probe solution had the NC probe:Ag^+:$NaBH_4$ molar ratio of 1:12:6.

Activation of NanoCluster Beacons. An NCB consists of an NC probe (originally carrying dark silver clusters) and an enhancer probe (guanine-rich). When the enhancer probe is brought close to the NC probe through hybridization, dark silver clusters are activated and become highly emissive through the interactions with the nearby guanine-rich enhancer sequence. We call this process "the guanine-proximity-induced activation of silver clusters" or, in short, "the activation of NCBs". To activate NCBs, 1.5 μl of 1.2 mM G-rich enhancer probe solution was added to a 120 μl aliquot of the previously prepared 15 μM NC probe solution. The mixture was vortexed, centrifuged, and immersed in a hot water bath (90-95 ^oC) for 1 min, followed by gradually cooling to room temperature for 1 hr. The activated NCB had the NC probe:enhancer probe molar ratio of 1:1. The fluorescence measurements started exactly at 1 hr after the addition of the enhancer probe.

Supplemental Note S3: Fluorescence and fluorescence correlation spectroscopy measurements

Fluorescence Measurements. All fluorescence emission and excitation scans were performed on a FluoroMax-4 spectrofluorometer from Horiba Scientific unless otherwise stated. Each 120 μL NCB sample was placed in a 100 μL quartz cuvette (16.100F-Q-10/Z15, Starna Cells) for fluorometer measurements.

For 1D emission scans, the excitation wavelength was set to 645 nm and the emission wavelength was scanned from 660 to 760 nm using 5 nm slit size, 1 nm increment step, 1000 detector gain, and 0.5 s integration time. A blank sample, 20 mM sodium phosphate buffer pH 6.7, was also measured.

For 2D measurements, both emission and excitation were scanned from 400 to 800 nm using 5 nm slit size, 5 nm increment step, and 0.1 s integration time. To avoid saturation, lower NCB concentration (10 μM) and detector gain (775) were used here. For each sample, the 2D scan was completed in 45 min. Color photos of inactivated (NC probe only) and activated (the duplex) NCBs were acquired with a digital camera (Canon PowerShot SX 500 IS) on a Syngene gel imager (365 nm excitation).

Fluorescence correlation spectroscopy setup. FCS measurements were carried out with a custom-built inverted confocal fluorescence Olympus IX-71 microscope, similar to the FCS setup described in previous publications[1-3]. A pulsed laser (repetition rate 10 MHz) from PicoQuant LDH-P-C-640B was reflected by the dichroic mirror (Semrock FF650-Di01-25x36), and then focused by a 60X NA=1.2, water immersion objective (Olympus, UPLSAPO 60XW) to excite the ATTO633 dyes and NCB. The fluorescence is collected by the same objective and filtered by ET700/75m (Chroma). An avalanche photo diode (APD, SPCM-AQ4C, Perkin-Elmer) was used to detect ATTO633 dyes and NCB emission and a hardware correlator (ALV-7002 /EPP, ALV-GmbH) was used to compute autocorrelation functions. Unless otherwise noted, the laser excitation intensity was kept low (100 μW before entering the aperture of objective) to avoid fluorescence saturation, triplet-state formation and photobleaching. The laser beam was focused 25 μm into the sample for all FCS measurements in this work.

FSC analysis. The one-component model is employed to fit the autocorrelation function $G(\tau)$[4] as follows,

$$G(\tau) = \frac{1}{N} g_d(\tau) X(\tau),$$

where τ represents the lag time, N is the average number of the molecules in the detection volume, $g_d(\tau)$ represents an autocorrelation function due to the translational diffusion[5], and $X(\tau)$ represents the fluctuation due to the fast-blinking kinetics[6]. $g_d(\tau)$, and $X(\tau)$ are characterized as follows[1],

$$g_d(\tau) = \frac{1}{1 + \left(\frac{\tau}{\tau_d}\right)^{\alpha}}, \quad X(\tau) = 1 + \frac{Fe^{-\left(\frac{\tau}{\tau_r}\right)^{\beta}}}{1 - F},$$

where τ_d and τ_r represents the characteristic diffusion time and the mean triplet state relaxation time[6] respectively, α and β are the anomalous factor and the stretch parameter individually, and F is the effective fraction of molecules in triplet states.

Supplemental Note S4: Purification strategy: Hydrophilic Streptavidin Magnetic Beads, NEB S1421S (surface affinity purification)

We utilized the duplex stabilities differences as a strategy for labeled NC probe purification. Biotinylated compC33_+n and streptavidin-coated magnet bead were used to pull down the unlabeled C33 NC probes. The supernatant was then analyzed by size exclusion chromatography.

The hydrophilic Streptavidin magnetic beads that we used are NEB S1421S, recommended for nucleic acids. Typically, 200 µL beads was washed with 160 µL wash buffer, vortexed for 2 s, and removed supernatant. The solution is added with 40 µL of 8 µM biotin-DNA (320 pmol, in wash buffer), vortexed, and centrifuged for 5min. The solution was washed with 160 µL wash buffer twice, and 160 µL 20mM SPB twice. It was then added 20 uL C33 (15 uM), vortexed, and centrifuged for 10 min. The supernatant was kept as purified sample 1 (P1). Another 20 uL C33 (15 uM) was added, vortexed and centrifuged for 10 min. The supernatant was kept again as purified sample 2 (P2).

Refer to NEB website for more information about the bead purification.

https://www.neb.com/products/s1421-hydrophilic-streptavidin-magnetic-beads#Product%20Information

Supplemental Note S5: High-resolution melting (HRM) analysis

High-resolution melting (HRM) analysis was carried out in a real-time thermal cycler (CFX Connect from Bio-Rad) as the standard protocol that we used before (Ref. 5 and 13 in the main text). We briefly explain here again the procedure.

1 µl of 120 µM single-stranded target and probe samples and 4 µl DI water (or 0.2 M urea or fetal bovine serum) were added to 6 µl of 2× Precision Melt Supermix® (Bio-Rad, USA) to obtain the final DNA and Supermix concentrations of 10 µM and 1×, respectively. All meltings were performed in 12 µl volume. The melting curve analysis was conducted on CFX Connect Real-Time System (CFX Manager Software version 1.6, Bio-Rad, USA) using the SYBR/FAM channel. Before melting, samples were denatured at 93 °C for 1 min and cooled down to room temperature for 30 min. During melting, the temperature was increased from 30 to 100 °C in 0.2 °C incremental steps, with each step held for 5 s. For melting analysis, the relative fluorescence units (RFU) of the resulting melting curves were first plotted against melting temperatures (Tm). Normalization regions were selected before and after the major decrease in fluorescence (Tm ± 4 °C). Comparisons between different DNA species were made in terms of Tm.

Supplemental Note S6: Size-exclusion chromatography analysis

Size-exclusion chromatography was conducted with a Shimadzu Prominence high performance liquid chromatography system using a 300 x 7.8 mm BioSep-SEC-S2000 column (Phenomenex), having 5 μm particles and a pore size of 145 Å. The mobile phase was buffered at pH = 6.5 with 10 mM cacodylate/cacodylic acid that was supplemented with 40mM sodium perchlorate to minimize solute interactions with the stationary phase[7]. To assess hydrodynamic radii, size standards were based on the thymine oligonucleotides dT_{10}, dT_{15}, dT_{20}, and dT_{30}[8,9].

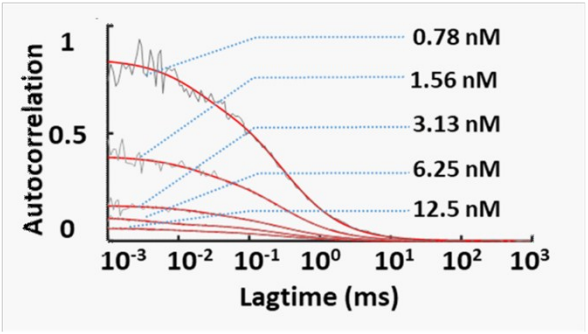
Supplemental Table S1: List of DNA sequences with a full set of comp_C33+n

NC probe C33	3’-TAA TTA TTT ATT ATA AAT TTT AAA TAA TAT-CCC TAATT CCC
Activator/Target G15	5’-ATT AAT AAA TAA TAT TTA AAA TTT ATT ATA-GGG TGGGG TGGGGTGGGG
compC33_+6	5’-ATT ATA-GGG ATTAA GGG
compC33_+9	5’-TTT ATT ATA-GGG ATTAA GGG
compC33_+12	5’-AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+15	5’-TTA AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+18	5’-TAT TTA AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+21	5’-TAA TAT TTA AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+24	5’-AAA TAA TAT TTA AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+27	5’-AAT AAA TAA TAT TTA AAA TTT ATT ATA-GGG ATTAA GGG
compC33_+30	5’-ATT AAT AAA TAA TAT TTA AAA TTT ATT ATA-GGG ATTAA GGG

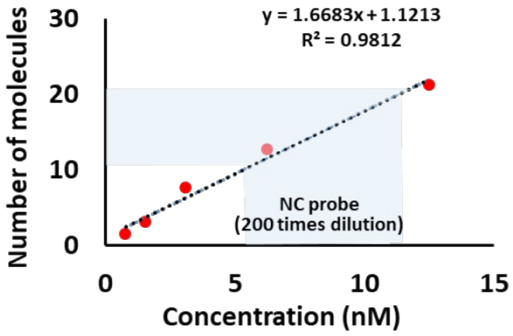
Sequences are highlighted with colors corresponding to the diagrams in Table of Contents, Figure 1 and Figure 2A of the main text.

Supplemental Figure S1: Fluorescence correlation spectroscopy (FCS) showed the synthesis yield of NC probe is around **10%**.

A



B



C

Concentration (nM)	[a]Ave p.c. (kHz)	[b]N	[c]tau	[d]SMB (kHz)	[e]chi²
12.5	78.72	21.23	0.253	3.70	0.72
6.25	46.45	12.71	0.242	3.65	0.75
3.13	26.12	7.57	0.27	3.44	1.3
1.56	10.16	3.06	0.263	3.31	1.53
0.78	4.58	1.44	0.28	3.16	1.27

[a]**Ave p.c.** (average photon count)
[b]**N** (average number of molecules in detection volume)
[c]**tau** (dwell time of molecules in detection volume
[d]**SMB** (single-molecule brightness)
[e]**SNR** (signal-to-noise ratio)

D

Dilution times	[a]Ave p.c. (kHz)	[b]N	[c]tau	[d]SMB (kHz)	[e]chi²
200	8.32	11.4	0.86	0.73	10.01

Based on the number of NC probes
The yield range of NC probe is around 8.25%:
N=11.4
⇒ NC probe concentration
=(11.4-1.122)/1.668 =6.19 nM
=> Yield = 6.19*200/15000 = 8.25%

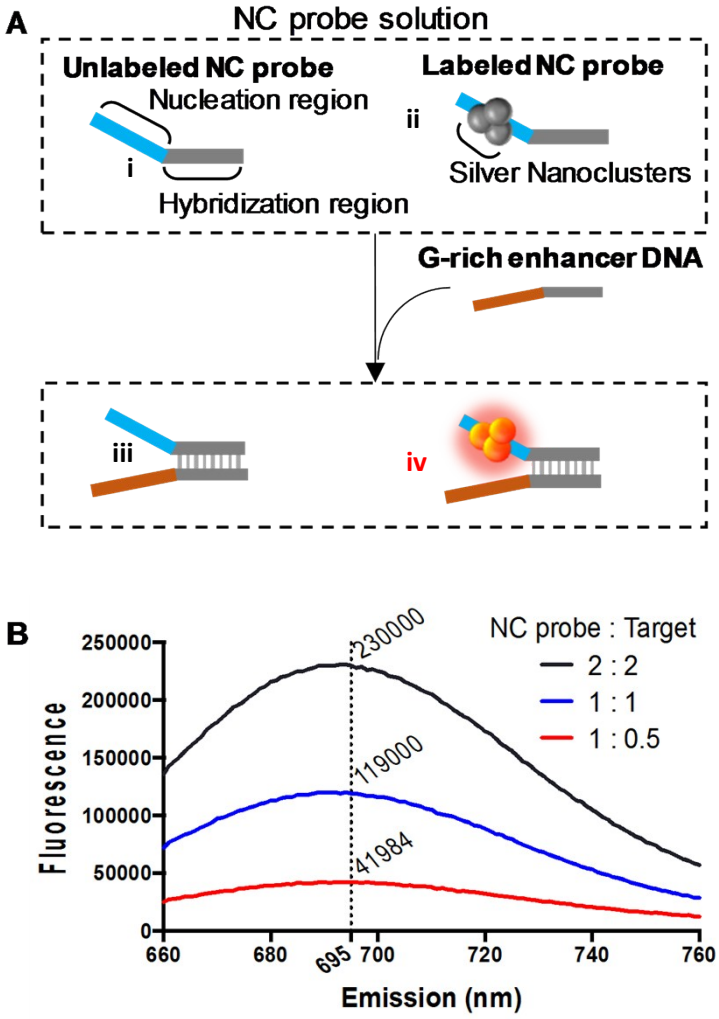
(**A**) Fluorescence autocorrelation functions as a function of time from several different concentrations of ATTO633-labeled ssDNA for calibrations. The concentrations used are (black curve, from the bottom): 12.5 nM, 6.25 nM, 3.13 nM, 1.56 nM and 0.78 nM. Fitting lines are in red. For FCS set up and analysis, refer to Note S3.

(**B**) The physical-relevant fitted parameters of ATTO633-label ssDNA are provided FCS (from the data shown in (A)). Number of molecules vs concentration is plotted as red dots in (C) for calibration.

(**C**) The calibration line of ATTO633-labeled single-strand DNA is used to predict the concentration of NC probe. The blue region is the range of NCB concentrations after 200 times dilution from the original 15000nM NC probe solution. Fluorescence correlation spectroscopy (FCS) showing the synthesis yield of NC probe is around **10%**.

(**D**) The physical-relevant fitted parameters of NC probe are provided FCS. The result falls into the blue region of (C).

Supplemental Figure S2: When the target concentration is lower than that of the NC probe, the decrease of target specific signal of NCB is disproportionate to the decrease of target concentration.

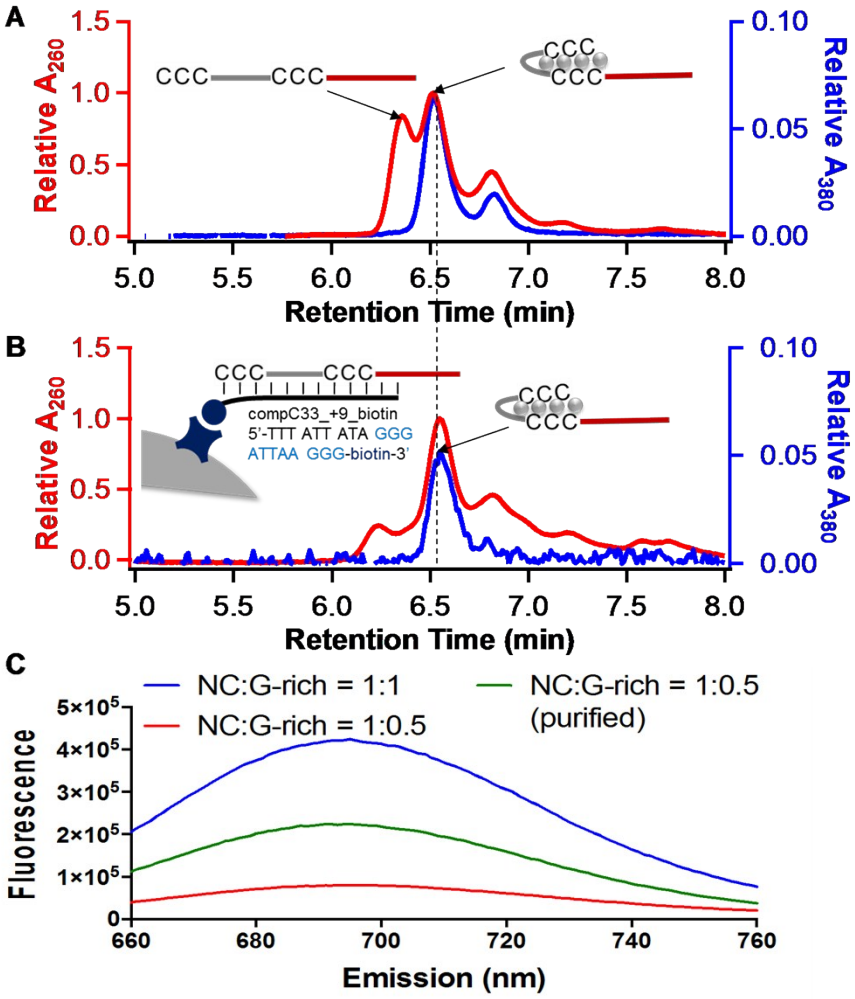


(A) Schematics of unlabeled and functional NC probes (denoted as **i** and **ii**) in the NC proble solution. After addition of G-rich enhancer DNA as targets, partially NC probes form hypothesized duplexes (denoted as **iii** and **iv**). Only **iv** species emit Fluorescence signal, which is highlighted with bright red color. Target-specific signal (TSS) is defined to be percentage of target strands that form the functional species **iv**.

(B) Fluorescence signals of NC probe solution with targets. In a typical NCB detection at 1:1 target-probe ratio, we expect 10% target-specific signal - TSS, which means that the Fluorescence signal (blue line) comes from 10% of the target strands. When we double the concentrations of both targets and probes, the Fluorescence signal is doubled as expected (black line). Assuming target binds with labeled and unlabeled NC probes with equal probability, when probes are twice more abundant for the target to bind at 1:0.5 target-probe ratio, we should see 10% TSS, which is corresponding to half Fluorescence signal compared to the 1:1 target-probe ratio in the blue line. Nevertheless, the observed Fluorescence is not half but 0.37 times (red line), which is corresponding to only 7.4% TSS. This indicate that target might preferentially

bind with unlabeled NC probes rather than labeled probes. We do not consider the trivial case when the TSS is low simply due to the lack of available probe (target/probe ratio greater than 1).

Supplemental Figure S3: The nucleation region of the labeled NC probe was less preferable for intermolecular base pairing.



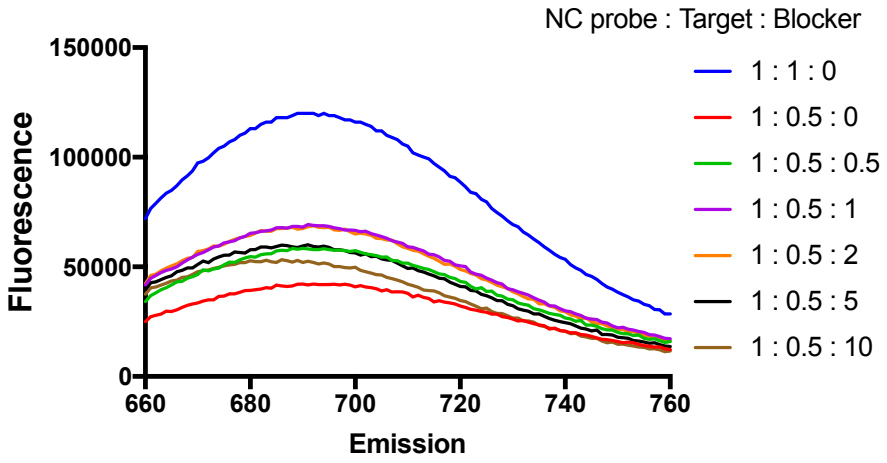
(A) Size exclusion chromatography of C33 NC probe. The absorbance at 260 nm illumination represented quantity of DNA and the 380 nm absorbance was used for measuring the amount of labeled NC probe. The unlabeled and labeled NC probe peaked at 6.38 minutes and 6.50 minutes, respectively. Smaller peaks are impurifies or partially folded variants of the C33 strand.

(B) SEC of C33 NC probe after purification with Biotinylated compC33_+9 and streptavidin-coated magnet beads. In the supernatant, the peak at ~6.3 minutes was greatly reduced (dashed line along the 2 graphs), and the majority remained in the supernatant was the labeled which exhibited an absorbance peak at ~6.5 min under 360-nm illumination. Hence, we were able to remove the unlabeled NC probe.

(C) Emission spectra of activated NCB excited by 645 nm light. All three samples contained same amount of starting NC probe (1.5 µM). The bottom two curves contained half amount of the G-rich

enhancer DNA as compared to the top curve. The NC probe in the middle curve was purified with compC33_+9 before adding the G-rich enhancer, showing higher emission spectra compared to the unpurified sample. This again demonstrates that we were successfully remove the unlabeled NC probe, leading to more target strand bound with the labeled NCB, enhancing the Fluorescence.

Supplemental Figure S4: Fluorescence recovery was maximized when the blocker concentration is similar to the NC probe concentration.



Fluorescence recovery of NCB with various blocker compC33_+9 concentration. The intensities are measured at 645 nm. The concentration of NC probe is at 2.5 μM, target DNA is either at 2.5 μM (blue line) or 1.25 μM. Blocker compC33_+9 ranging from 1.25 μM to 25 μM was added and showed different level of fluorescence recovery of NCB. The recovery peaked when blocker DNA are at 2.5 μM and 5 μM.

References

(1) Yeh, H.-C.; Puleo, C. M.; Ho, Y.-P.; Bailey, V. J.; Lim, T. C.; Liu, K.; Wang, T.-H. *Biophys J* **2008**, *95*, 729-737.
(2) Yeh, H. C.; Ho, Y. P.; Shih, I. M.; Wang, T. H. *Nucleic Acids Res* **2006**, *34*, e35.
(3) Yeh, H.-C.; Puleo, C. M.; Lim, T. C.; Ho, Y.-P.; Giza, P. E.; Huang, R. C. C.; Wang, T.-H. *Nucleic Acids Res* **2006**, *34*, e144-e144.
(4) Widengren, J.; Mets, U.; Rigler, R. *The Journal of Physical Chemistry* **1995**, *99*, 13368-13379.
(5) Meseth, U.; Wohland, T.; Rigler, R.; Vogel, H. *Biophys J* **1999**, *76*, 1619-1631.
(6) Kim, J.; Doose, S.; Neuweiler, H.; Sauer, M. *Nucleic Acids Res* **2006**, *34*, 2516-2527.
(7) Leroy, J. L.; Gehring, K.; Kettani, A.; Gueron, M. *Biochemistry-Us* **1993**, *32*, 6019-6031.
(8) Doose, S.; Barsch, H.; Sauer, M. *Biophys J* **2007**, *93*, 1224-1234.
(9) Sengupta, B.; Springer, K.; Buckman, J. G.; Story, S. P.; Abe, O. H.; Hasan, Z. W.; Prudowsky, Z. D.; Rudisill, S. E.; Degtyareva, N. N.; Petty, J. T. *The Journal of Physical Chemistry C* **2009**, *113*, 19518-19524.