Supporting Information

Construct Real-Time, Wash-Free, and Reiterative Sensors for Cell Surface Proteins Using Binding-Induced Dynamic DNA Assembly

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Materials and Reagents

Recombinant human epidermal growth factor receptor 2 (HER2), biotinylated anti-HER2 polyclonal antibody (goat IgG), phycoerythrin labeled anti-HER2 monoclonal antibody (mouse IgG2B, clone191924), fluorescein labeled streptavidin, and biotinylated normal goat IgG control were purchased from R&D Systems (Minneapolis, MN). Streptavidin from *Streptomyces avidinii*, biotin, bovine serum albumin (BSA), and magnesium chloride hexahydrate ($MgCl_2\ 6H_2O$) were purchased from Sigma (Oakville, ON, Canada). Phosphate buffer saline (PBS), Tween 20, diamidino-2-phenylindole (DAPI) solution (1 mg/ml), McCoy's 5A modified medium, penicillin/streptomycin (100×), and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA). NANOpure H_2O (> 18.0 MΩ), purified using an Ultrapure Milli-Q water system, was used for all experiments. All DNA samples were purchased from Integrated DNA Technologies (Coralville, IA) and purified by high performance liquid chromatography (HPLC). The DNA sequences and modifications are listed in Table S1.

Probe preparation for real-time HER2 sensor

To prepare DNA probes for the detection of HER2, we mixed 25 μL of 2.5 μM biotinylated probe TB or probe B*C with equal volume of 2.5 μM streptavidin and then incubated the solution at 37 ℃ for 30 min, followed by incubation at 25 ℃ for another 30 min. To this reaction mixture, 50 μL of 1.25 μM biotinylated HER2 polyclonal antibodies was then added. The solution was incubated at 25 ℃ for 30 min. The prepared DNA probe was then diluted to 250 nM with a solution containing 1 × PBS, 0.01% BSA, and 1 mM biotin. A pair of isotype control (I.C.) probes was also prepared the same way as for the HER2 sensor, except that nonspecific polyclonal normal goat IgG antibodies were used instead of HER2 specific antibodies. Reporter DNA duplex (T*C*:C) was prepared at a final concentration of 5 μM by mixing 20 μL of 50 μM FAM-labeled T*C* with 20 μL of 50 μM dark quencher-labeled C in 160 μL of PBS-Mg buffer (1 × PBS, 10 mM $MgCl_2$, 0.05% Tween20). The mixture was heated to 90 ℃ for 5 min and then the solution was allowed to cool down slowly to 25 ℃ in a period of 3 h.

Real-time detection of recombinant HER2 in buffer

For the real-time detection of HER2 in buffer, the reaction mixture contained 10 nM antibody-modified TB, 10 nM antibody-modified B*C, 20 nM reporter duplex T*C*:C, 10 nM target HER2, and PBS-Mg buffer. Once mixing HER2 with all sensor components, fluorescence was measured every 5 min for 50 min. Fluorescence was measured directly from the microplate using a multimode microplate reader (SpectraMax i3, Molecular Devices). The excitation was 485 nm and emission was 535 nm. The measured fluorescent signal was normalized so that 1 normalized unit (n. u.) of fluorescence corresponded to the fluorescent signal generated by 1 nM positive control TC. This normalization was achieved using a positive control containing 10 nM TC and 20 nM T*C*:C in PBS-Mg buffer and a negative control containing identical reagents in positive control except that there was no TC added.

Cell culture and fixation

The human breast adenocarcinoma cell line SK-BR-3, was obtained from ATCC (Manassas, VA) and cultured in McCoy's 5A medium (ATCC) plus 10% FBS and 1% penicillin/streptomycin (100 U/100 μg/ml) at 37 ℃ in a humidified atmosphere of 5% CO_2. The human bladder epithelial carcinoma cell line, T24, was also obtained from ATCC and cultured in McCoy's 5A modified medium plus 10% FBS and 1% penicillin/streptomycin at 37 ℃ in a humidified atmosphere of 5% CO_2. For a typical fluorescent imaging experiment, cells were seeded to 35 mm well plate with a confocal window at a concentration of 5×10^4 cells per well. When cultured to 90% confluence (a lower confluence was used for real-time imaging experiments to obtain single cells), cells were fixed using 4% paraformaldehyde for 30 min and permeabilized at room temperature for 10 min using 0.05% Tween20 in PBS (PBST) buffer. After a brief wash using PBST buffer, cells were stored in PBST buffer containing 1% BSA at 4 ℃.

Cell imaging using HER2 DNA sensor and confocal fluorescence microscopy

To image cellular HER2 using our strategy, fixed SK-BR-3 cells were incubated with a reaction mixture containing 25 nM antibody modified DNA probes TB and B*C, 50 nM reporter duplex T*C*:C, 100 nM DAPI. After adding all sensor components, cells were directly observed under confocal fluorescence microscope without any washing steps.

Fluorescence imaging of fixed cells was performed on an Olympus IX-81 microscope that was coupled with a Yokagawa CSU × 1 spinning disk confocal scan-head and Hamamatsu EMCCD cameras with 40×/1.3 Oil and 20×/0.85 Oil objective lenses. A 405 nm pumped diode laser was used for the excitation of nucleus staining dye DAPI. A 491 nm pumped diode laser was used for the excitation of FAM labeled HER2 sensor. The exposure time was set to be 200 ms for DAPI and 600 ms for FAM-labeled sensor for both samples and controls.

Live cell analysis using HER2 DNA sensor and flow cytometry

To detect HER2 from live SK-BR-3 cells using HER2 DNA sensor, cells that were cultured to 90% confluence were detached from culture dish using 1× trypsin and diluted to 10^6 cells/ml in PBS-Mg buffer containing 25 nM probe TB, 25 nM probe B*C, 50 nM reporter T*C*:C, and 1% FBS. After incubation for 15 min, cells were analyzed using BD LSRFortessa digital flow cytometer at a rate of 5000 cells/sec. Data analysis was performed using FlowJo 7.6.

Table S1. DNA sequences and modifications

DNA name	Sequences
TB	5'-Biotin-TTT TTT TTT TTT TTT T-GTG AGG-TT-CGT GTG ATG-3'
B*C	5'-AA GCG TGT ATC CCA TGT GTC-CCT CAC TTT TTT TTT TTT TTT T-Biotin-3'
T*C*	5'-CAT CAC ACG GAC ACA TGG GAT ACA CGC TT-FAM-3'
C	5'-Dabcyl-AA GCG TGT ATC CCA TGT GTC-3'
E*T*C*	5'-CTA GAG CAT CAC ACG GAC ACA TGG GAT ACA CGC TT-FAM-3'
ETC	5'-Dabcyl-AA GCG TGT ATC CCA TGT GTC CGT GTG ATG CTC TAG-3'
TC	5'-AA GCG TGT ATC CCA TGT GTC CGTGTGAT-3'

Design of DNA Sequences for Binding-Induced DNA Three-Way Junction Sensor

All DNA sequences are rationally designed and melting temperatures T_m of the designed DNA probes were estimated using OligoAnalyzer (free software from IDT). Generally, the fluorogenic beacon (T*C*:C) is designed as a DNA duplex containing a short sticky end (DNA toehold). The duplex part is designed to have a melting temperature T_m = 55 ℃, so that it will form a stably DNA duplex at all assay and imaging conditions (room temperature or 37 ℃). The DNA toehold is designed to be 9 nt long, because it has been well established that this length is long enough to maximize the kinetics of a toehold-mediated DNA strand displacement reaction between T*C*:C and TC (Figure S1A and S1B).[1] DNA probes TB and B*C are designed by splitting a TC into two parts (the toehold region T and the competing region C) and extended with a short complementary domain B and B*. B and B* are designed to have a T_m = 10 ℃, so that they are not able to form a stable DNA duplex at room temperature or 37 ℃. Consequently, TB and B*C are not able to trigger a strand displacement reaction with beacon T*C*:C. TB and B*C are then each conjugated with an affinity ligand that can bind to the same target protein but at diffident epitopes through a flexible linker. Upon the target binding that assembled TB and B*C to form a loop (Figure S1C), the T_m of the B:B* duplex was increased from 10 ℃ to 45 ℃. As a result, the target-TB:B*C complex reassembles T and C into an "intact" single-strand DNA (Figure S1C) and regenerates its activity to trigger DNA strand displacement between T*C*:C (Figure S1D). The T_m value of B:B* was estimated by considering the binding-induced DNA assembly as a DNA hairpin structure with 6 complementary base pairs as the stem and 60 dNTP as the loop. This loop length (60 dNTP) was chosen for T_m estimation because of its similar size to that of the affinity complex.

Supporting Figures

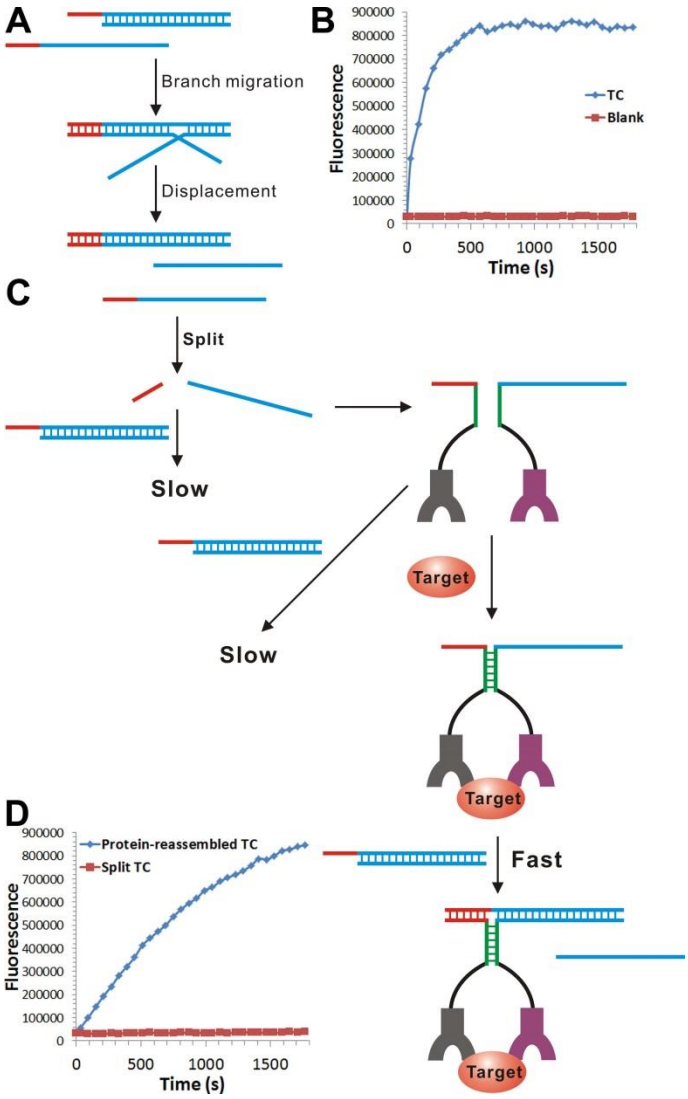

Figure S1. Design principle of DNA sequences for binding-induced DNA three-way junction (TWJ) sensors. (A) Schematic illustrating of toehold-mediated DNA strand displacement, which is used as a template for designing binding-induced DNA TWJ sensor; (B) Fluorescence increase of 50 nM DNA strand displacement beacon as a function of time in the presence of 10 nM TC; (C) Schematic illustrating of principles to design DNA sequences for binding-induced DNA TWJ sensor through splitting TC and reassembling TC using a target protein; (D) Fluorescence increase of 50 nM DNA strand displacement beacon as a function of time in the presence of 10 nM streptavidin-reassembled TC (blue curve) or 25 nM split TC (red curve).

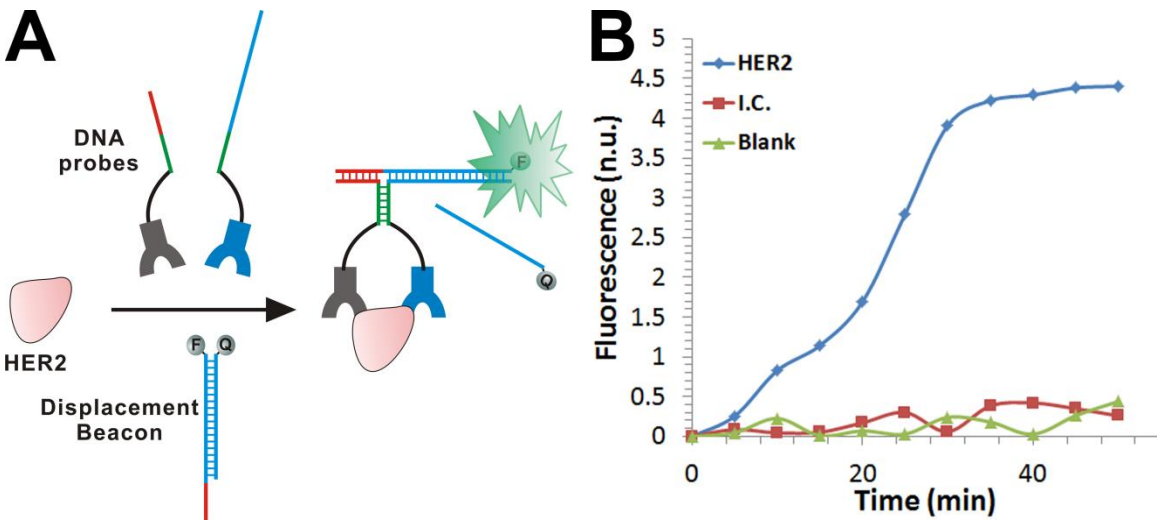

Figure S2. (A) Schematic illustrating the principle of real-time HER2 sensor for detecting recombinant HER2 in buffer; (B) Fluorescence increases as a function of time when 10 nM target HER2 mixed with 10 nM HER2 sensor (HER2, blue curve) or 10 nM target HER2 mixed with 10 nM Isotype control probes (I.C., red curve). The blank (green curve) contained the same amount of HER2 sensor as for the target, except that there was no HER2 added.

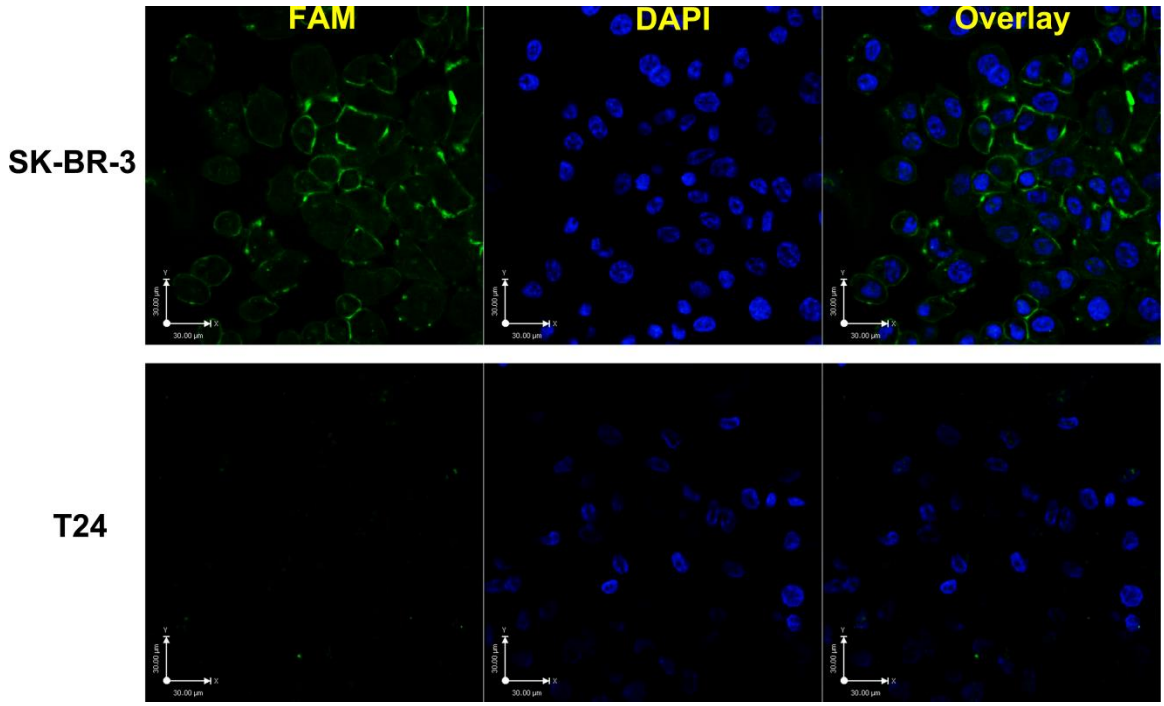

Figure S3. Specificity of HER2 sensor for SK-BR-3 cells (HER2 overexpression) versus T24 cells (non-HER2 expression). Scale bars represent 30 μm.

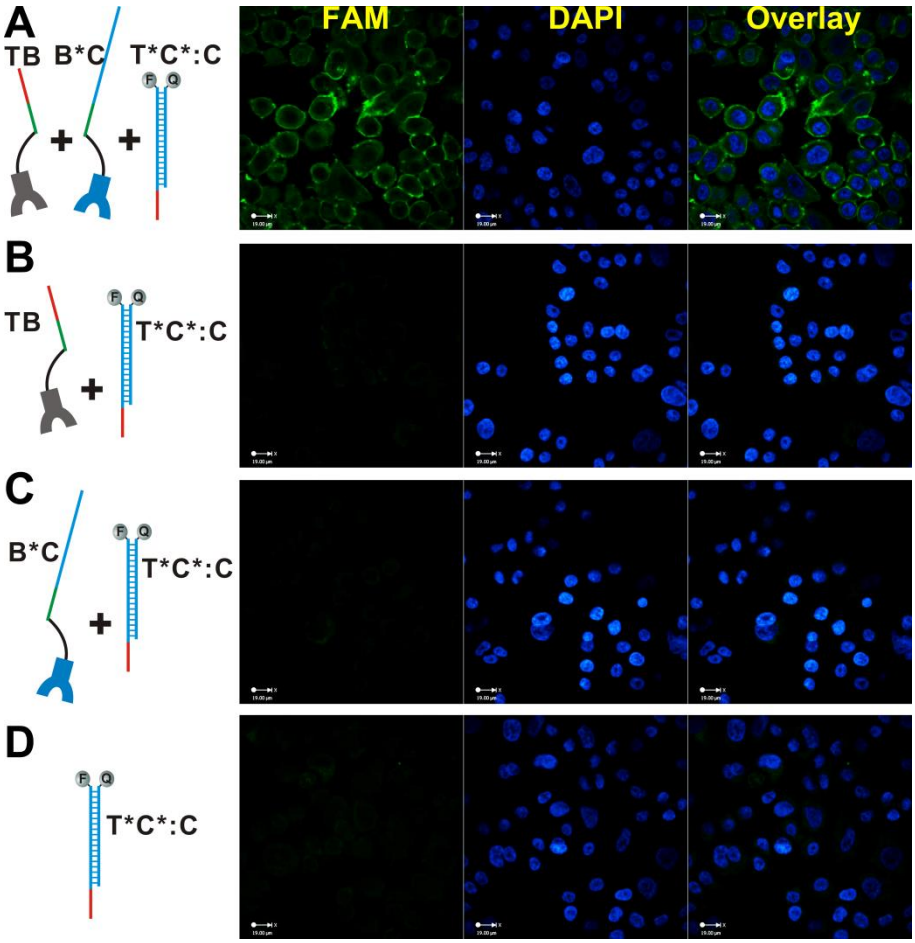

Figure S4. Confocal fluorescence microscopy images of SK-BR-3 cells that were labeled with the complete HER2 sensor that contained TB, B*C, and T*C*:C (A) versus cells that were labeled with DNA probes TB and T*C*:C (B), B*C and T*C*:C (C), and T*C*:C only (D). Scale bars represent 19 μm.

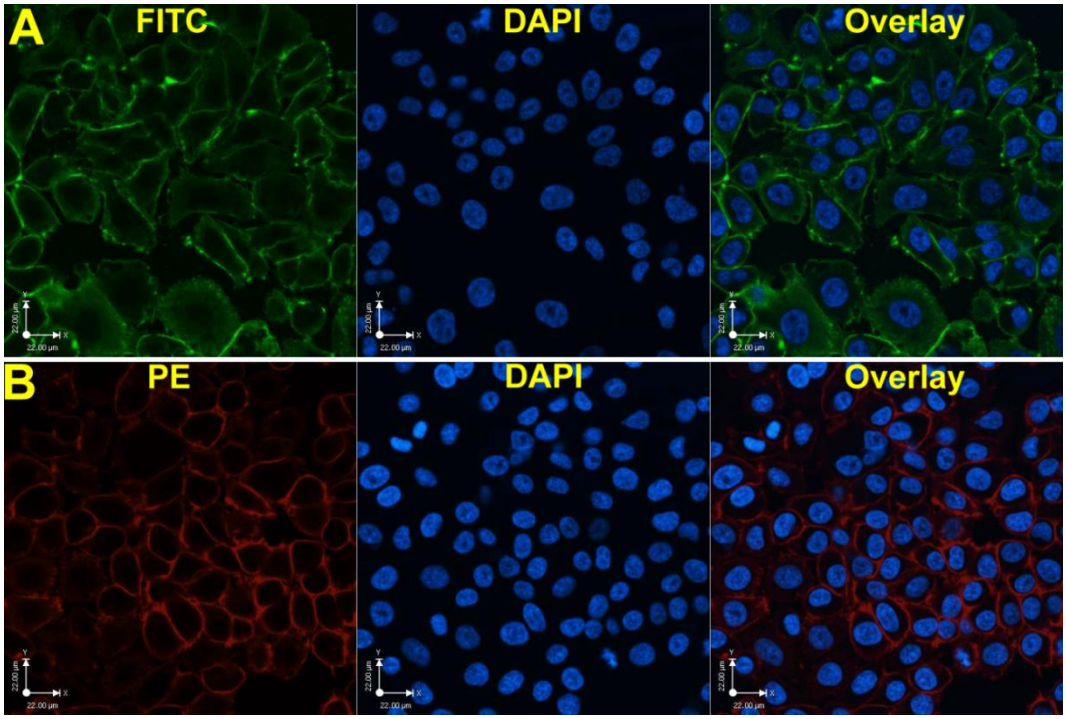

Figure S5. Immunofluorescence staining of SK-BR-3 cells using (A) Biotinylated HER2-specific polyclonal antibody coupled with FITC-labeled streptavidin, and (B) PE-labeled monoclonal HER2-specfic antibody. Scale bars represent 22 μm.

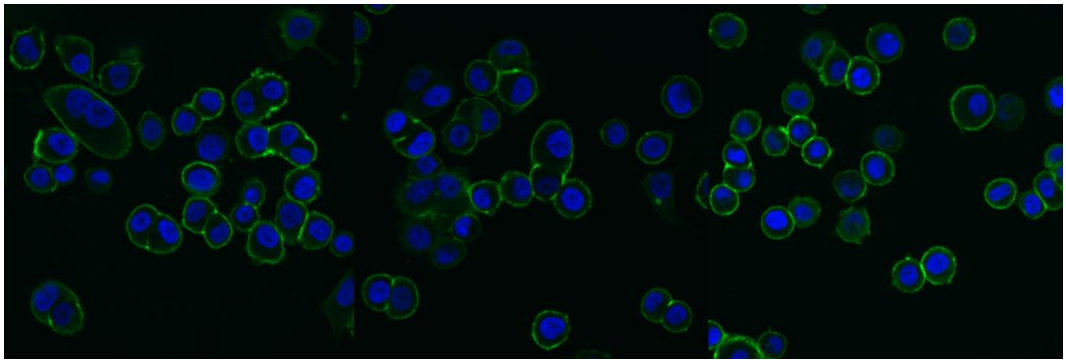

Figure S6. Quantitative determination of signal-to-background ratio (SBR) using three representative cell imaging results obtained by incubating HER2 sensor with fixed SK-BR-3 cells for 10 min. Ten cells from each image were randomly selected and used to determine SBR. All fluorescence measurements were carried out using ImageJ 1.47t. For each cell, SBR = Integrated density / (Area of elected cell × Mean fluorescence of background readings).

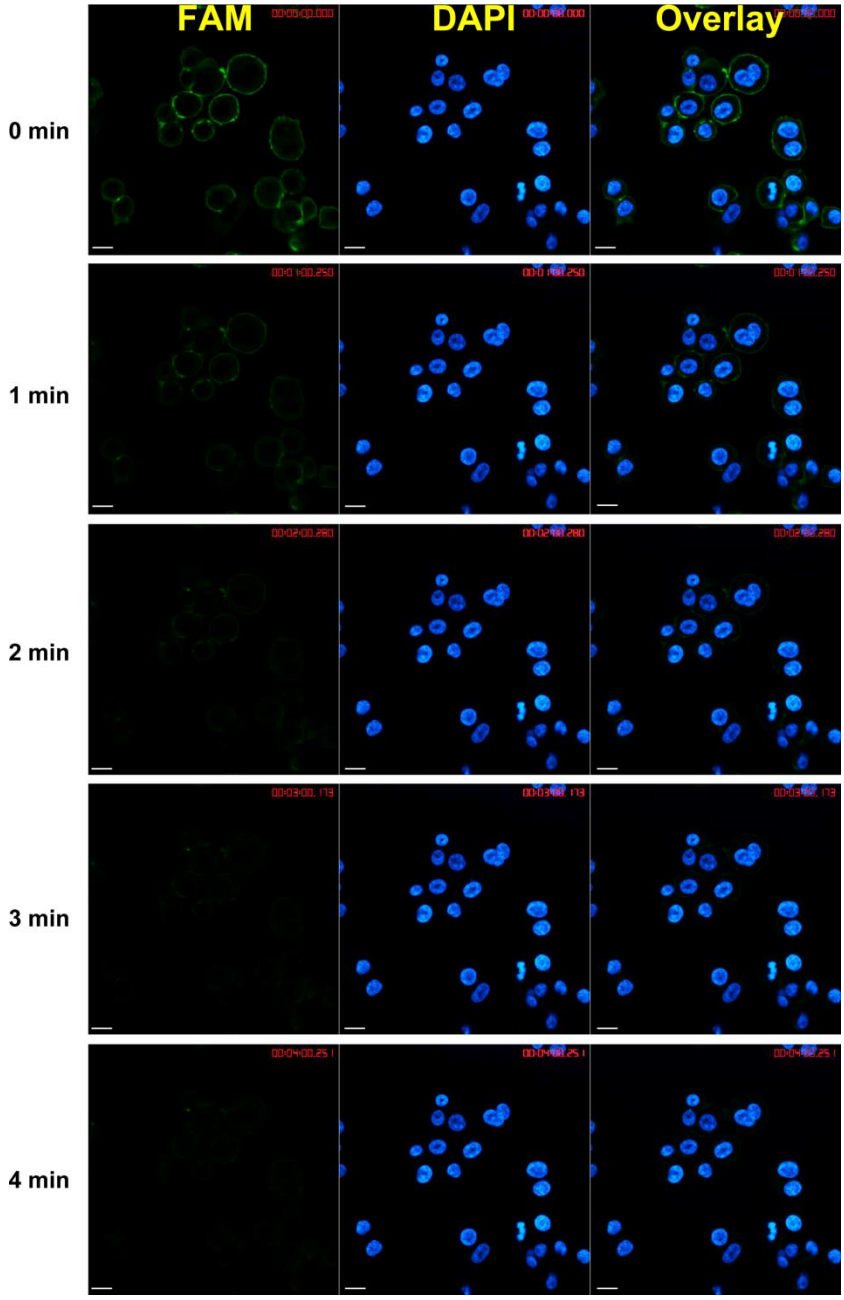

Figure S7. Time-lapse confocal fluorescent microscopy images of SK-BR-3 cells were taken at a 1 min interval immediately after mixing cells that were labeled with HER2-specific DNA sensors (25 nM TB, 25 nM B*C, and 50 nM T*C*:C) and then with 50 nM erasing DNA probe ETC. Scale bars represent 20 μm.

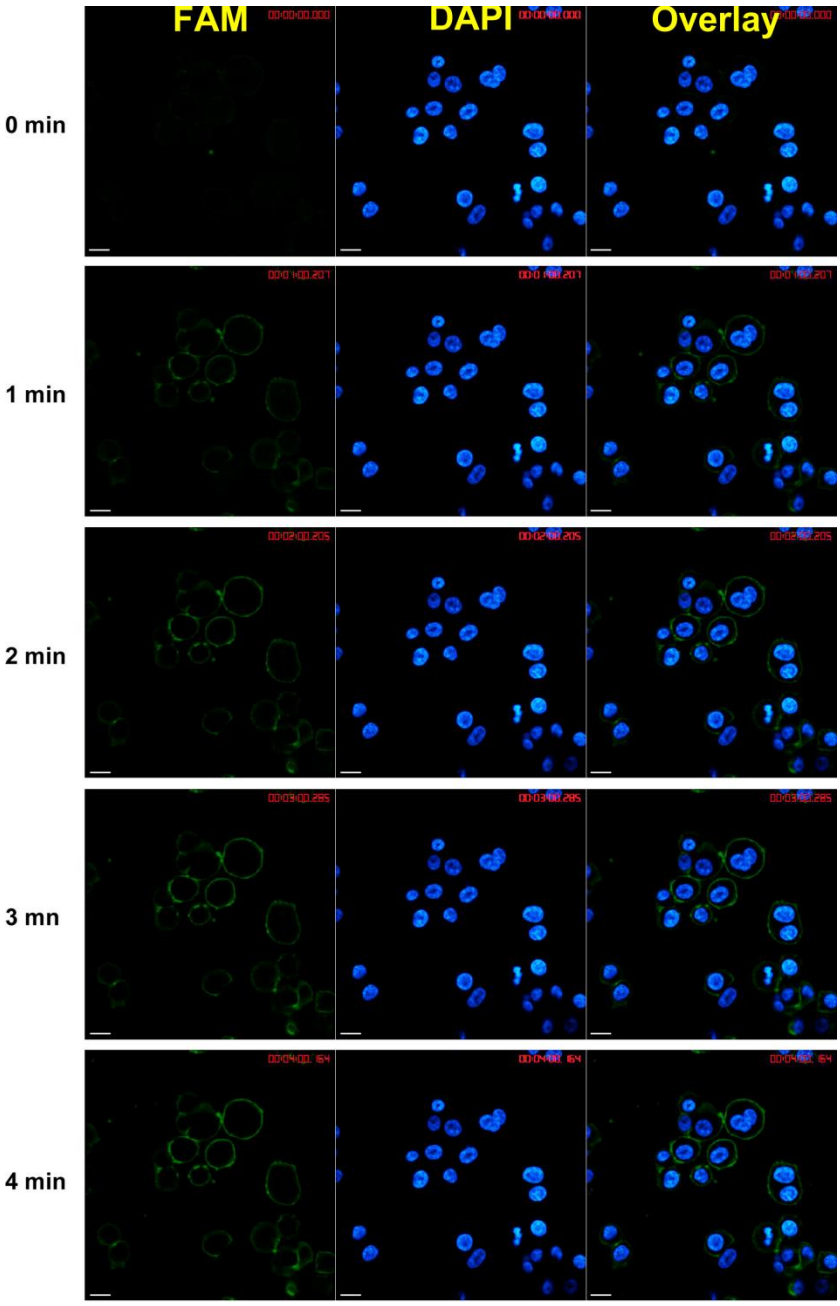

Figure S8. Time-lapse confocal fluorescent microscopy images of SK-BR-3 cells that were turned on by re-staining cells with HER2 sensors after turning off the fluorescence using erasing DNA probes. Fluorescent images were taken at a 1 min interval immediately after adding 50 nM reporter duplex E*T*C*:C to the cells that have gone through a labeling and erasing cycle as shown in Figure 4 in the main content. Scale bars represent 20 μm.

References

1. D. Y. Zhang, E. Winfree, *J. Am. Chem. Soc.* **2009**, *131*, 17303-17314.