Supporting information for

An Enhanced Ascorbate Peroxidase 2/Antibody-Binding Domain Fusion Protein (APEX2-ABD) as a Recombinant Target-Specific Signal Amplifier

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Materials and Methods

Construction and purification of APEX2 fused with antibody-binding domain

The oligodeoxynucleotide which encodes the antibody-binding domain (ABD, 58 amino acid) was synthesized and introduced to the C-terminus of APEX2, which is in the pTRC99A bacterial expression vector, with 27 extra amino acids as a flexible linker. The engineered plasmid DNA was transformed into competent *E. coli* strain BL21 (DE3). One single colony was chosen and amplified in 5 mL of Luria Broth and subsequently seeded into 1L LB media. The expression of APEX2-ABD was induced by the addition of 420 μM isopropyl p-D-1-thiogalactopyranoside (IPTG) at 30 °C overnight. The bacterial cells were pelleted from media by centrifugation and resuspended in 30 mL of lysis buffer (50 mM sodium phosphate and 100 mM sodium chloride, pH 6.5). To catalyze the hydrolysis of the bacterial cell wall, the suspension was treated with lysozyme (50 μg/ml) and incubated for 30 min at 4 °C. The solution was sonicated for 10 min in 30 s intervals, followed by centrifugation at 12000 g for 1 h at 4 °C. APEX2-ABD was further purified by using an immobilized metal affinity chromatography (IMAC). The filtered extract was loaded into 1 mL Ni-NTA agarose affinity column HisTrap^{TM} (GE healthcare, code number 17-5319-01) and eluted by a linear gradient from 5 to 100 % of elution buffer (20 mM sodium phosphate, 500 mM sodium chloride and 1 M imidazole pH 7.4). The free imidazole was removed by extensive dialysis with a buffer solution (50 mM sodium phosphate and 100 mM

sodium chloride, pH 6.5) and the purified APEX2-ABD was characterized by UV/visible spectrophotometry and SDS-PAGE.

Quartz crystal microbalance (QCM) measurements

To check the binding capability of APEX2-ABD and APEX2 to immunoglobulin, Q-Sense E4 and standard gold QCM sensors (Q-Sense, Sweden) were used as described in a previous study.[1] Flow mode with a peristatic pump was used and the temperature of the chamber was maintained at 25.0 ± 0.1 °C. Each sample solution was introduced to the measurement chamber by a peristaltic pump with constant flow. First, the measurement chambers were kept at an equilibrium with a phosphate buffer (100 mM NaCl and 50 mM sodium phosphate, pH 7.4). Subsequently either 1 mg/ml APEX2 or APEX2-ABD solution was introduced to form a monolayer. After maintaining the signal balance using the buffer, 0.2 mg/ml rabbit, mouse, or rat IgG in phosphate buffer were introduced. The QCM chips were extensively washed with phosphate buffer to remove weakly or non-specifically bound materials at each intervals. Resonance frequencies were measured simultaneously at seven harmonics (5, 15, 25, 35, 45, 55 and 65 MHz). For clarity, only the normalized frequency of the third overtone is shown.

Surface plasmon resonance (SPR) analysis

SPR experiments were performed with standard CM-5 gold chips on a Biacore 3000 device at 25 °C using a filtered PBS buffer as a running solution. The CM-5 sensor chips were coated with carboxymethylated dextran to which molecules can be coupled with rabbit, mouse, or rat IgG to the surface of a sensor chip by standard amine-coupling chemistry.[1a, 2] For immobilization of antibodies on the surface of the sensor chip, 0.1 mg/mL of rabbit, mouse, or rat IgG was injected for a short period of time and subsequently 60 μL of a 1:1 mixture of EDC (0.5 mg/mL) and NHS (0.5 mg/mL) was infused onto the chip at a flow rate of 10 μL/min to activate the carboxyl groups on the dextran surface. 0.1 mg/mL of rabbit, mouse, or rat IgG was added (volume was determined by desired RU). Excess reactive carboxylated groups were blocked with 1 M ethanolamine (pH 8.0) and the IgG immobilized CM-5 sensor chips were equilibrated with running buffer. Various amounts of APEX2-ABD or APEX2 (0.08, 0.16, 0.3, 0.6 and 1.2 μM) were loaded to the IgG immobilized CM-5 sensor chips at a flow rate of 5 μL/min for 3 min, followed by washing with running buffer at the same flow rate for 7 min. Responsive units were monitored real-time and the dissociation constants were derived by globally fitting the SPR data to a Langmuir (1:1) model using BIAevaluation software provided by the manufacturer.[2]

Cell culture and fluorescence confocal microscopy

SKBR-3 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum, 1% streptomycin, and 25mM HEPES and 25mM NaHCO3 in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. SCC-7 cells were cultured in RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin. SK-BR-3 and SCC-7 cells (8×10^4/well) were grown in a 12-well culture plate (SPL, 30012) by attaching on microscope cover glass (18 mm Φ). For fluorescence confocal imaging, the cells were fixed with 4% paraformaldehyde in PBS for 20 min and washed three times with PBS containing 0.1% Tween-20. APEX2-ABD and APEX2 were labeled with fluorescein-5-maleimide (F5M) as previously described.[3] To prevent non-specific binding of fAPEX2-ABD or fAPEX2 to the background, the blocking reagent (5% BSA, 5% FBS, and 0.5% Tween-20 in PBS) was introduced and incubated at 4 °C for 12 h prior to sample treatment. Anti-HER2 rabbit IgG (Abcam), anti-CD44 mouse IgG, and anti-CD44 rat IgG (Biolegend) were mix either with fAPEX2-ABD or fAPEX2 and as-formed fAPEX2-ABD/IgG complexes were added to the cell culture plate and incubated at 4 °C for 1 h. The free IgGs and weakly bound proteins were washed three times with PBS buffer containing 0.1% Tween-20. The sample-treated cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) prior to sealing. Fluorescence cell images were obtained by using an Olympus Fluoview FV1000 (UOBC) confocal microscope.[1a]

Amplex® Red hydrogen peroxide/peroxidase assay

To verify peroxidase activity of APEX2-ABD, a plate-level assay was carried out using Amplex® Red reagent (Molecular probe®, cat# A12222) which is converted into red fluorescent product, resorufin, when it is oxidized by hydrogen peroxide. To determine the optimum concentration of APEX2-ABD for an assay, 100 μL samples of 11 different concentrations of APEX2-ABD (0, 15, 31, 62.5 125, 250, and 500 pM and 1, 2, and 4 nM) were aliquotted to 96 well-plate and subsequently 100 μL of a reaction solution (50 μM Amplex Red® and 50 μM H_2O_2) was added to each well. To determine the minimum amount of H_2O_2 that APEX2-ABD can detect, the reaction solution was serially diluted (0, 195, 390, 781 nM and 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 μM of H_2O_2) and each of these dilutions was loaded into the 96 well-plate. Finally, 100 μL of 1 μM APEX2-ABD (final concentration was 500 pM) was added. Fluorescence intensities were monitored for 30 min with a fluorescence microplate reader (Tecan Group Ltd., Infinite® 200) which has a detection limit of fluorescence intensity of 70,000 units. All data were acquired from three independent experiments.

Tyramide Signal Amplification assay

All cells used in the TSA assay were prepared as described above (cell culture section) with some modifications. Primary antibody binding and APEX2-ABD/IgG complex binding were performed for 1 h at room temperature and the cells were washed three times with PBS containing 0.1 % Tween-20. The samples were treated with tyramide working solution (tyramide stock and 0.0015% H_2O_2 in amplification buffer) and incubated for 15 min at room temperature as the manufacturer suggested.

References

1(a) H. J. Kang, Y. J. Kang, Y.-M. Lee, H.-H. Shin, S. J. Chung and S. Kang, *Biomaterials*, 2012, **33**, 5423; (b) Y. J. Kang, M. Uchida, H. H. Shin, T. Douglas and S. Kang, *Soft Matter*, 2011, **7**, 11078.

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3(a) J. Min, S. Kim, J. Lee and S. Kang, *RSC Advances*, 2014, **4**, 48596; (b) H. Moon, J. Lee, J. Min and S. Kang, *Biomacromolecules*, 2014.

Supporting figures

Figure S1

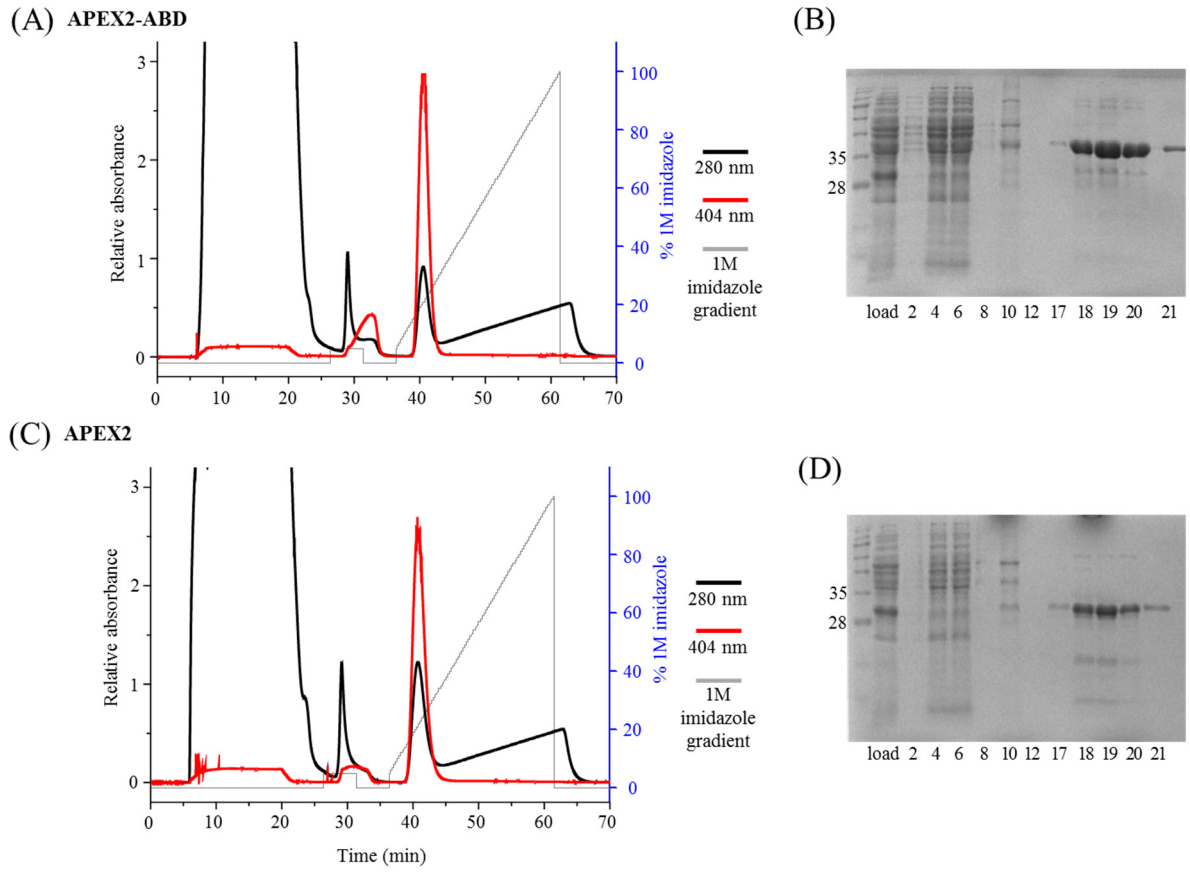

Figure S1. IMAC Chromatograms of APEX2-ABD (A) and APEX2 (C) and their corresponding SDS-PAGEs of collected fractions (B and D). APEX2-ABD and APEX2 were captured with a Ni ion-charged Histrap column and eluted with a linear gradient (grey lines) of a 5-100% elution buffer (1 M imidazole in 20 mM sodium phosphate and 500 mM sodium chloride, pH 7.4). Absorptions were recorded both at 280 (black lines) and 404 (red lines) nm.

Figure S2

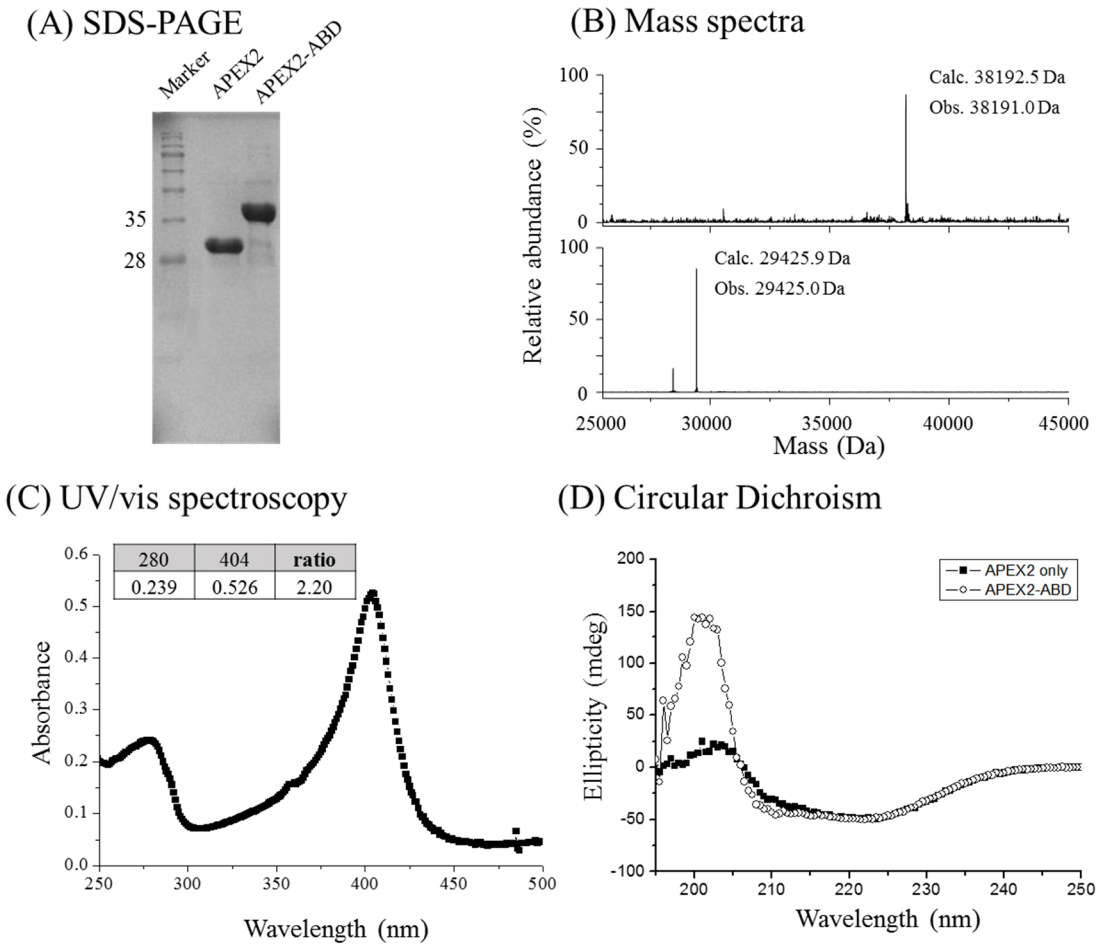

Figure S2. (A) SDS-PAGE of purified APEX2 and APEX2-ABD. (B) Molecular mass measurements of APEX2 (bottom) and APEX2-ABD (top) using ESI-MS. Calculated and observed masses were indicated. Heme groups were dissociated during mass analysis and calculated masses were not included the molecular mass of heme. (C) UV/Vis spectrum of purified APEX2-ABD. Absorption ratio of 280 and 404 nm was determined. (D) The circular dichroism (CD) spectra of APEX2 (closed squares) and APEX2-ABD.

Figure S3

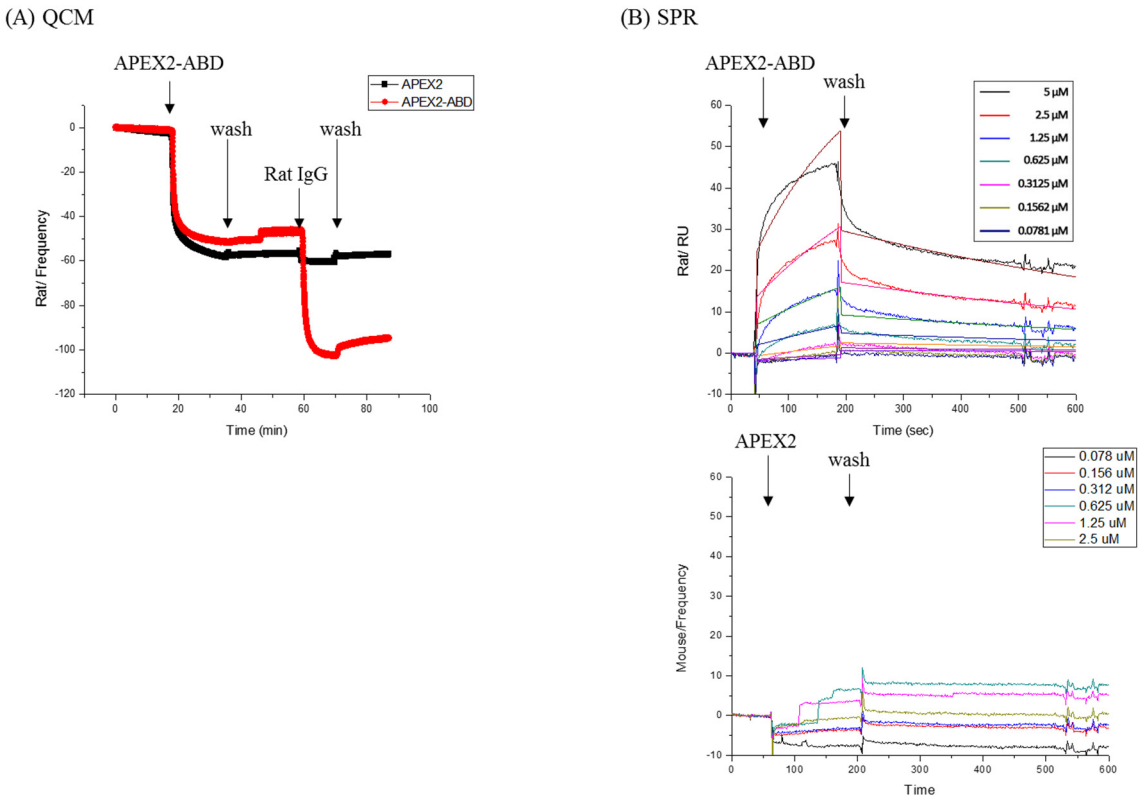

Figure S3. (A) QCM resonance frequency change (-Δ*F*) profiles of either APEX2-ABD (red lines) or APEX2 (black lines) on the gold QCM sensor and subsequent deposition of rat IgG on the monolayer of either APEX2-ABD (red lines) or APEX2 (black lines). (B) SPR analyses of APEX2-ABD (top) and APEX2 (bottom) bindings to rat IgG immobilized gold SPR sensors. Introduction of APEX2-ABD or APEX2 and buffer washing are indicated.

Figure S4

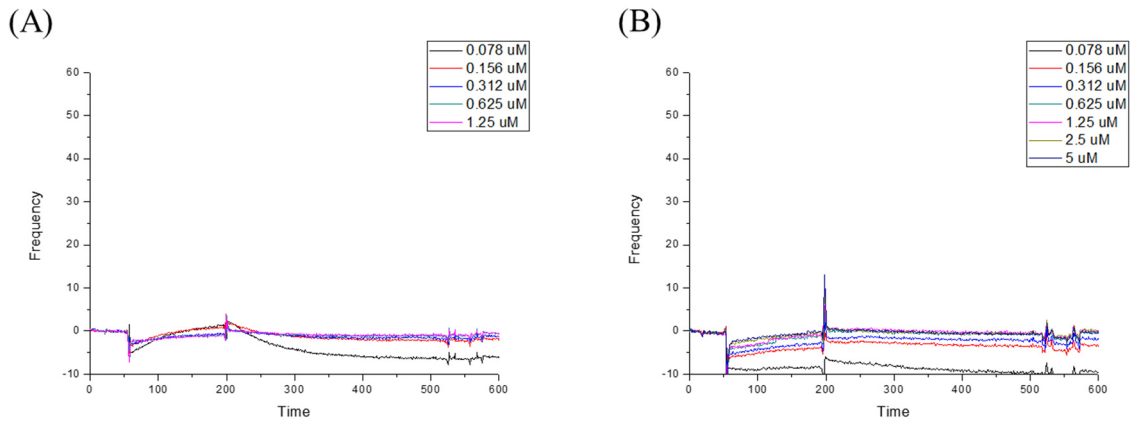

Figure S4. SPR analyses of APEX2 binding to rabbit (A) or mouse (B) IgG immobilized gold SPR sensors.

Figure S5

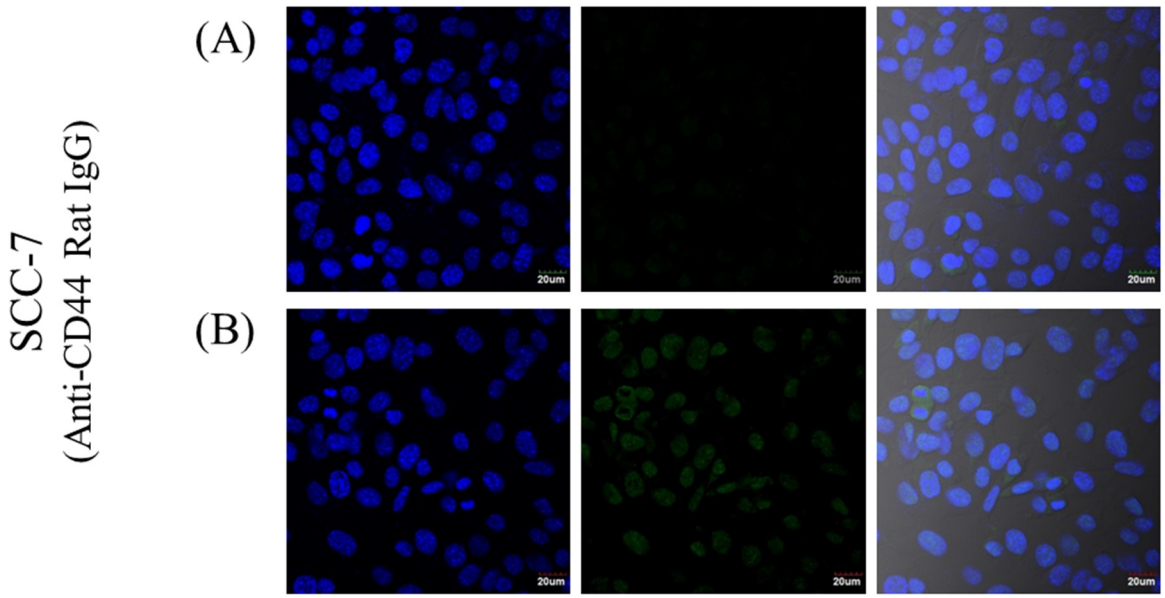

Figure S5. Fluorescent microscopic images of SCC-7 cells treated with fAPEX2-ABD (A) and fAPEX2-ABD/Anti-CD44 rat IgG (B). DAPI (left rows), fluorescein (middle rows), and merged (right rows) images are presented.

Figure S6

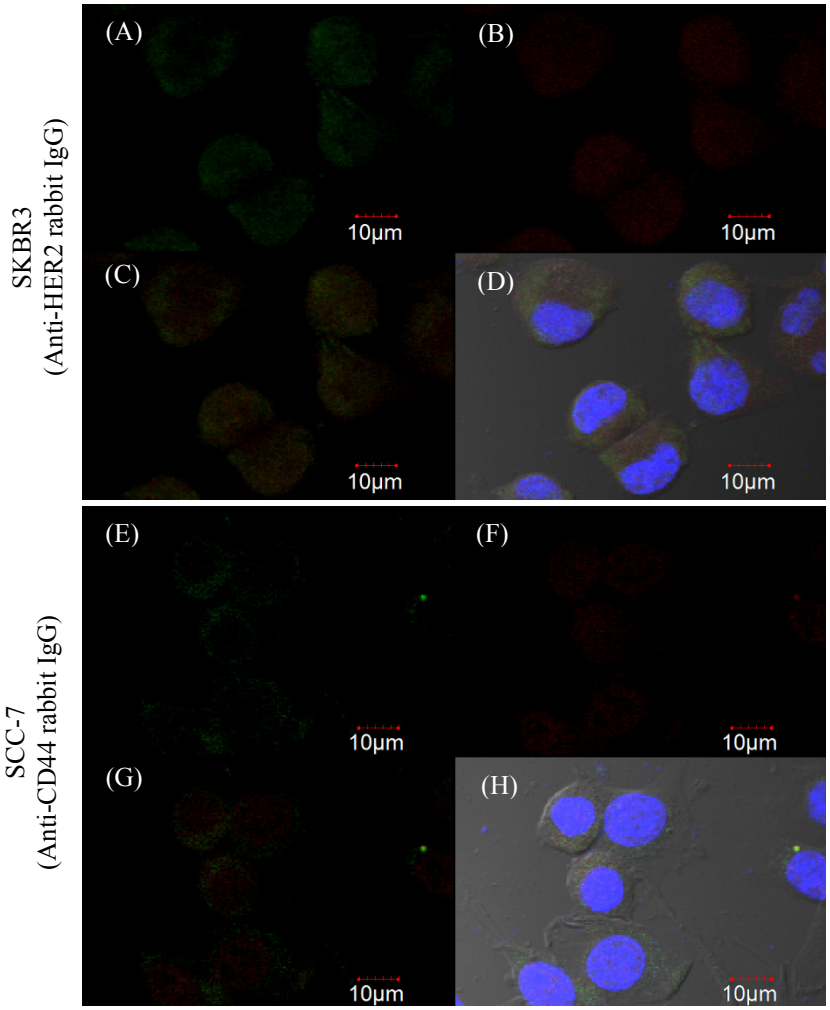

Figure S6. Magnified fluorescent microscopic images of SKBR3 cells (A-D) and SCC-7 cells (E-H) treated with fAPEX2-ABD/Anti-Her2 rabbit IgGs (A-D) and fAPEX2-ABD/Anti-CD44 mouse IgGs (E-H), respectively, before TSA treatment (A, E) and after TSA treatment (B, F) are shown. Fluorescein (A, E), Alexa-555 (B, F), merged (C, G), and DAPI (D, H) images are presented.