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Supplementary information for

Plug-and-Playable Fluorescent Cell Imaging Modular Toolkits Using the Bacterial Superglue, SpyTag/SpyCatcher

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Materials and Methods

Genetic modification and purification of engineered monomeric proteins

SpyTag peptide (AHIVMVDAYKPTK) with extra amino acids was genetically added to the N-termini of affibodies (HER2 and EGFR affibodies) and fluorescent proteins (mCherry and eYFP) by an established polymerase chain reaction (PCR). SpyCatcher protein with extra amino acids also was genetically fused to the C-termini of fluorescent proteins and N-termini of affibodies by PCR. Eight types of proteins (ST-HER2Afb/ST-EGFRAfb, mCherry-SC/eYFP-SC, SC-HER2Afb/SC-EGFRAfb and ST-mCherry/ST-EGFRAfb) are transformed into competent *E. coli* strain BL21 (DE3), and the proteins were overexpressed. All types of proteins were purified with immobilized metal affinity chromatograpy (IMAC, 1mL HisTrap FF column / GE HealthCare).

Formation and characterization of affibody-fluorescent protein conjugates (AFPCs)

ST-affibody (ST-HER2Afb, ST-EGFRAfb, and ST-GST-HER2Afb), SC- affibody (SC-HER2Afb, SC-EGFRAfb), fluorescent protein-SC (eYFP-SC, mCherry-SC) and ST-fluorescent protein (ST-eYFP, ST-mCherry) were stable in PBS (pH 7.4) and their concentrations were examined by BCA assay. ST-proteins and their corresponding SC-proteins were simply mixed in PBS and incubated for 5 min to overnight at room temperature with gentle shake. 35 μM of each protein was used for AFPC formation

and approximately 5 μM of AFPCs were used for fluorescent cell imaging. Reaction products were subsequently analyzed with SDS-PAGE and MS. Reaction was stopped at indicated time by adding SDS loading buffer and boiling at 110 °C. Each monomeric protein or reaction product was loaded onto a MassPREP microdesalting column (Waters) with aqueous acetonitrile solvents (buffer A: 5% acetonitrile, 1% formic acid in water) and eluted with buffer B (5% water, 1% formic acid in acetonitrile) at a flow rate of 0.5 mL/min. The molecular masses of each species can be determined from the charges and the observed m/z ratio values. Mass spectra were acquired in the range of m/z 500-3000 and deconvoluted using MaxEnt1 from MassLynx program to obtain the average mass from multiple charge state distributions.

Cell culture and fluorescent cell microscopy

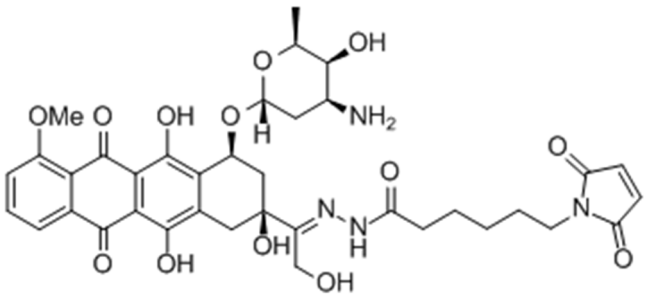
SK-BR-3 cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic, MCF-10A cells were grown in DMEM/F12 with 5% horse serum, 1% penicillin/streptomycin, EGF, hydrocortizone, insulin and 25mM sodium bicarbonate. MDA-MB-468 cells were grown in Leibovitz's L-15 medium with 10% FBS, 1% antibiotic-antimycotic, 25mM HEPES and sodium bicarbonate. MCF-7 cells were cultured in RPMI-1640 with 10%FBS and 1% antibiotic-antimycotic. SK-BR-3, MCF-10A, MDA-MB-468 and MCF-7 cells were incubated in humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were grown on microscope cover glass in 12-well plate and treated with 5 μM of each AFPC sample for 1 hr at 37 °C. Subsequently, cells were fixed with 4% paraformaldehyde in PBS. Negative controls (only fluorescent proteins) also were added to cells under the same condition. Before sealing, the cells were washed two times with PBS, and nuclei were stained with DAPI. Images of AFPC samples were collected with Olympus Fluoview FV1000 fluorescent microscope (Olympus, UOBC).

Cytotoxicity assay

The *in vitro* cytotoxicity was measured using thiazolyl blue tetrazolium bromide (MTT, sigma-Aldrich) assay. SK-BR-3 and MCF10A cells were seeded into 96-well cell culture plate at 1×10^4 cells/well and then incubated in grow medium for 24 hr at 37 °C under 5% CO_2. After cells were treated with AFPC, AlDox loaded AFPC, free AlDox and PBS in cells for 1 hr, they were washed with fresh medium and grown for 7 days. The cells were treated with 200 μL of media containing of 0.5 mg/mL of MTT for 4 hr, media were removed, and the cells were resuspended with 200 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals formed by viable cells. Absorbances of 96-well plate were measured

at 595 nm using multiscanner (TECAN).

The Structure of AlDoxorubicin and Fluorescein-5-maleimide



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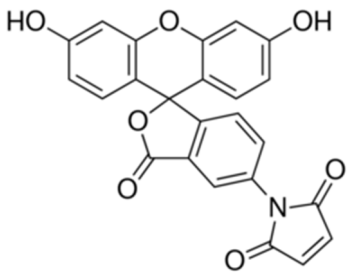

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2. eYFP_SpyCatcher

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2) Amino acid sequence

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3. SpyTag_mCherry

1) DNA sequence

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4. SpyTag_eYFP

1) DNA sequence

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5. SpyCatcher_HER2Afb

1) DNA sequence

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6. SpyCatcher_EGFRAfb

1) DNA sequence

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2) Amino acid sequence

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7. SpyTag_HER2Afb

1) DNA sequence

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2) Amino acid sequence

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8. SpyTag_EGFRAfb

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9. SpyTag_GST_HER2Afb

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2) Amino acid sequence

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Supplementary Figures

Figure S1

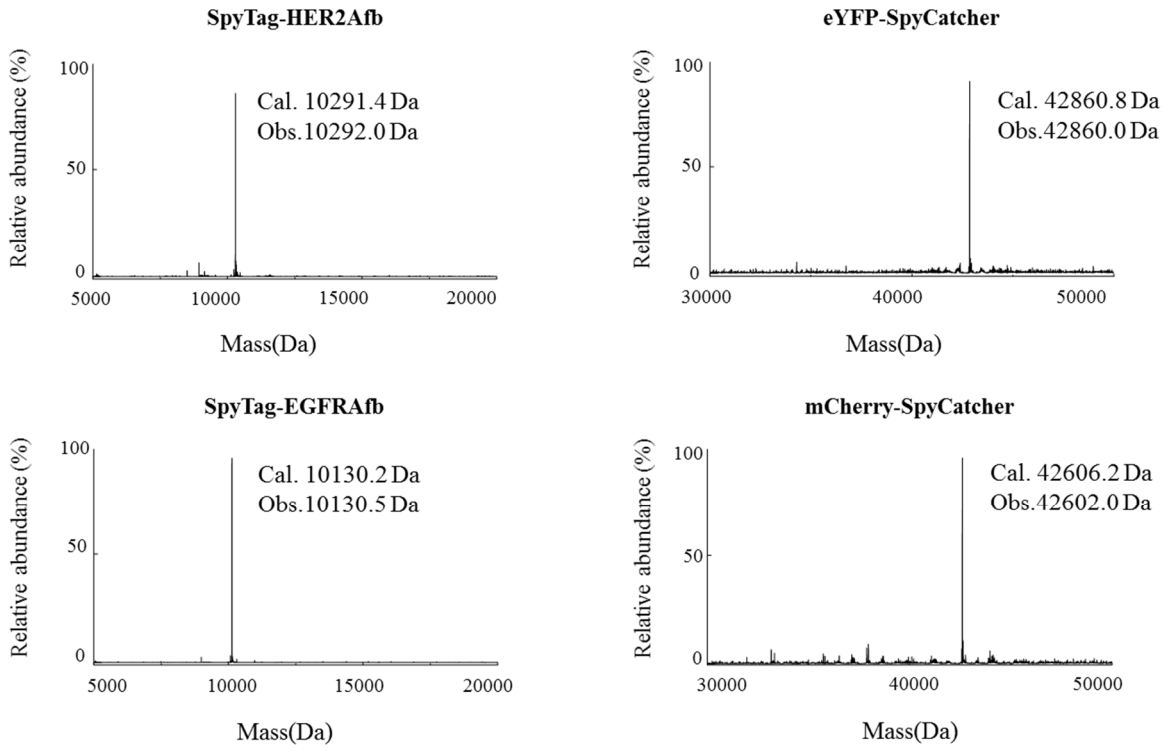


Figure S1. Molecular mass measurements of SpyTag-affibodies and fluorescent proteins-SpyCatcher.

FigureS2

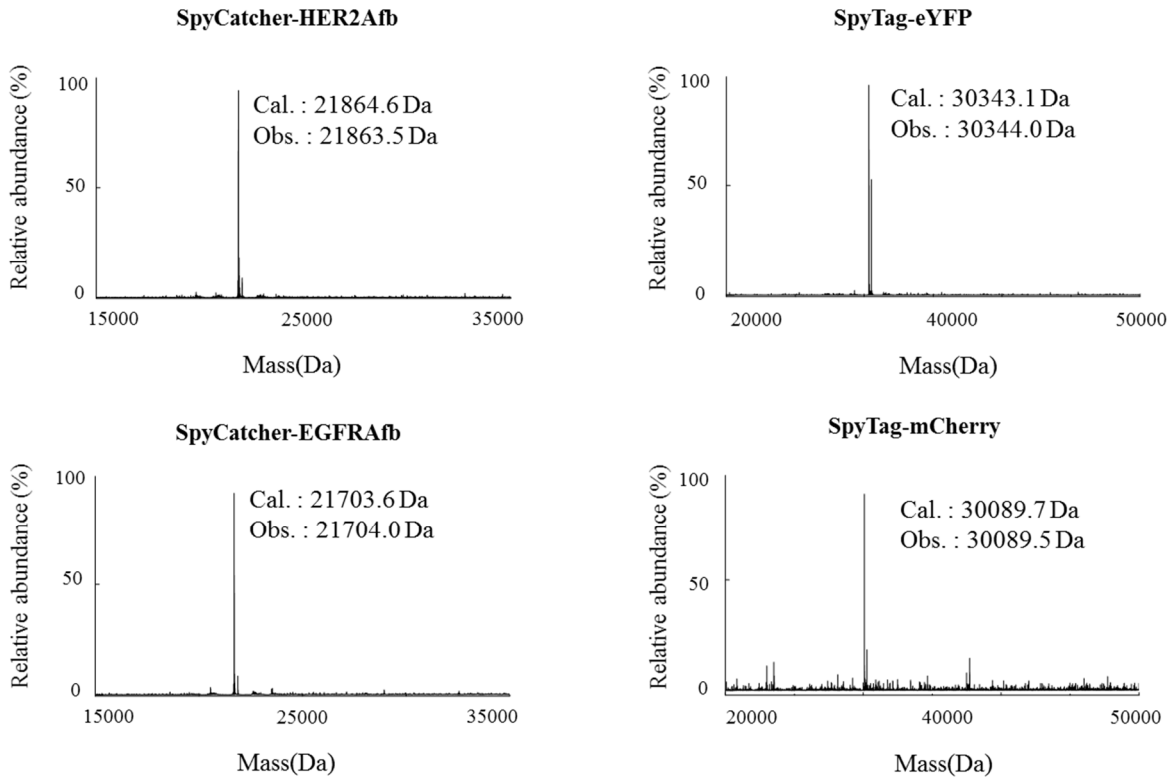
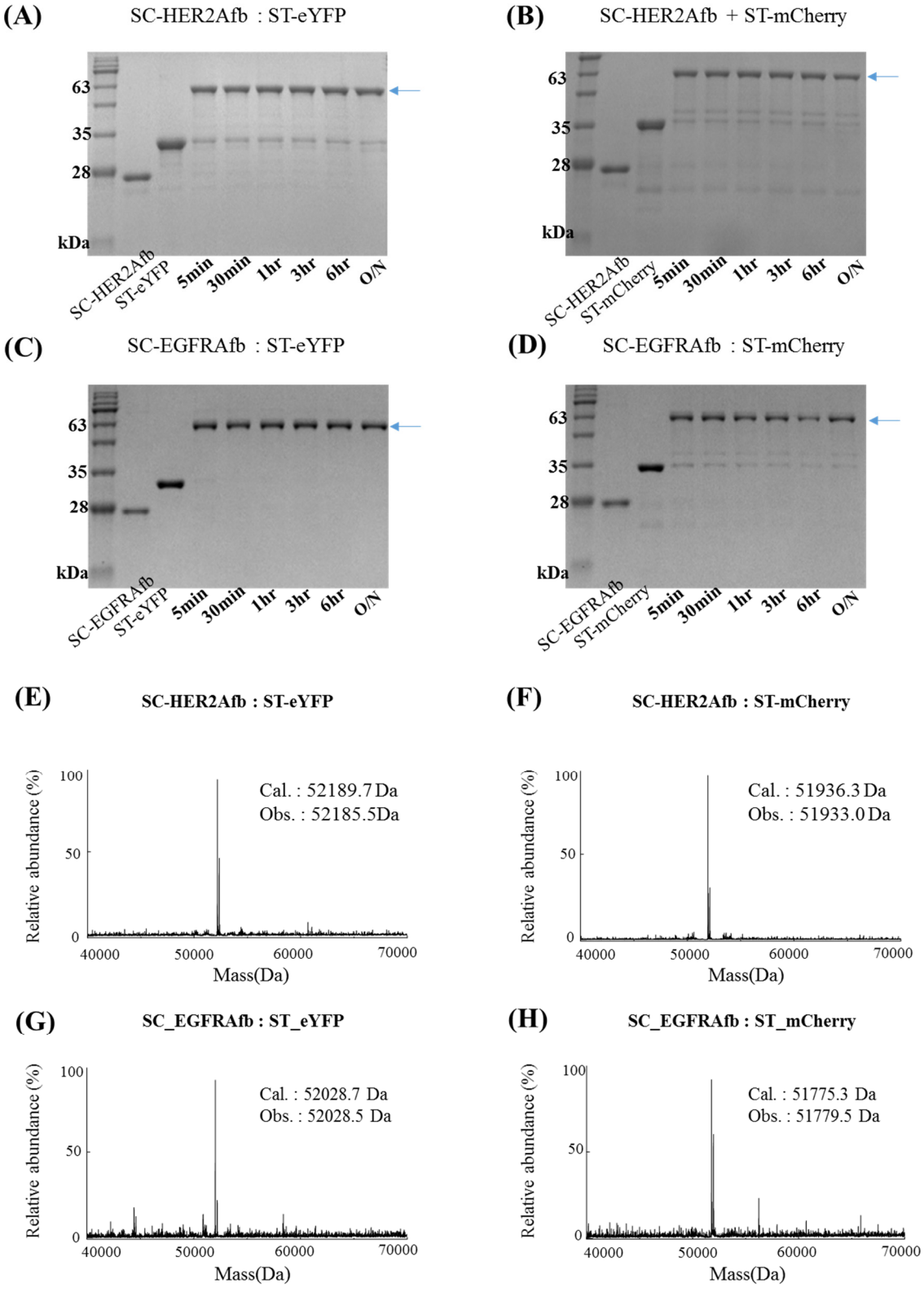


Figure S2. Molecular mass measurements of SpyCatcher-affibodies and SpyTag-fluorescent proteins.

FigureS3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28
kDa
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1hr
3hr
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O/N
(B)
SC-HER2Afb + ST-mCherry
63
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28
kDa
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63
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SC-HER2Afb : ST-eYFP
Relative abundance (%)
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Obs. : 52185.5Da
Mass(Da)
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SC-HER2Afb : ST-mCherry
Relative abundance (%)
Cal. : 51936.3 Da
Obs. : 51933.0 Da
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Relative abundance (%)
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Relative abundance (%)
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Obs. : 51779.5 Da
Mass(Da)

Figure S3. Characterization of affibody–fluorescent protein conjugates (AFPC) as results of the isopeptide bond formation between SpyCatcher-affibody and SpyTag-fluorescent protein. Reaction products of SC-HER2 and ST-eYFP (A, E), SC-HER2 and ST-mCherry (B, F), SC-EGFR and ST-eYFP (C, G), and SC-EGFR and ST-mCherry (D, H) are analyzed with SDS-PAGE (A-D) and ESI-TOF MS (E-H). Reaction times and molecular weight markers are indicated (A-D). Calculated and observed molecular masses are indicated (E-H). (Blue arrow: conjugated product of AFPCs)

FigureS4

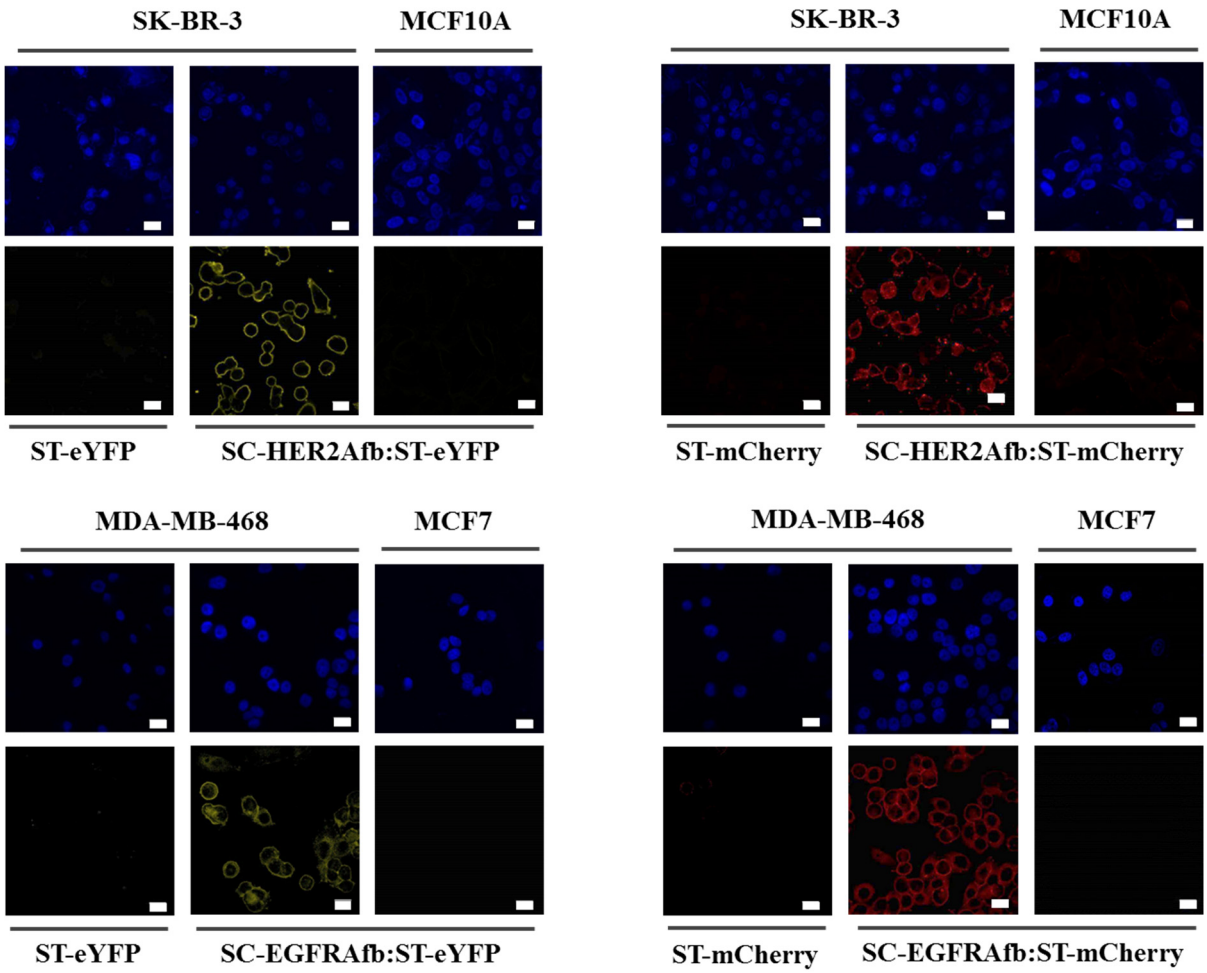

Figure S4. Fluorescent microscopic images of various cells treated with AFPCs and ST-fluorescent proteins. Cells and AFPCs are indicated on top and the bottom of image panels, respectively. Nuclei are stained with DAPI (blue, top panels), and eYFP and mCherry are visualized with yellow and red, respectively (bottom panels).

FigureS5

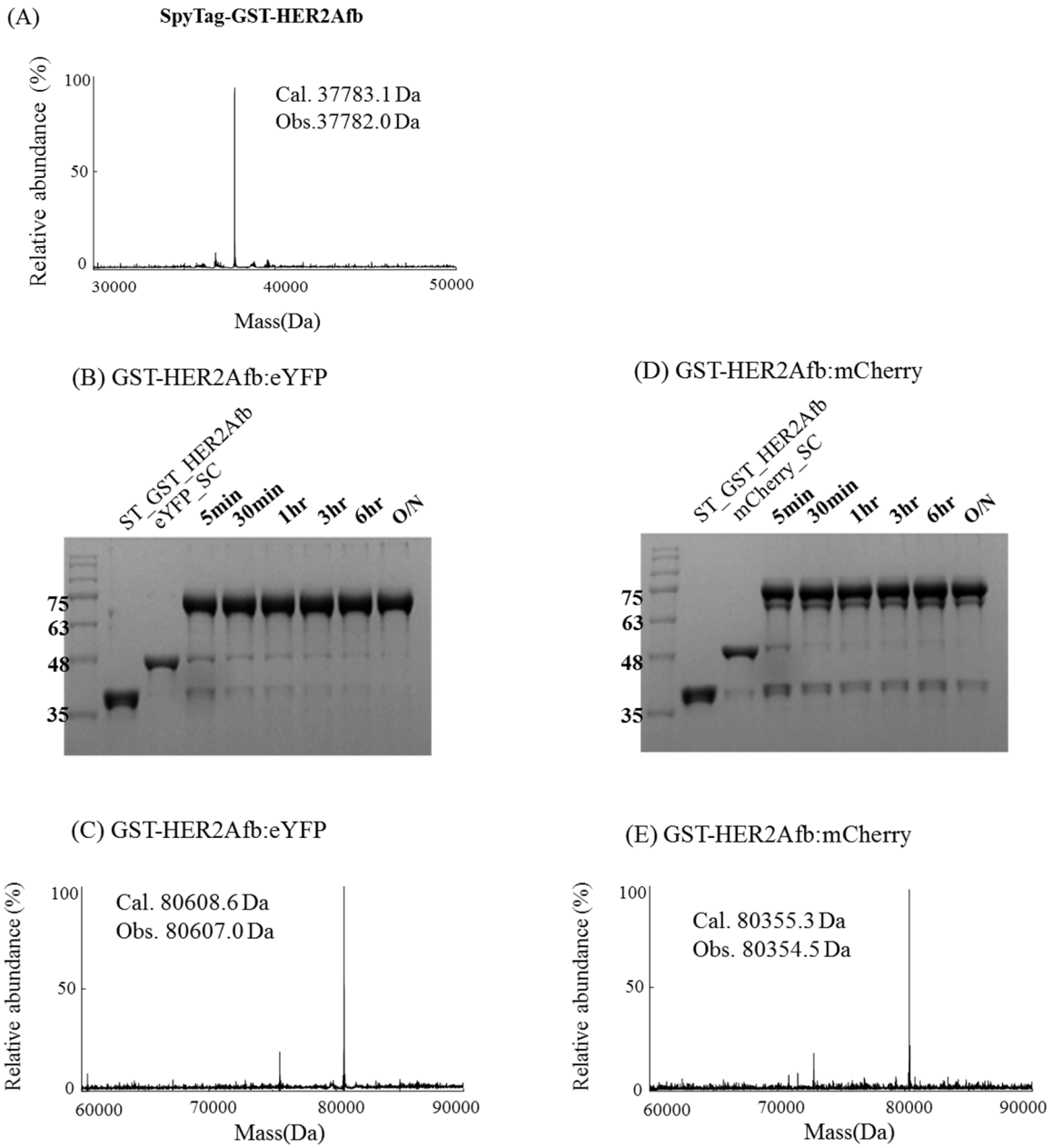

Figure S5. Characterization of ST-GST-HER2 and GST-HER2:fluorescent proteins-SC. Molecular mass of SpyTag-GST-HER2 is measured with EST-TOF MS (A). Reaction products between ST-GST-HER2 and fluorescent proteins-SC are analyzed via SDS-PAGE (B, D) and ESI-TOF MS (C, E).

Figure S6

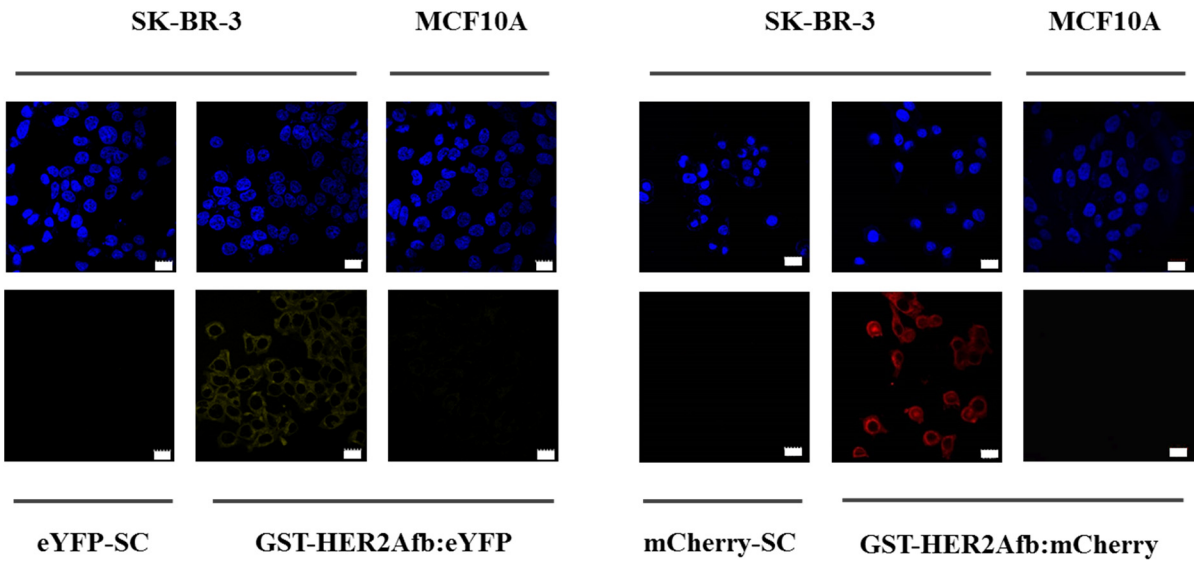

Figure S6. Fluorescent microscopic images of SK-BR-3 and MCF 10A cells treated with fluorescent proteins-SC, GST-HER2:fluorescent proteins (GST-HER2:eYFP or GST-HER2:mCherry). Cells and AFPCs are indicated on top and the bottom of image panels, respectively. Nuclei are stained with DAPI (blue, top panels), and eYFP and mCherry are visualized with yellow and red, respectively (bottom panels). (Scale bar: 20μM).

Figure S7

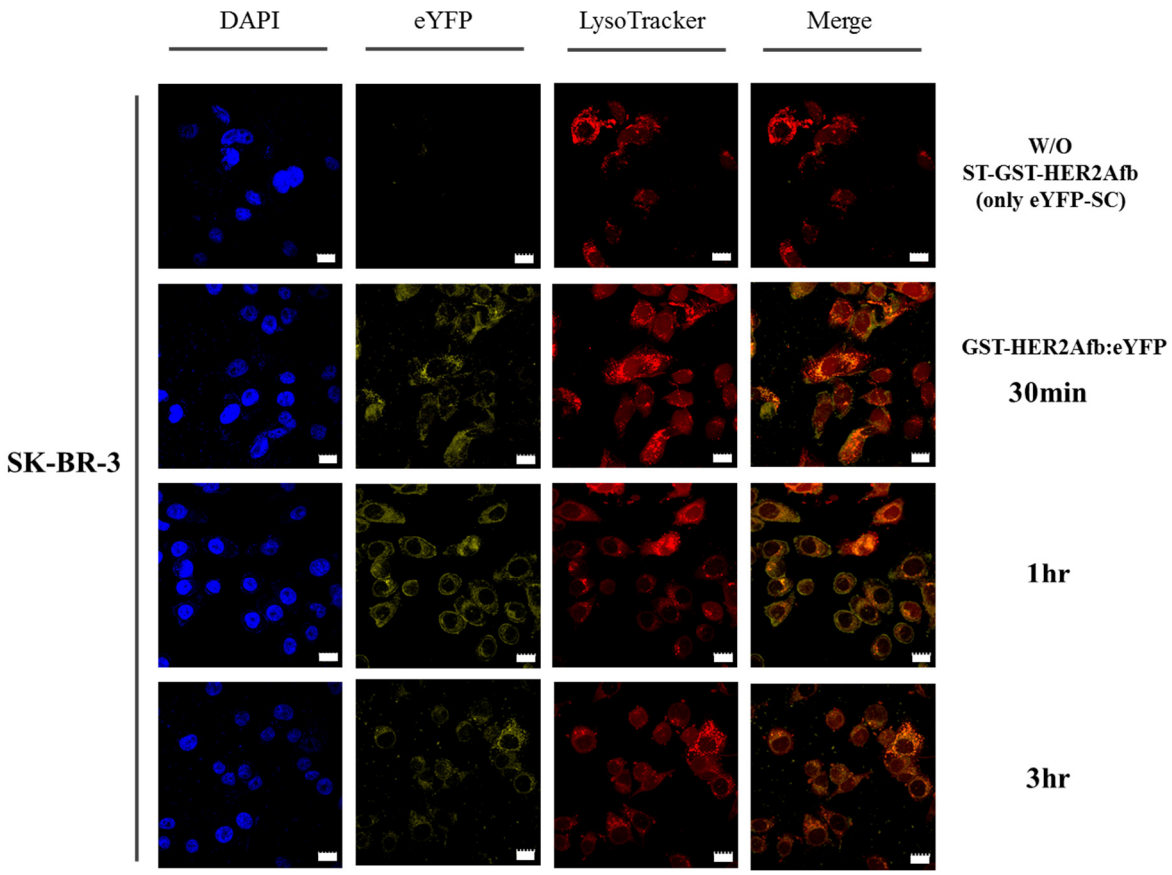

Figure S7. Subcellular localization of GST–HER2:eYFP in SK-BR-3 cells are visualized according to incubation times. Nuclei are stained with DAPI (blue, left column), and eYFP and lysotracker are visualized with yellow and red, respectively (middle two columns). Merged images of eYFP and lysotracker are presented in right column. (Scale bar: 20μM)

Figure S8

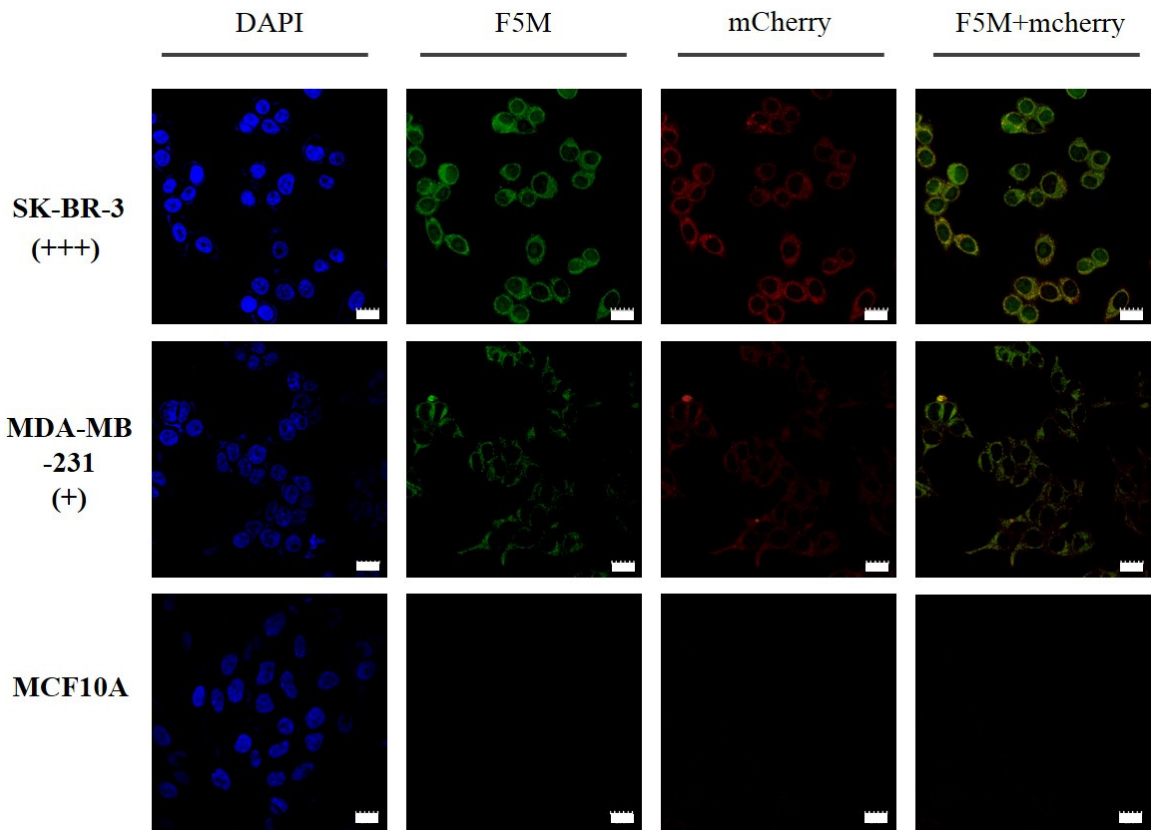

Figure S8**.** Fluorescent microscopic images of various cells treated with F5M-GST-HER2:mCherry. High (SK-BR-3, top panels), low (MDA-MB-231, middle panels), or no (MCF10A, bottom panels) fluorescent signals are observed in the cytosol, depending on the levels of HER2 expression and green and red fluorescent signals overlap to each other. Nuclei are stained with DAPI (blue, left columns), and F5M and mCherry are visualized with green and red, respectively (second and third columns). Merged images of F5M and mCherry channels are presented in right columns. (Scale bar: 20μM)