N-terminal Labeling of Filamentous Phage to Create Cancer Marker Imaging Agents

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Supporting Information

Materials

Unless otherwise noted, all chemical reagents were purchased from Aldrich. Alexa Fluor® 488 C5-aminooxyacetamide, bis(triethylammonium) salt (AF488-ONH_2), Alexa Fluor® 647 C5-aminooxyacetamide, bis(triethylammonium) salt (AF647-ONH_2), *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine, trifluoroacetic acid salt, and neutravidin-HRP were purchased from Invitrogen. M13KE and Anti-M13 p3 antibodies were purchased from New England Biolabs. *O*-(Methoxypoly(ethylene glycol))-hydroxylamine (PEG2k-ONH_2) was prepared as previously described.[1] Water used in biological procedures and chemical reactions was deionized using a NANOpure purification system (Barnstead, USA). All cell culture reagents were obtained from Gibco/Invitrogen Corp (Carlsbad, CA) unless otherwise noted.

Instrumentation

High performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, USA). Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and an Agilent Zorbax 300 SB-CN column. 0.1% TFA/water (A) and 0.1% TFA/acetonitrile (B) were used as HPLC solvents. The following method was used: 35% B for the first 4 min, ramping to 70% B over 15 min, then to 95% B over the next 30 s, and a 5.5 min wash with 95% B.

Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was performed on a Voyager-DETM system (PerSeptiveBiosystems, USA) in the QB3/Chemistry Mass Spectrometry Facility. Sinipinic acid was used as the matrix.

Transmission electron microscopy (TEM). TEM images were obtained at the UC Berkeley Electron Microscope Lab (www.em-lab.berkeley.edu) using an FEI Tecnai 12 transmission electron microscope with a 100 kV accelerating voltage. Samples were prepared for TEM analysis by pipetting 8 μL of 0.1 nM fd solutions onto grids and allowing them to equilibrate for 3 min. The samples were wicked dry with filter paper, and the grids exposed to 8 μL of 10 mg/mL aqueous uranyl acetate solution for 90 s as a negative stain. The excess stain was removed by wicking, and the grid was allowed to dry in air.

Flow cytometry. A FACSCalibur flow cytometer (BD Biosciences, USA) equipped with 488 and 633 nm lasers were used for all flow cytometry measurements, usage courtesy of Prof. Carolyn Bertozzi (UC Berkeley).

Confocal microscopy. Images were acquired on a Zeiss 510 NLO Axiovert 200M Tsunami microscope equipped with 488 and 633 nm lasers, usage courtesy of Prof. Christopher Chang (UC Berkeley).

Zeta potential. Zeta potential measurements were obtained using a Malvern Instruments Zetasizer Nano ZS and DTS1060 cuvettes, usage courtesy of Prof. Jean M. J. Fréchet (UC Berkeley). Measurements were taken in water. Thirty measurements were taken per sample.

Calculation of phage concentration

phage/mL = ((absorbance at 269 nm – absorbance at 320 nm) / 6 x 10^6) / (number of single stranded DNA bases in the phage genome)[2]

Detailed Experimental Procedures

fd and M13KE phage growth and purification. fd phage displaying anti-EGFR, -HER2, and -BoNT scFvs were propagated in and reacted under identical conditions.[3–5] A tetracycline resistance gene was previously introduced into the fd phage genome to allow measurement of *E. coli* infectivity in colony forming units (cfu) using LB-agar plates containing 20 μg/mL tetracycline. A colony of *E. coli* TG1 cells infected with fd were inoculated into 2 mL of LB growth media containing 20 μg/mL tetracycline, incubated at 37 °C with 250 rpm shaking. After approximately 6 h, 1 mL culture was added to 1 L of 2xYT media containing 20 μg/mL tetracycline. The culture was incubated at 30 °C for approximately 13 h with 250 rpm shaking. Cells were removed via centrifugation at 6,000 rpm for 10-30 min at 4 °C. The supernatant was collected and the fd were precipitated for 1 h at 4 °C after thorough mixing with 0.15 volumes of 20% PEG8k/2.5M NaCl solution. The resulting suspension was centrifuged at 8,000 rpm for 20 min at 4 °C, and the recovered pellet was resuspended in 30 mL of 4 °C PBS. This solution of fd was centrifuged at 6,000 rpm for 10 min at 4 °C to remove additional cell debris. The supernatant was collected, and fd were precipitated for 40 min at 4 °C after thorough mixing with 0.15 volumes of a solution of 20% PEG8k and 2.5 M NaCl. The samples were then centrifuged at 9,000 rpm for 30 min to isolate the precipitated fd. The resulting pellet was resuspended in 5 mL of 4 °C PBS.

M13KE filamentous phage were used for western blotting experiments. Because M13KE lacks antibiotic resistance, it was grown from plaques rather than colonies. It was grown in media lacking antibiotics at 37 ºC and its purification was identical to that used for fd.

Transamination. fd and M13KE were transaminated using 100 mM pyridoxal-5'-phosphate (PLP) in 100 mM phosphate buffer, pH 6.5, for 13 h at rt. Concentrations of 75-128 nM fd were used in these experiments, typically at total volumes of 5-20 mL. Due to the large excess of PLP used, fd concentration was not found to be critical for successful transamination. As an example reaction: 4.7 mL of water was added to 3.3 mL of 128 nM anti-EGFR fd, followed by 1 mL of 250 mM phosphate buffer at pH 6.5, and 1 mL of 1 M PLP in 125 mM phosphate buffer, pH 6.5. The transamination was allowed to proceed for approximately 13 h, at which point the excess PLP was removed by a series of precipitations and resuspensions in PBS. After thorough mixing with 0.15 volumes of a 20% PEG8k/2.5 M NaCl solution, the fd were precipitated for 1 h at 4 °C. The fd were then isolated by centrifugation at 9,000 rpm for 30 min. The fd pellet was resuspended in 30 mL of PBS, and then the precipitation, centrifugation, and

resuspension cycle was repeated two additional times. The final pellet was resuspended in PBS to yield a final volume of approximately 2 mL. To prepare the PLP solution used in these reactions, a 2 M solution of PLP in 250 mM phosphate buffer (pH 6.5) was made. The pH was adjusted to 6.5 with 3 M NaOH, and the solution was diluted with 250 mM phosphate buffer, pH 6.5 to give a 1 M PLP solution. The PLP solution must be freshly made before use.

Reaction with 2-(aminooxy)acetic acid. 24 nM transaminated fd was reacted with 5 mM 2-(aminooxy)acetic acid in 100 mM anilinium acetate, pH 4.7, for 21 h at rt. For M13KE, 25 nM transaminated M13KE was reacted with 7 mM 2-(aminooxy)acetic acid in 100 mM phosphate buffer, pH 4 with 1 mM aniline for 21 h at rt. Both reactions conditions yield approximately equivalent percentages of oxime product (Supporting Information Figure S1 and S3).

M13KE reaction with biotin and western blotting. M13KE filamentous phage were used for biotin labeling as an fd surrogate because fd could not be sufficiently concentrated to observe all minor coat proteins by western blot. M13KE is a one amino acid variant of fd, and is shorter because its genome has not been enlarged by genetic engineering. This decrease in length enables higher minor coat protein concentrations to be obtained.[2] The final reaction concentrations were: 296 nM M13KE, 10 mM phosphate buffer (pH 6.2), 10 mM aniline, and 16 mM biotin-ONH_2. After 15 h at rt, the reaction was quenched by adding DL-glyceraldehyde to a final concentration of 33.3 μM, followed by SDS-PAGE. A 1:10,000 dilution of anti-p3 antibodies and a 1:2,000 dilution of Neutravidin-HRP were used for blotting. A Genscript 1-hour western kit was used for detection.

Conjugation of fluorophores to ketone-modified fd phage. A sample of ketone-labeled fd phage prepared as described above was exposed to the appropriate alkoxyamine and an aniline catalyst[6] in an Eppendorf tube. The final reaction concentrations were: 185 nM fd, 20 mM phosphate buffer pH 6.2, aniline (10 mM for AF-488 or 100 mM for AF-647, as a catalyst for the oxime formation), and 1 mM fluorophore. Total reaction volumes were typically <100 μL. The reaction was quenched by fd precipitation and solution removal after 45 min at rt, resulting in 2% p8 labeling with the fluorophore. For higher levels of modification, the reactions were allowed to proceed for up to 18 h. Levels of modification were calculated using the extinction coefficients of the fluorophores (AF488: 71,000 M^{-1} cm^{-1}, AF647: 237,000 M^{-1} cm^{-1} according to Molecular Probes/Invitrogen) to determine the fluorophore concentration. After the fluorophore contribution to the 269 and 320 nm absorbance has been subtracted from the total 269 and 320 nm absorbance, the fd concentration can be calculated. Excess fluorophore was removed in an analogous fashion to the removal of excess PLP.

Conjugation of PEG2k to ketone-modified fd phage. Fluorophore labeled fd were reacted with PEG2k-ONH_2 for varying lengths of time. The conditions for the PEG2k-ONH_2 reaction were: 37 nM phage, 20 mM PEG2k-ONH_2, 20 mM phosphate buffer pH 6.2, and 10 mM aniline. After 1.5, 4, and 22 h at rt, aliquots from the reaction mixture were washed over an Illustra Nap-5 gel filtration column (GE Healthcare). If desired, DL-glyceraldehyde can also be used to quench the reaction before passing it over an Illustra Nap-5 gel filtration column; however, this quench was not used for the samples described in this report. The extent of PEG2k labeling was quantified using reverse phase HPLC. The typical p8 coat protein elution time was 10-13 min.

Zeta potential measurement. Zeta potential measurements were performed using a Zetasizer Nano-DS and DTS1060 cuvettes. fd labeled with PEG2k, but not with fluorophore, were used. Following the PEG conjugation reaction, the fd were eluted from NAP-5 columns in water to improve the reproducibility of the zeta-potential measurements.

Cell culture. Immortalized human breast cancer cells were maintained according to ATCC guidelines. SUM52PE cells were from the Tissue Culture Facility, Department of Molecular & Cell Biology, UC Berkeley, and were grow in Ham's F-12 media supplemented with 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 10 mM HEPES (pH 7.4). MCF-7 clone 18 cells were from the Preclinical Therapeutics Core Facility, UCSF. All cells were grown at 37 °C in 5% CO_2.

Cell microscopy. Cells were washed with PBS, trypsinized, and trypsin was quenched with growth media. Cells were centrifuged at 125 rcf for 5 min, and resuspended in growth media. Following counting via hemocytometer, the cells were centrifuged again, and resuspended in normal growth media at a concentration of 25,000 cells/mL. 2 mL was added to each 35 mm glass bottom dish (MatTek Corp.). For MCF-7 clone 18/MDA-MB-231 co-cultures, 1 mL (25,000 cells/mL) of each cell line was added to a centrifuge tube and mixed by pipetting prior to plating in dishes together. Cells were allowed to grow at 37 °C with 5% CO_2 for 72-96 h. All media was removed from the dishes, and cells were washed once with 1 mL PBS. 150 μL of 0.8 nM fd in flow cytometry buffer (FCB, see below) was added to each well of the plate, and the dishes were incubated at 37 °C with 5% CO_2. After 1 h, 1 mL of PBS was added to wash the cells gently, and was then removed. Two more washes with 1 mL of PBS were performed, and then 1 mL of phenol red-free media with 10% FBS was added to the cells. DAPI was added to 1 μM prior to imaging.

Flow cytometry. Following the harvesting and counting of cells as above, cells were resuspended in flow cytometry buffer (FCB; 1% FBS in DPBS). The cells were aliquotted into Eppendorf tubes at 100 μL (500,000 cells) per tube and kept on ice. 100 μL of 0.8 nM fd in FCB was added and incubated at 4 ºC. After 1 h, each sample was diluted to 1 mL with FCB, and the tubes were centrifuged at 2,000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in 1 mL of FCB, followed by centrifugation, and removal of the supernatant. The cells were finally resuspended in 200 μL of FCB. Data were analyzed using FlowJo analysis software (Tree Star Inc.). Gating was performed by applying the autogating tool in FlowJo onto the major population of cells in the FSC x SSC (forward versus side scatter plots) of untreated samples; additional (agent treated) samples were subject to the same gating as the untreated populations for that respective cell line.

Supporting Information References

[1] Schlick, T. L.; Ding, Z.; Kovacs, E. W.; Francis, M. B. Dual-Surface Modification of the Tobacco Mosaic Virus. *J. Am. Chem. Soc.* **2005**, *127*, 3718-3723.

[2] Barbas, C. F.; Burton, D. R.; Scott, J. K.; Silverman, G. J. *Phage Display: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, **2001**.

[3] Zhou, Y.; Drummond, D. C.; Zou, H.; Hayes, M. E.; Adams, G. P.; Kirpotin, D. B.; Marks, J. D. Impact of Single-Chain Fv Antibody Fragment Affinity on Nanoparticle Targeting of Epidermal Growth Factor Receptor-Expressing Tumor Cells. *J. Mol. Biol.* **2007**, *371*, 934-947.

[4] Amersdorfer, P.; Wong, C.; Smith, T.; Chen, S.; Deshpande, S.; Sheridan, R.; Marks, J. D. Genetic and Immunological Comparison of Anti-Botulinum Type A Antibodies from Immune and Non-Immune Human Phage Libraries. *Vaccine* **2002**, *20*, 1640-1648.

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[6] Dirksen, A.; Hackeng, T. M.; Dawson, P. E. Nucleophilic Catalysis of Oxime Ligation. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581-7584.

Figure S1

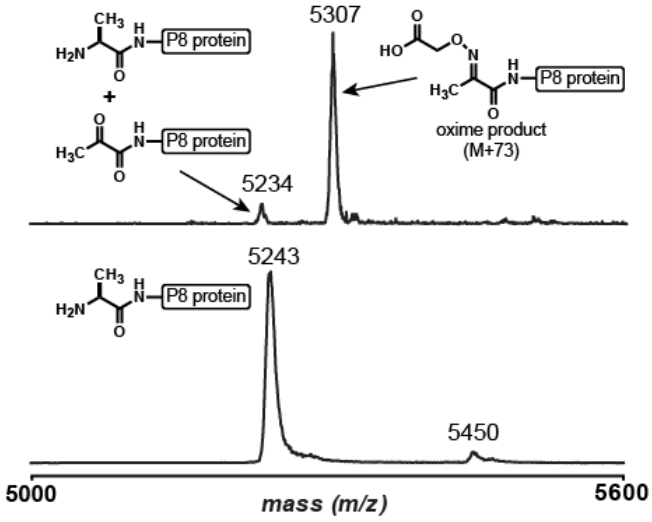

Figure S1. MALDI-TOF spectrum of transaminated fd (top) and non-transaminated fd (bottom) following reaction with 2-(aminooxy)acetic acid. The expected molecular weight of p8 is 5240 Da. The observed 5234 *m*/*z* peak (top) and the 5243 m/z peak (bottom) correspond to the transaminated and/or unmodified p8 proteins. The peak at 5307 m/z (top) corresponds to the oxime product (expected mass increase: 73 m/z, observed 73 *m*/*z*).

Figure S2

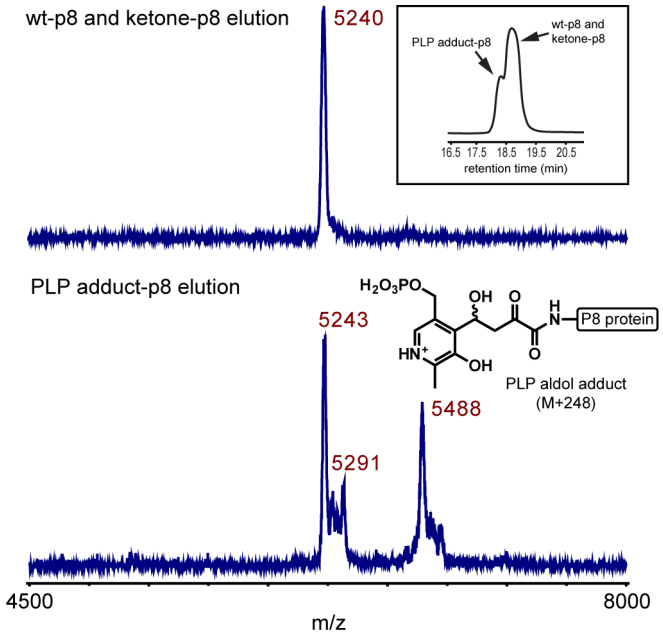

Figure S2. Characterization of modified M13KE phage p8 using MALDI-TOF MS after M13KE transamination and separation of p8 modifications via reversed phase HPLC**.** (Inset) HPLC chromatogram of modified M13KE. (Top) MALDI-TOF spectrum corresponding to the major peak of the p8 elution (see Figure S13, top spectrum for the analogous elution profile of fd). The expected molecular weight of wt-p8 is 5239 Da and overlaps with that of the expected ketone-p8 mass of 5238 Da. (Bottom) MALDI-TOF spectrum of the shoulder-peak, which elutes slightly before the main peak, corresponding to the PLP adduct-P8; some of the major elution peak bleeds into this peak, explaining the 5243 Da signature. The 5291 peak is of unknown origin. The observed mass difference between the two major MALDI peaks is 245 Da, which is presumably an aldol addition of the ketone group to the PLP aldehyde (expected change: 248 Da).

Figure S3

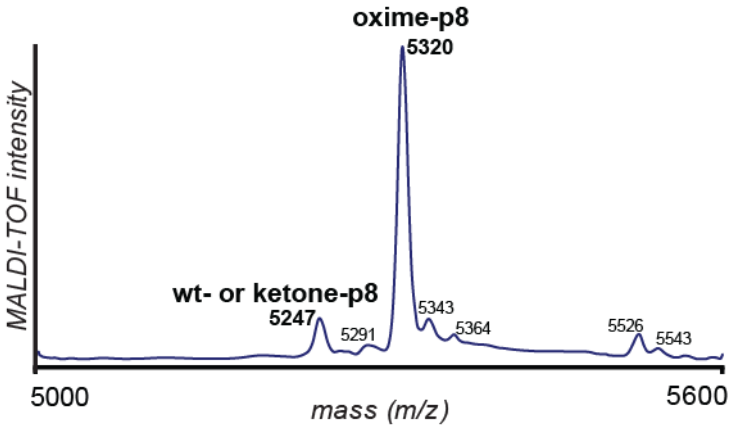

***Figure S3*.** Characterization of modified M13KE phage using MALDI-TOF MS. The spectrum shows p8 oxime formation following reaction with 2-(aminooxy)acetic acid (expected mass increase: 73 m/z, observed: 73 m/z). The smaller peaks cannot be attributed to any coat protein and are of unknown origin.

Figure S4

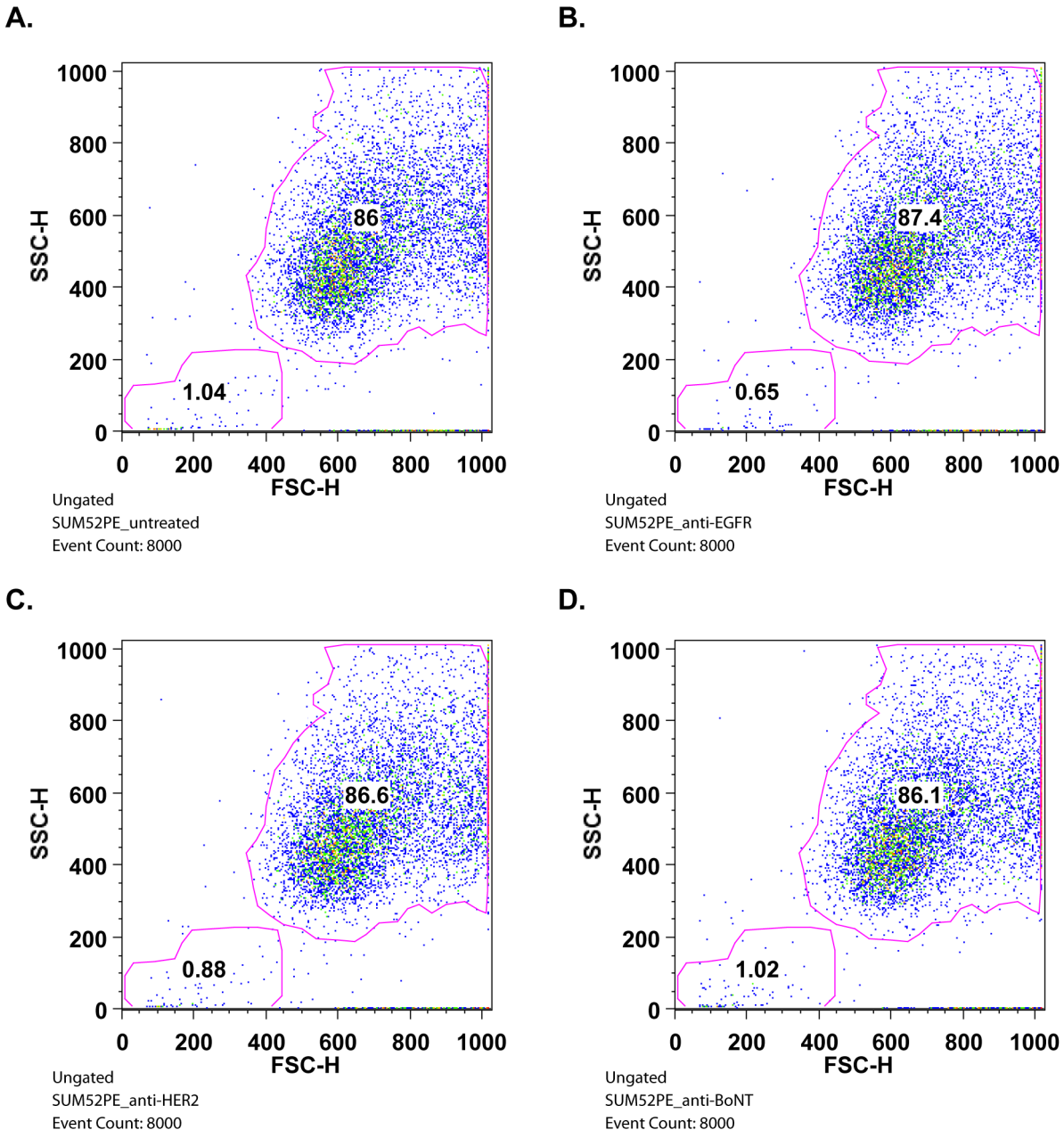

Figure S4. Forward and side scatter plots for flow cytometry with SUM52PE cells. Plots are shown for (**A**) untreated, (**B**) anti-EGFR phage treated, (**C**) anti-HER2 phage treated, and (**D**) anti-BoNT treated cells. The gating of the majority population was used for histogram generation (Figure 3a) as indicated by the pink outline; the gating of the minority population reflects dead or dying cells. Number inside of the plot reflects percentage of cells within each gate.

Figure S5

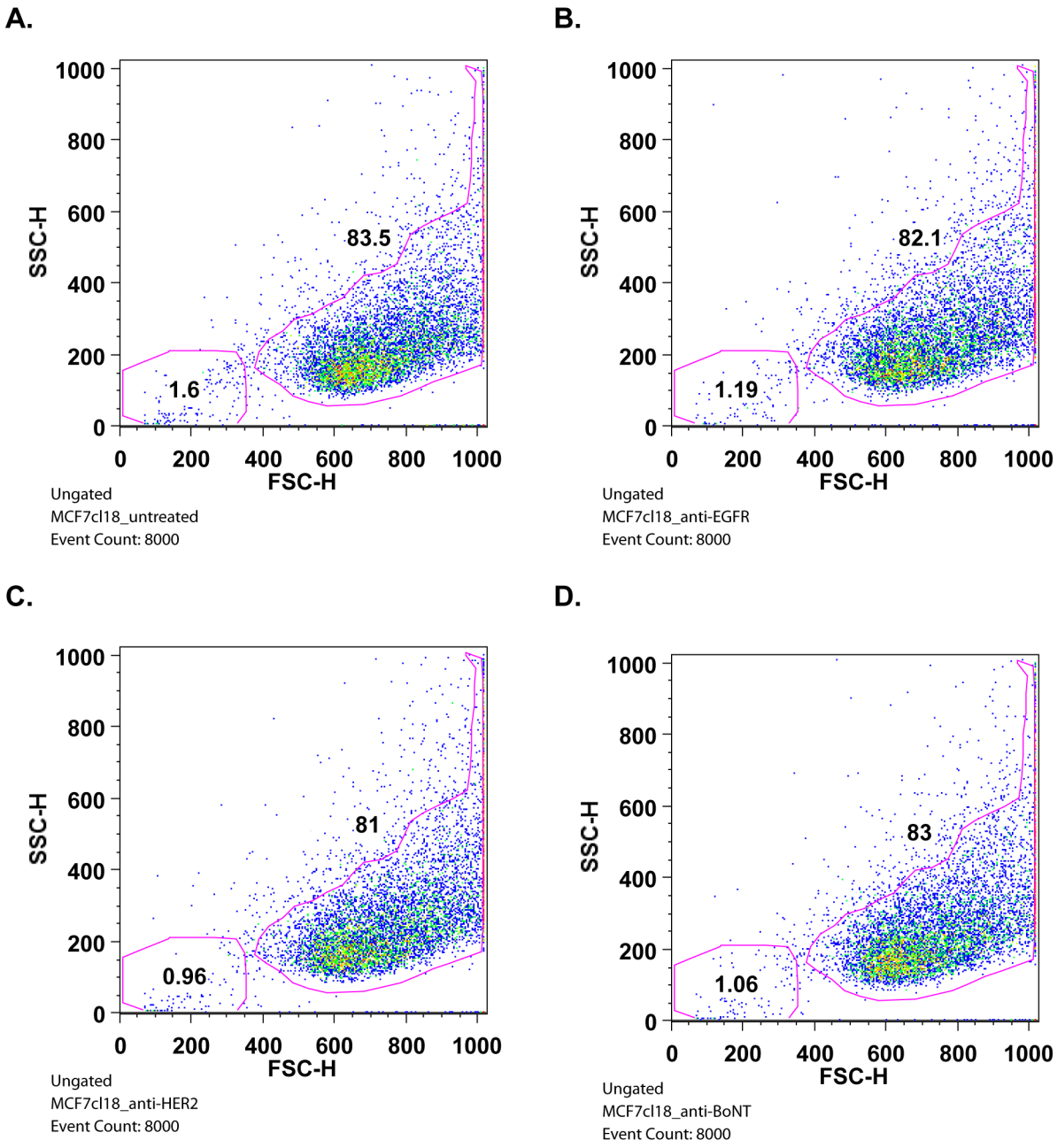

Figure S5. Forward and side scatter plots for flow cytometry with MCF-7 clone 18 cells. Plots are shown for (**A**) untreated, (**B**) anti-EGFR phage treated, (**C**) anti-HER2 phage treated, and (**D**) anti-BoNT treated cells. The gating of the majority population was used for histogram generation (Figure 3a) as indicated by the pink outline; the gating of the minority population reflects dead or dying cells. Number inside of the plot reflects percentage of cells within each gate.

Figure S6

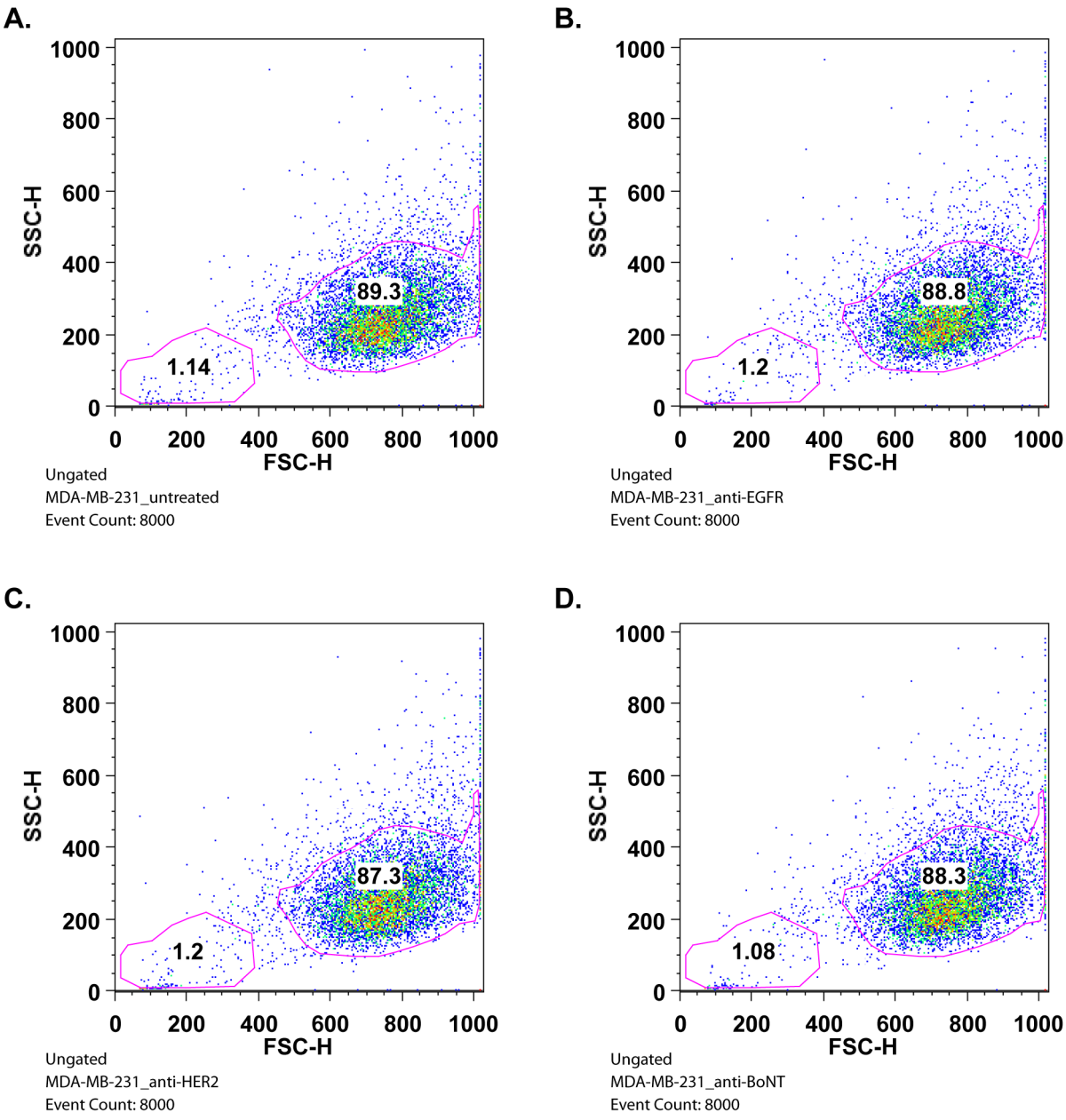

Figure S6. Forward and side scatter plots for flow cytometry with MDA-MB-231 cells. Plots are shown for (**A**) untreated, (**B**) anti-EGFR phage treated, (**C**) anti-HER2 phage treated, and (**D**) anti-BoNT treated cells. The gating of the majority population was used for histogram generation (Figure 3a) as indicated by the pink outline; the gating of the minority population reflects dead or dying cells. Number inside of the plot reflects percentage of cells within each gate.

Figure S7

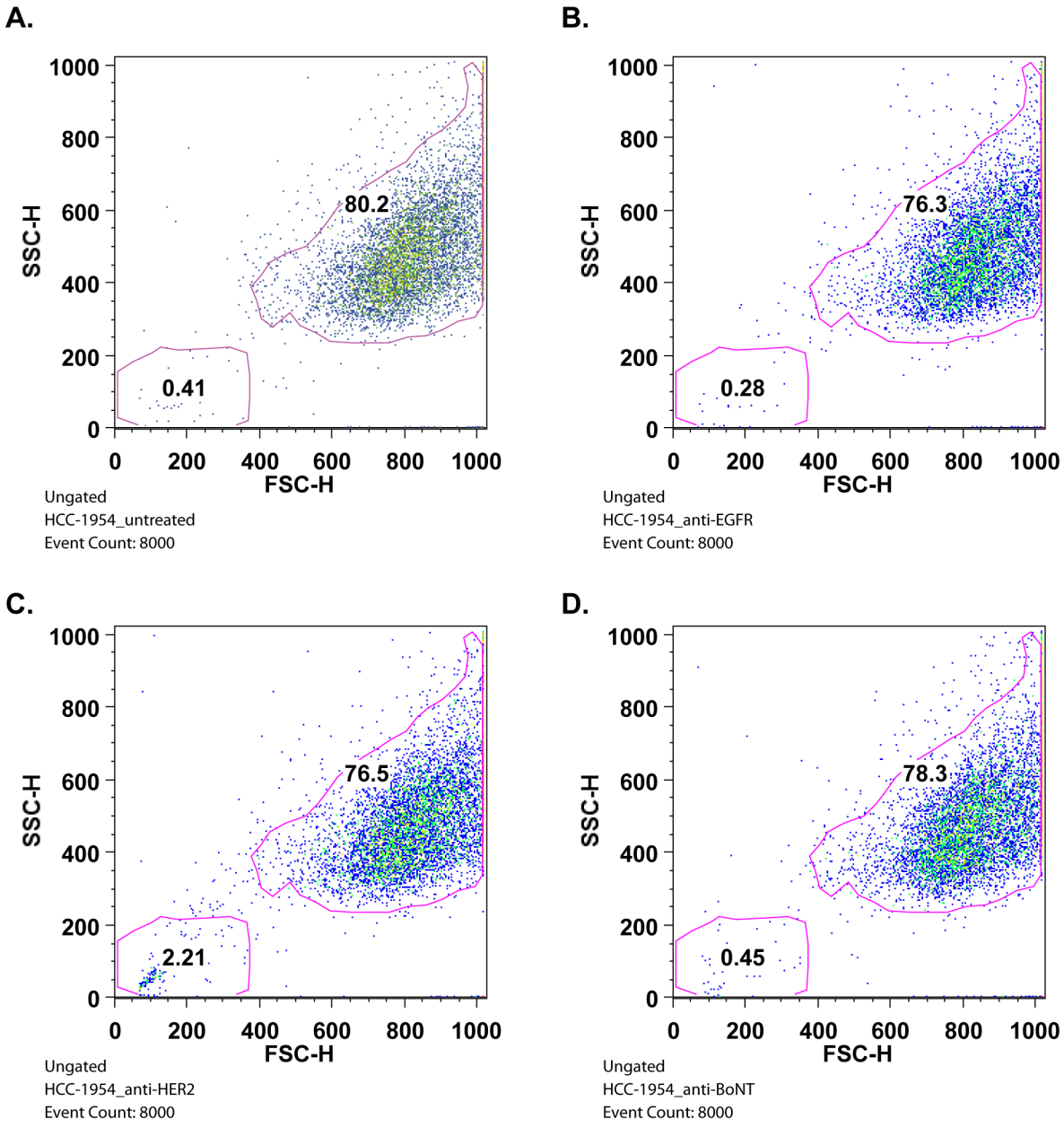

Figure S7. Forward and side scatter plots for flow cytometry with HCC1954 cells. Plots are shown for (**A**) untreated, (**B**) anti-EGFR phage treated, (**C**) anti-HER2 phage treated, and (**D**) anti-BoNT treated cells. The gating of the majority population was used for histogram generation (Figure 3a) as indicated by the pink outline; the gating of the minority population reflects dead or dying cells. Number inside of the plot reflects percentage of cells within each gate.

Figure S8

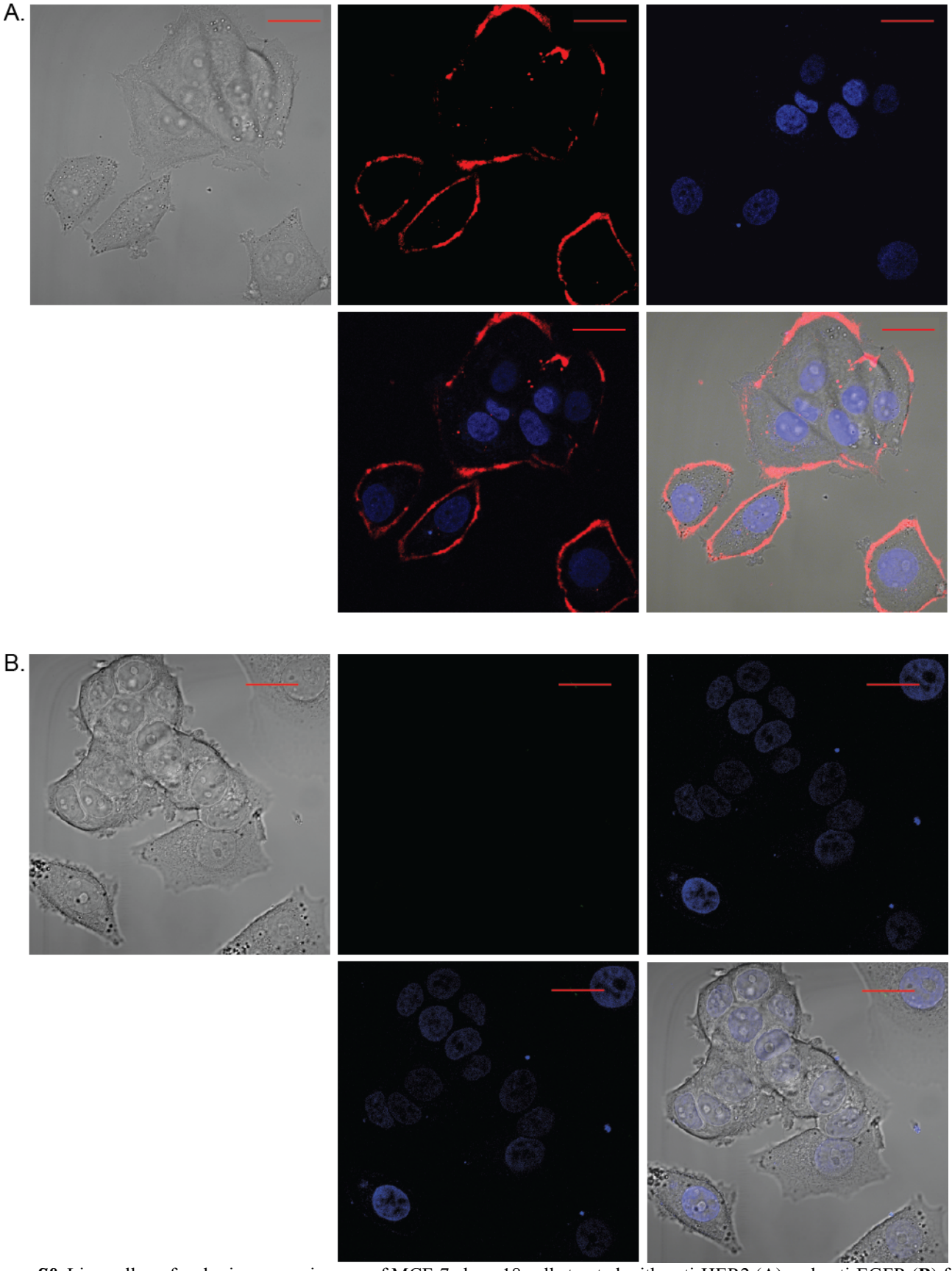

Figure S8. Live cell confocal microscopy images of MCF-7 clone 18 cells treated with anti-HER2 (**A**) and anti-EGFR (**B**) fd. Fd were added at 0.8 nM in 150 µL of PBS. Scale bars indicate 20 µm. Fluorescence is as follows: DAPI (blue), anti-HER2

(red), anti-EGFR (green). Top row (L to R): bright field image, red (**A**) or green (**B**) channel only, blue channel only; bottom row (L to R): all fluorescence channels, merge.

Figure S9

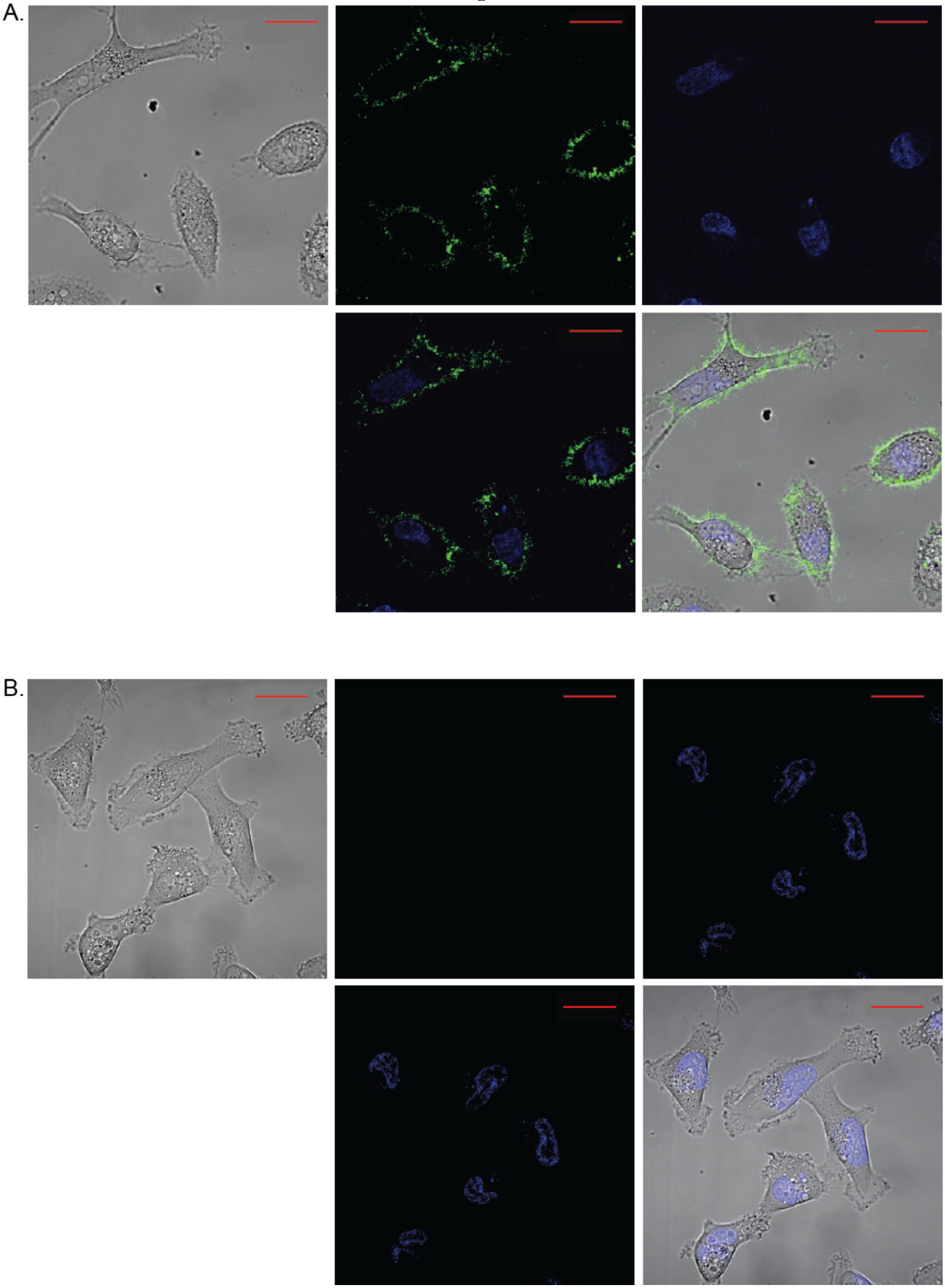

Figure S9. Live cell confocal microscopy images of MDA-MB-231 cells treated with anti-EGFR (**A**) and anti-HER2 (**B**) fd. Fd were added at 0.8 nM in 150 µL of PBS. Scale bars indicate 20 µm. Fluorescence is as follows: DAPI (blue), anti-HER2 (red), anti-EGFR (green). Top row (L to R): bright field image, green (**A**) or red (**B**) channel only, blue channel only; bottom row (L to R): all fluorescence channels, merge.

Figure S10

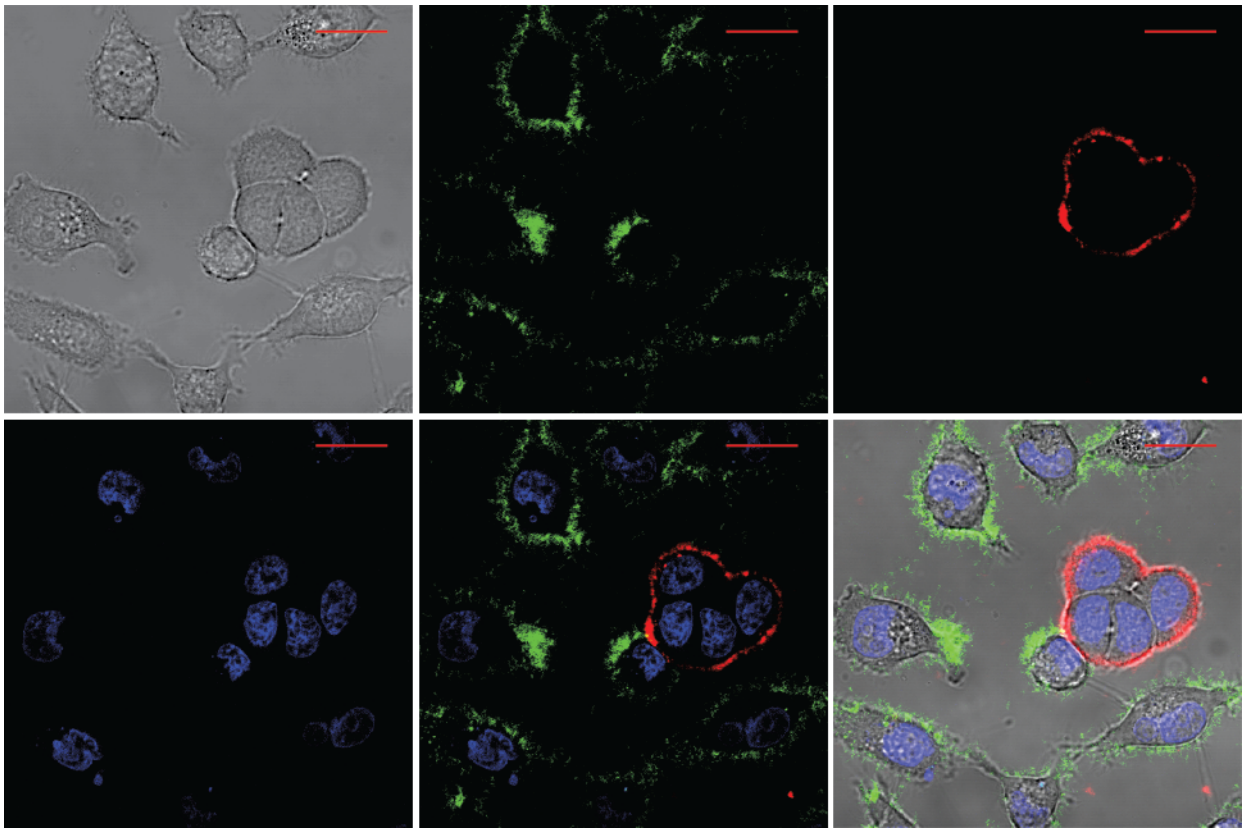

Figure S10. Live cell confocal microscopy images of MDA-MB-231 and MCF7 clone 18 cells treated with anti-HER2 and anti-EGFR fd. Fd were added at 0.8 nM in 150 µL of PBS. Scale bars indicate 20 µm. Fluorescence is as follows: DAPI (blue), anti-HER2 (red), anti-EGFR (green). Top row (L to R): bright field image, green channel only, red channel only; bottom row (L to R): blue channel only, all fluorescence channels, merge.

Figure S11

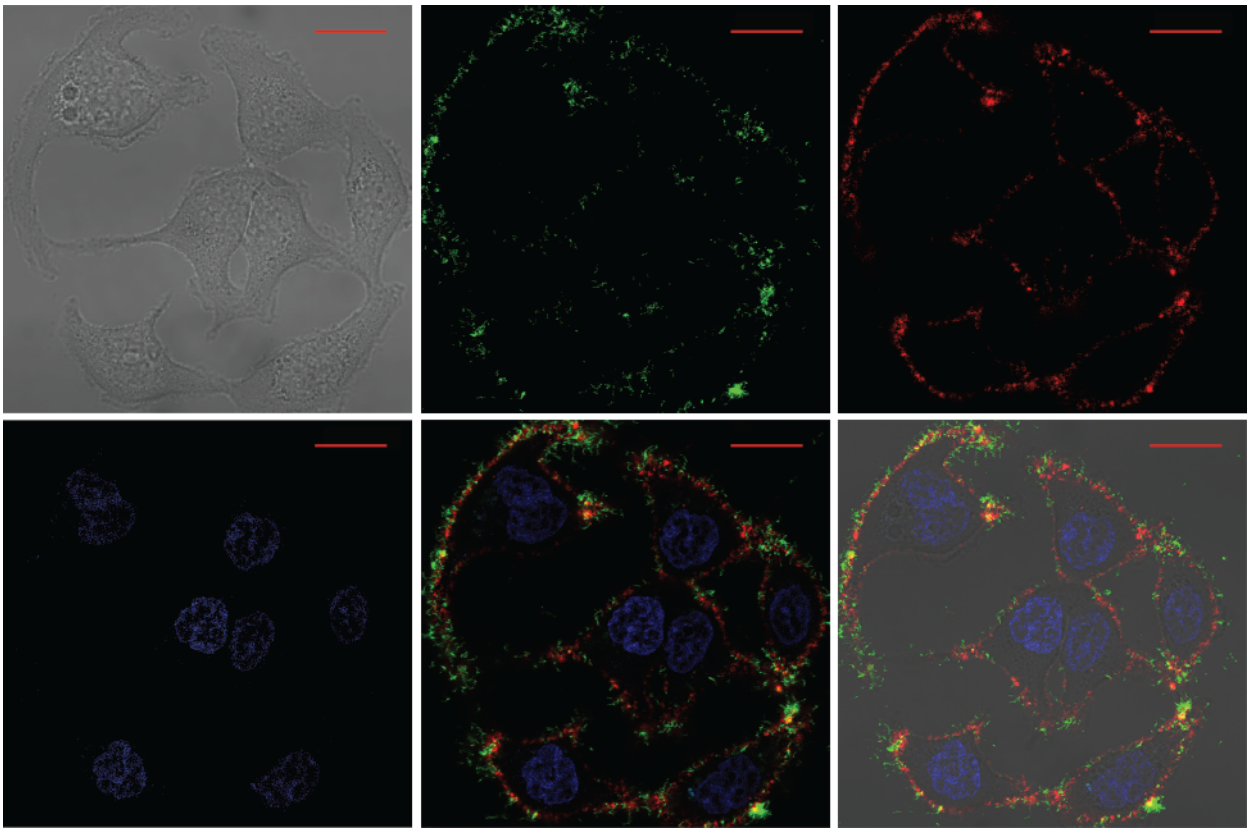

Figure S11. Live cell confocal microscopy images of HCC1954 cells treated with anti-HER2 and anti-EGFR phage. Phage were added at 0.8 nM in 150 µL of PBS. Scale bars indicate 20 µm. Fluorescence is as follows: DAPI (blue), anti-HER2 (red), anti-EGFR (green). Top row (L to R): bright field image, green channel only, red channel only; bottom row (L to R): blue channel only, all fluorescence channels, merge.

Figure S12

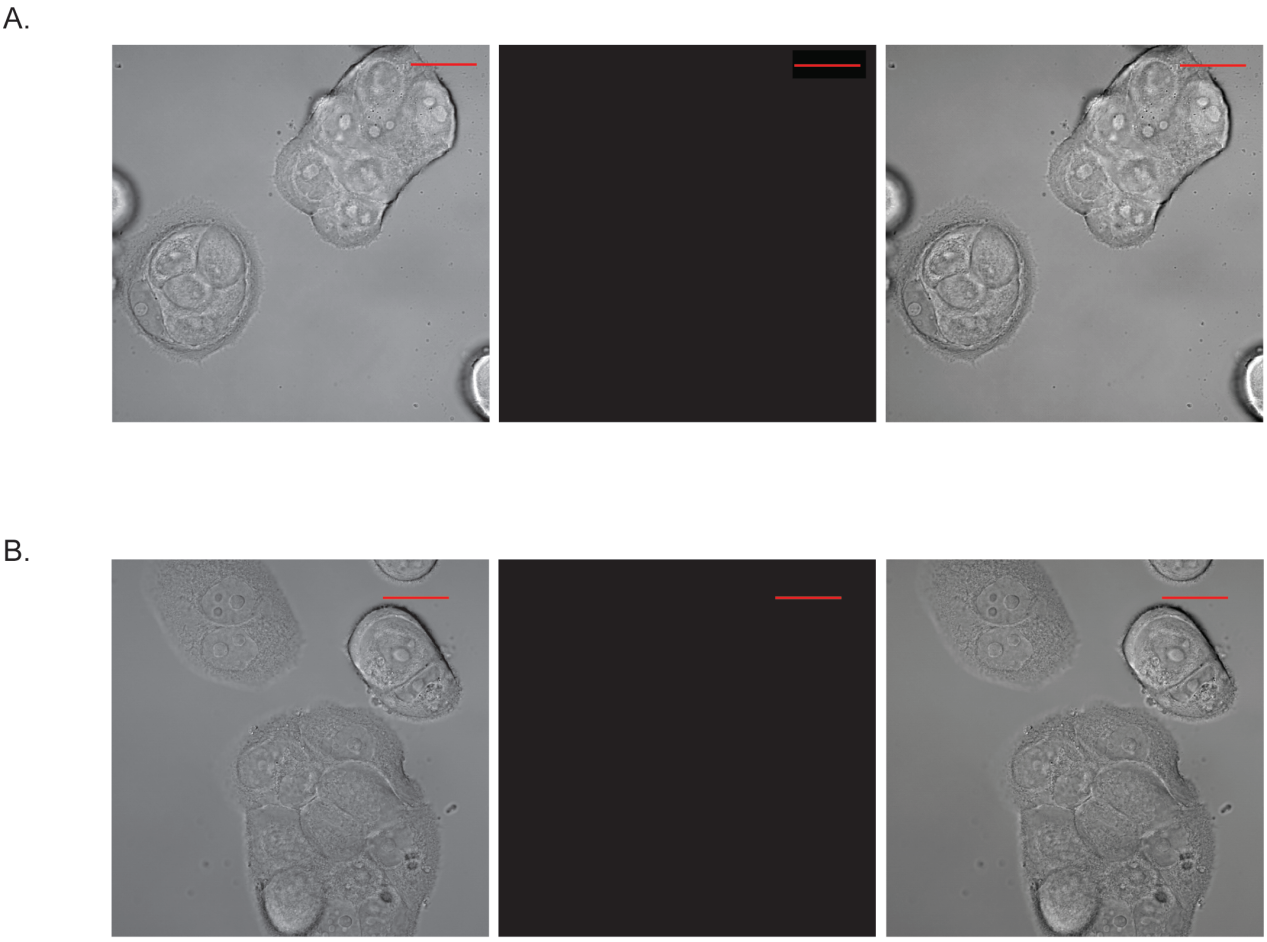

Figure S12. Live cell confocal microscopy images of SUM52PE cells treated with anti-HER2 (**A**) and anti-EGFR phage (**B**). Phage were added at 0.8 nM in 150 µL of PBS. Scale bars indicate 20 µm. Fluorescence is as follows: anti-HER2 (red), anti-EGFR (green). (L to R): bright field image, red channel only (**A**) or green channel only (**B**), merge. Due to cellular toxicity, nuclear staining was not used with these cells.

Figure S13

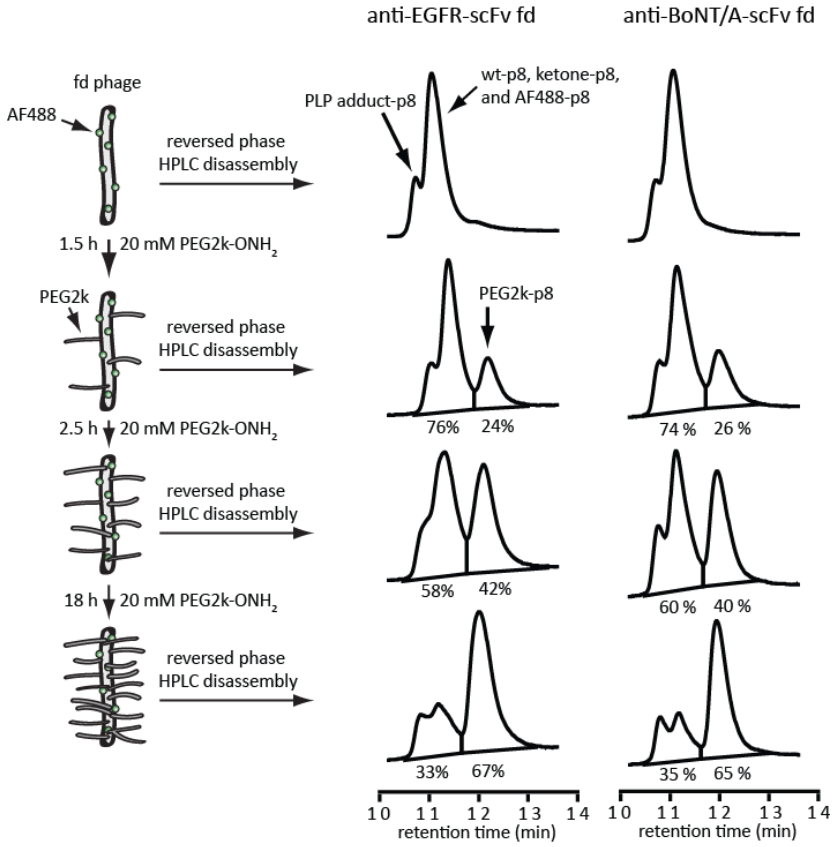

***Figure S13*.** PEG2k modification of fd**.** After transamination using PLP and modification with AF488-ONH_2, the fd were reacted with 20 mM PEG2k-ONH_2 for 1.5, 4, and 22 h. During reversed phase chromatography, the fd disassembled into coat proteins and DNA. The absorbance at 280 nm is shown; p8 was the only coat protein observed. The first peak to elute corresponds to the PLP adduct of p8 (see Figure S2). The second peak is composed of wt-p8, ketone-p8, and AF488-p8 (absorbance at 488 nm is observed), and the last peak to elute (PEG-treated samples only) is PEG2k-p8.

Figure S14

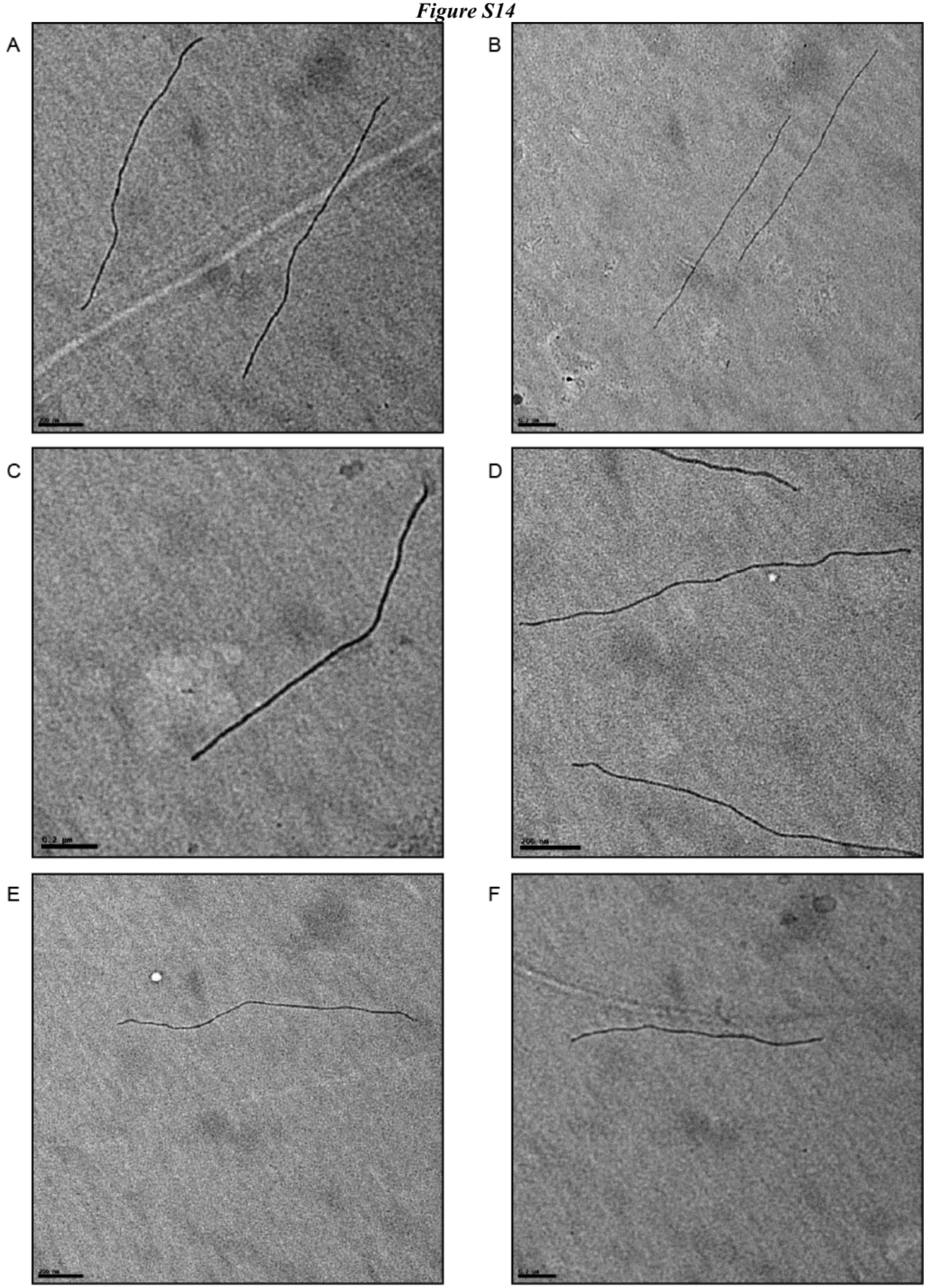

***Figure S14*.** Transmission electron microscope (TEM) images of fd bearing anti-EGFR-scFv molecules**.** (**A**) Wt-fd. (**B**) Fd following PLP-mediated transamination. (**C**) Fd with 2% of the p8 proteins fluorescently labeled with AF488-ONH_2, **(D)** 2% AF488 and 24% PEG2k, (**E**) 2% AF488 and 42% PEG2k, and (**F**) 2% AF488 and 67% PEG2k. All scale bars represent 200 nm.

Figure S15

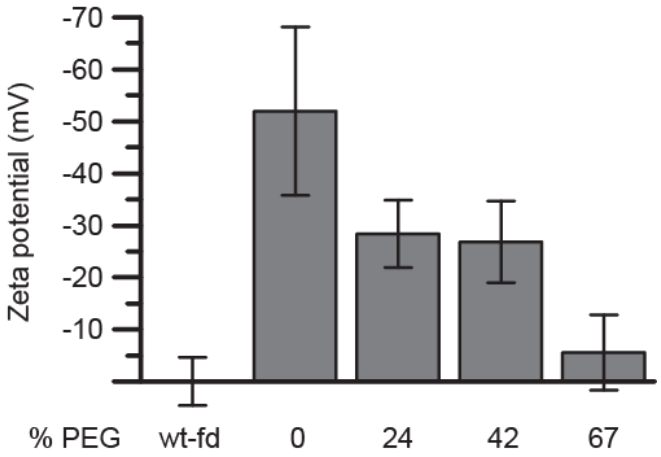

Figure S15. Zeta potential measurements of anti-EGFR fd as a function of the percent of p8 proteins labeled with PEG2k**.** These fd phage have not been modified with AF488.

Figure S16

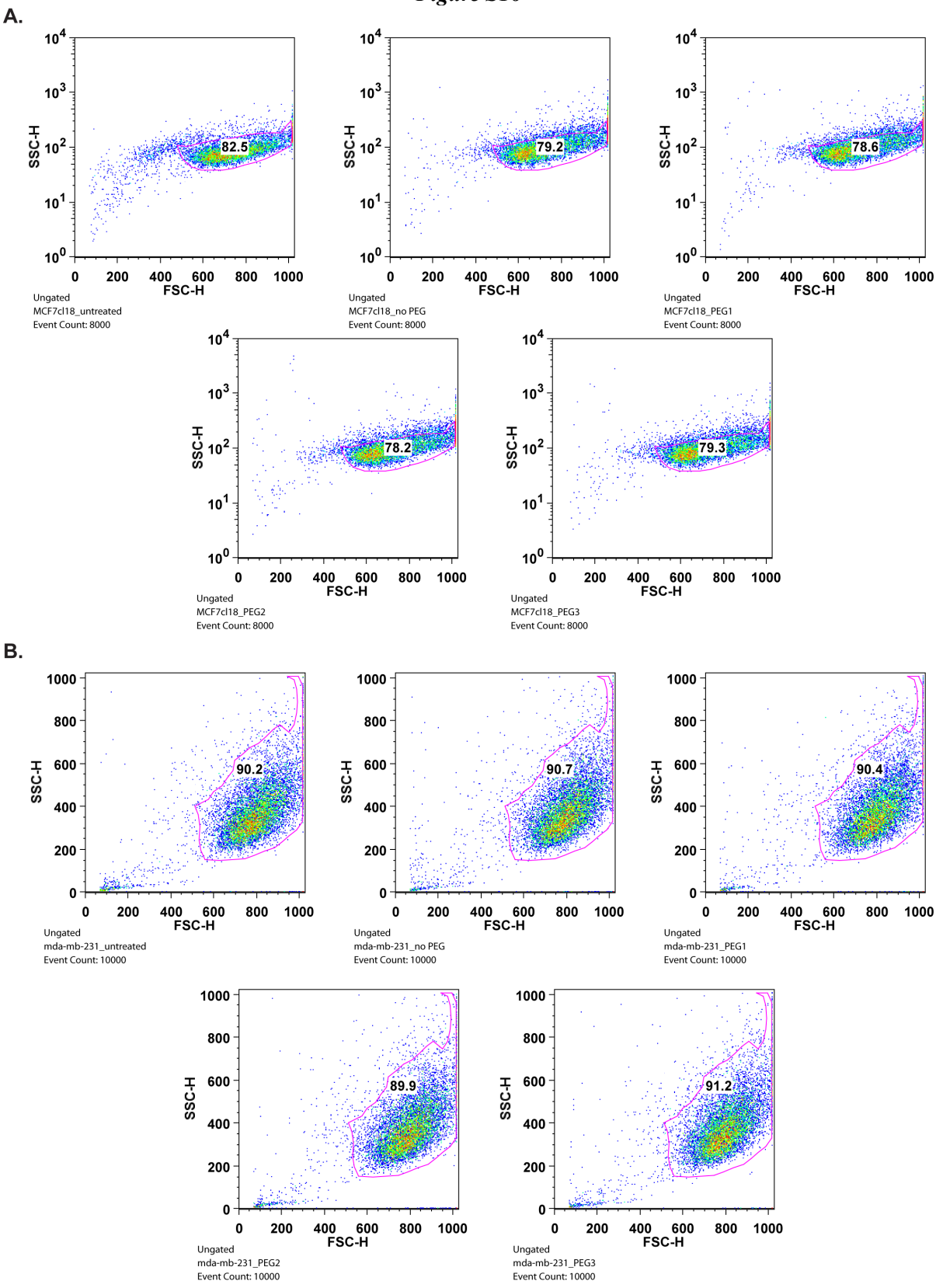

Figure S16. Forward and side scatter plots for flow cytometry with MCF-7 clone 18 (**A**) and MDA-MB-231 (**B**) cells. Plots are shown for untreated, no-PEG (0% modified), PEG1 (15% modified), PEG2 (48% modified), and PEG3 (74% modified) anti-EGFR treated cells. The gating of the majority population was used for histogram generation (Figure 4 in main text) as indicated by the pink outline. The number inside of the plot reflects percentage of cells within each gate.

Figure S17

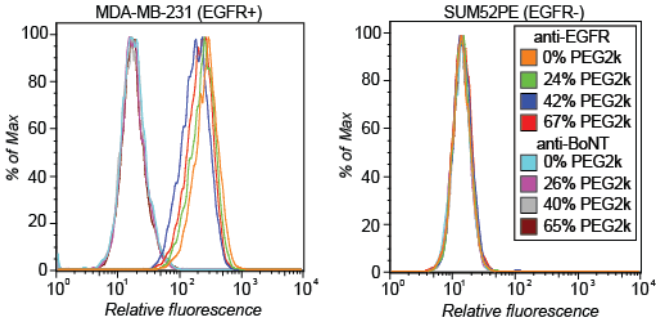

Figure S17. Histograms of MDA-MB-231 (left) and SUM52PE (right) cell-binding by anti-EGFR and anti-BoNT fd with various percentages of p8 proteins labeled with PEG2k.

Figure S18

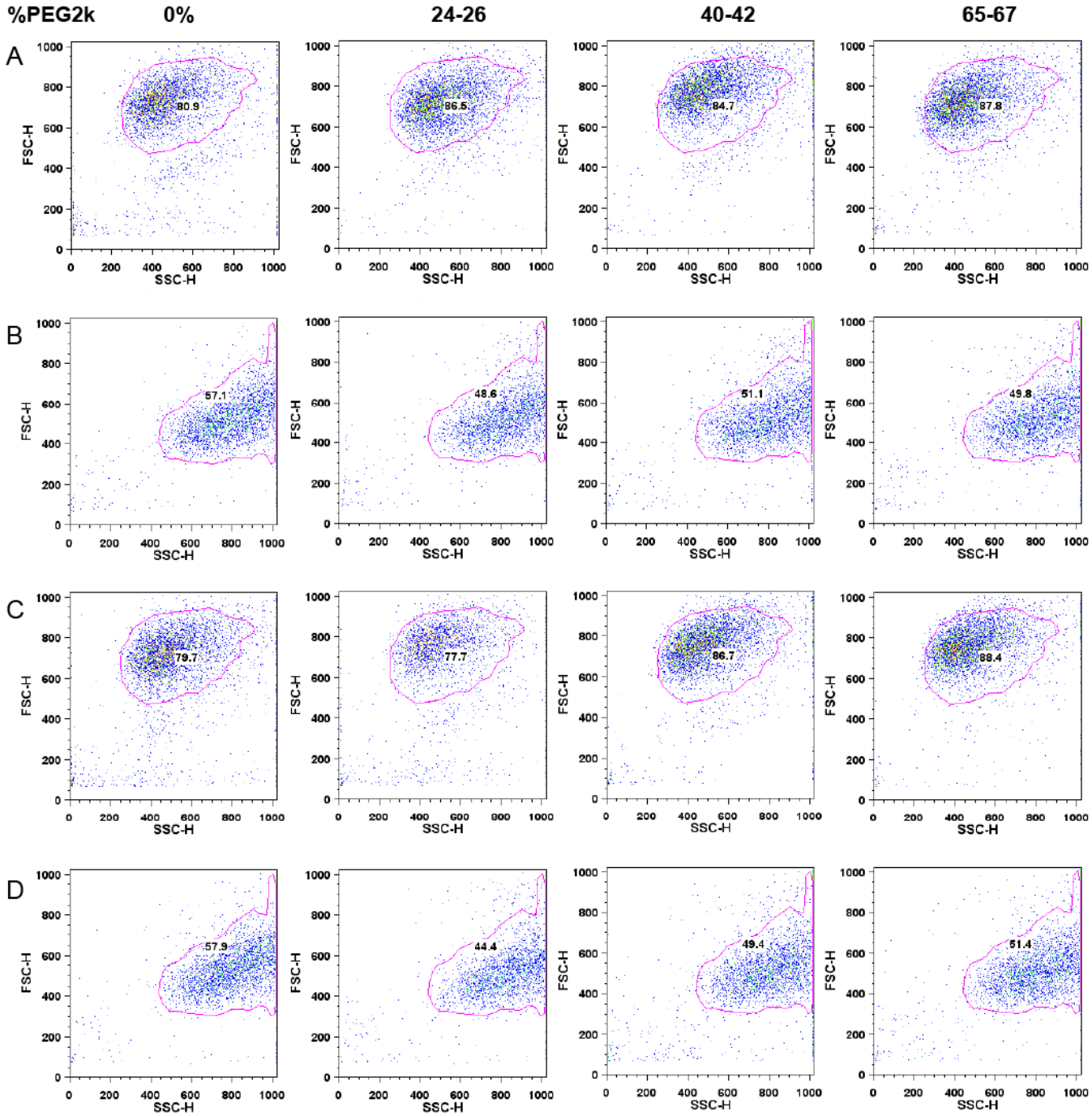

Figure S18. Forward and side scatter plots for flow cytometry with PEG-modified phage. Columns (L to R) indicate p8 proteins modified with various percentages of PEG2k. Plots are shown for (**A**) anti-EGFR fd treated MDA-MB-231 cells, (**B**) anti-EGFR fd treated SUM52PE cells, (**C**) anti-BoNT fd treated MDA-MB-231 cells, and (**D**) anti-BoNT fd treated SUM52PE cells. Gating used for histogram generation (Figure S17) is indicated by the pink outline. The number inside of the plot reflects percentage of cells within each gate.