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Analysis of Cell-Surface Receptor Dynamics through Covalent Labeling by Catalyst-Tethered Antibody

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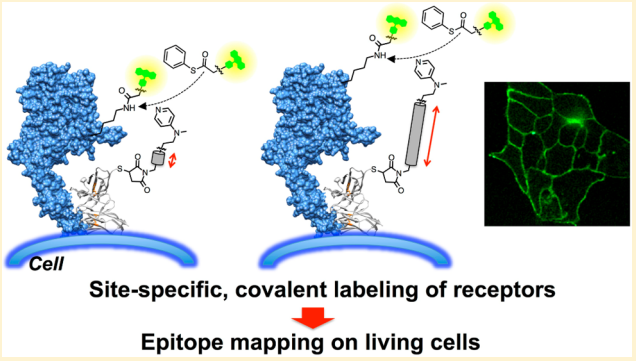
Supporting Information

ABSTRACT: A general technique for introducing biophysical probes into selected receptors in their native environment is valuable for the study of their structure, dynamics, function, and molecular interactions. A number of such techniques rely on genetic engineering, which is not applicable for the study of endogenous proteins, and such approaches often suffer from artifacts due to the overexpression and bulky size of the probes/protein tags used. Here we designed novel catalyst-antibody conjugates capable of introducing small chemical probes into receptor proteins such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) in a selective manner on the surface of living cells. Because of the selectivity and efficiency of this labeling technique, we were able to monitor the cellular dynamics and lifetime of HER2 endogenously expressed on cancer cells. More significantly, the current labeling technique comprises a stable covalent bond, which combined with a peptide mass fingerprinting analysis allowed epitope mapping of antibodies on living cells and identification of potential binding sites of anti-EGFR affibody. Although as yet unreported in the literature, the binding sites predicted by our labeling method were consistently supported by the subsequent mutation and binding assay experiments. In addition, this covalent labeling method provided experimental evidence that HER2 exhibits a more dynamic structure than expected on the basis of crystallographic analysis alone. Our novel catalyst-antibody conjugates are expected to provide a general tool for investigating the protein trafficking, fluctuation, and molecular interactions of an important class of cell-surface receptors on live cell surfaces.



INTRODUCTION

Cell-surface receptors play key roles in many physiological and pathological events,[1−3] and therefore, detailed characterization of their cellular and structural dynamics is of great importance. Ideally, these proteins should be studied in the presence of all relevant interaction partners under live-cell conditions for a precise understanding of their functions.[4] A number of powerful methods for such studies have been reported to date, but these rely largely on genetically encoded fluorescent proteins or peptide/protein tags that are selectively labeled with a variety of biophysical probes.[5−7] Alternatively, nonsense suppression techniques are used for site-specific incorporation of unnatural amino acids into proteins.[8] While these methods continue to be valuable, they often suffer from artifacts due to overexpression.[9,10] In addition, these methods cannot be applied to proteins that are endogenously expressed. Therefore, techniques for labeling selected endogenous receptors in their native environment are in high demand.

The specific recognition and binding of proteins by antibodies and their derivatives, e.g. fragment antigen-binding (Fab), single-chain variable fragment (scFv), and single-variable domain (VHH), are often used for the study of cell-surface receptors.[11] For instance, probe-conjugated antibodies provide a means of analyzing the localization and dynamics of these proteins in cultures of living cells as well as *in vivo*.[9,12] However, these methods offer indirect measurements of protein motion because reporter probes are attached to the antibodies rather than to the receptors themselves, thereby limiting the scope of their applications. Furthermore, antibodies bind their antigens to form noncovalent antigen/antibody complexes that can dissociate with a half-life of minutes to hours, which presents additional challenges to the study of their interactions.[13]

We recently reported that 4-dimethylaminopyridine (DMAP) tethered to lectins (sugar-binding proteins) is a powerful tool for the global labeling and profiling of glycoproteins on living animal cells.[14] The rational coupling of the sugar-binding ability of lectins and the reactivity of the organocatalyst DMAP facilitated the acylation of a variety of glycoproteins driven by sugar-lectin recognition on living

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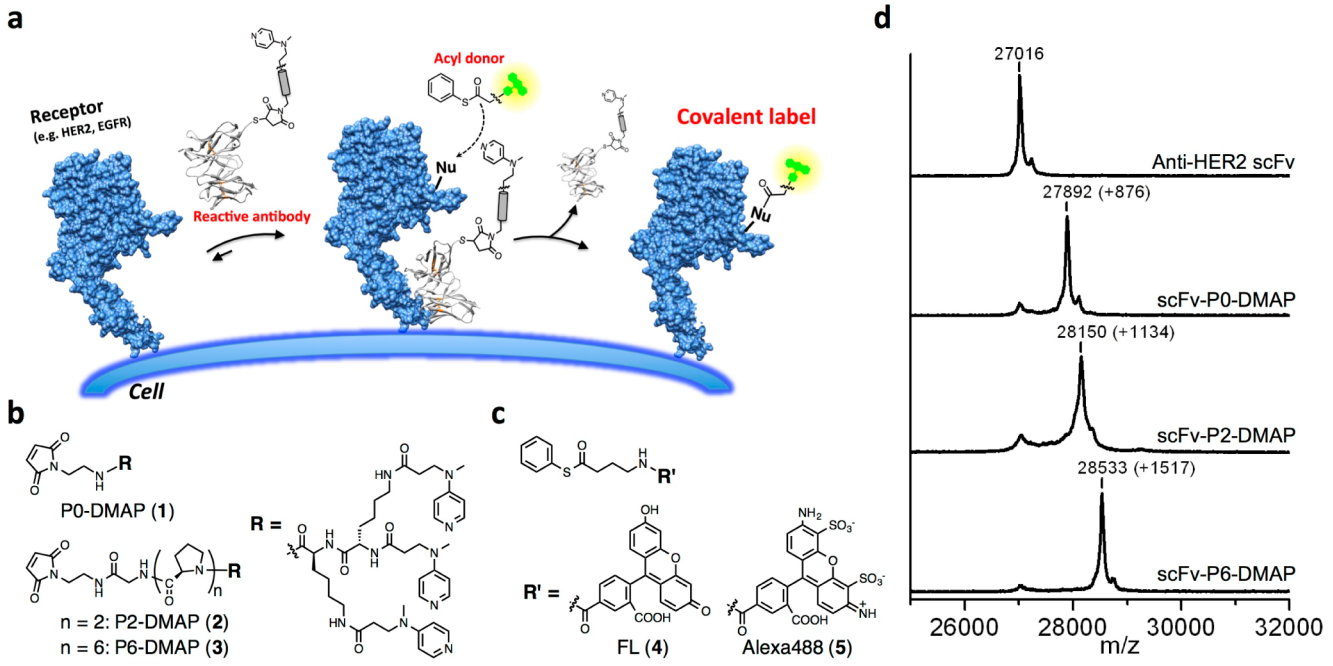

Figure 1. Chemical modification of cell-surface receptors by DMAP-antibody conjugates: model, chemical tools and characterization of anti-HER2 scFv-DMAP. (a) Schematic representation of DMAP-tethered antibody for selective labeling of cell surface receptors. (b) Maleimide-appended DMAP catalysts **1**, **2**, and **3** and (c) fluorescein (FL)-type (**4**) and Alexa488-type (**5**) acyl donors. (d) MALDI-TOF-MS of unconjugated anti-HER2 scFv and scFv-DMAP conjugates prepared with compound **1**, **2**, and **3**.

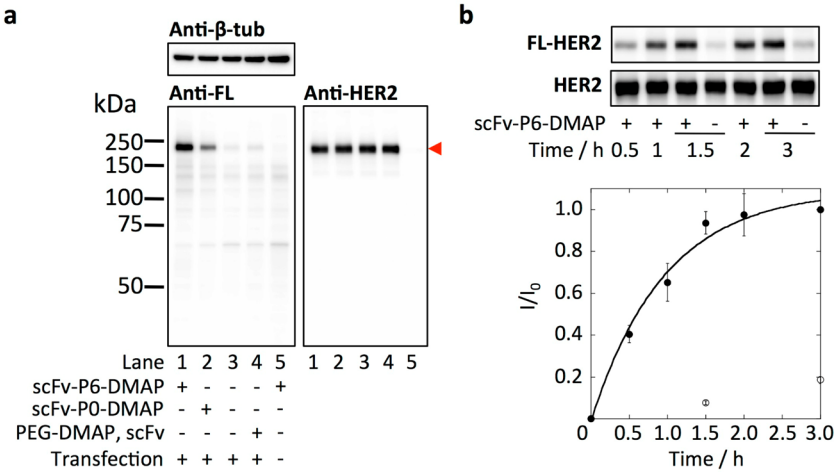

Figure 2. On-cell labeling of HER2 and its time profile. (a) Western blot analysis of HER2-expressing HEK293T labeled by DMAP-tethered anti-HER2 scFv catalysts in the presence of acyl donor **4** for 90 min at 4 °C, (b) time profile of the labeling reaction of HER2 in the presence of acyl donor **4** with (filled circle) or without (circle) anti-HER2 scFv-P6-DMAP. I and I_0 are band intensities of FL-labeled HER2 at the indicated time point and at 3 h, respectively. Error bars represent SD, n = 3.

cells.[14−16] This previous study established the general concept that noncovalent protein−protein interactions can be transformed to stable covalent bonds, which enables the detailed characterization of proteins of interest. Here we report the construction of semisynthetic catalyst-antibody conjugates capable of selectively labeling proteins of interest in a covalent manner on living cells (Figure 1a). The conjugates were prepared by bioconjugation of the DMAP catalysts with anti-HER2 scFv or anti-EGFR affibody molecules via a conventional cysteine-maleimide coupling reaction. Selective labeling of HER2 and EGFR on the surface of living cells was therefore facilitated by the DMAP-tethered anti-HER2 scFv and anti-EGFR affibody, respectively. With the selectivity and efficiency of the labeling reaction employed, we succeeded in monitoring the internalization and degradation of endogenously expressed HER2. Furthermore, our covalent labeling methods combined with peptide mass-fingerprinting analysis allowed epitope mapping of antibodies on living cells and identification of potential binding sites of anti-EGFR affibody. Our approach also provided experimental evidence that HER2 exhibits a more dynamic structure on live cell surfaces than expected from crystallographic analyses. Our semisynthetic DMAP-tethered antibodies could therefore provide dynamic and structural insights into protein−protein interactions, and expand the current repertoire of techniques available for a deeper understanding of an important class of cell-surface receptors.

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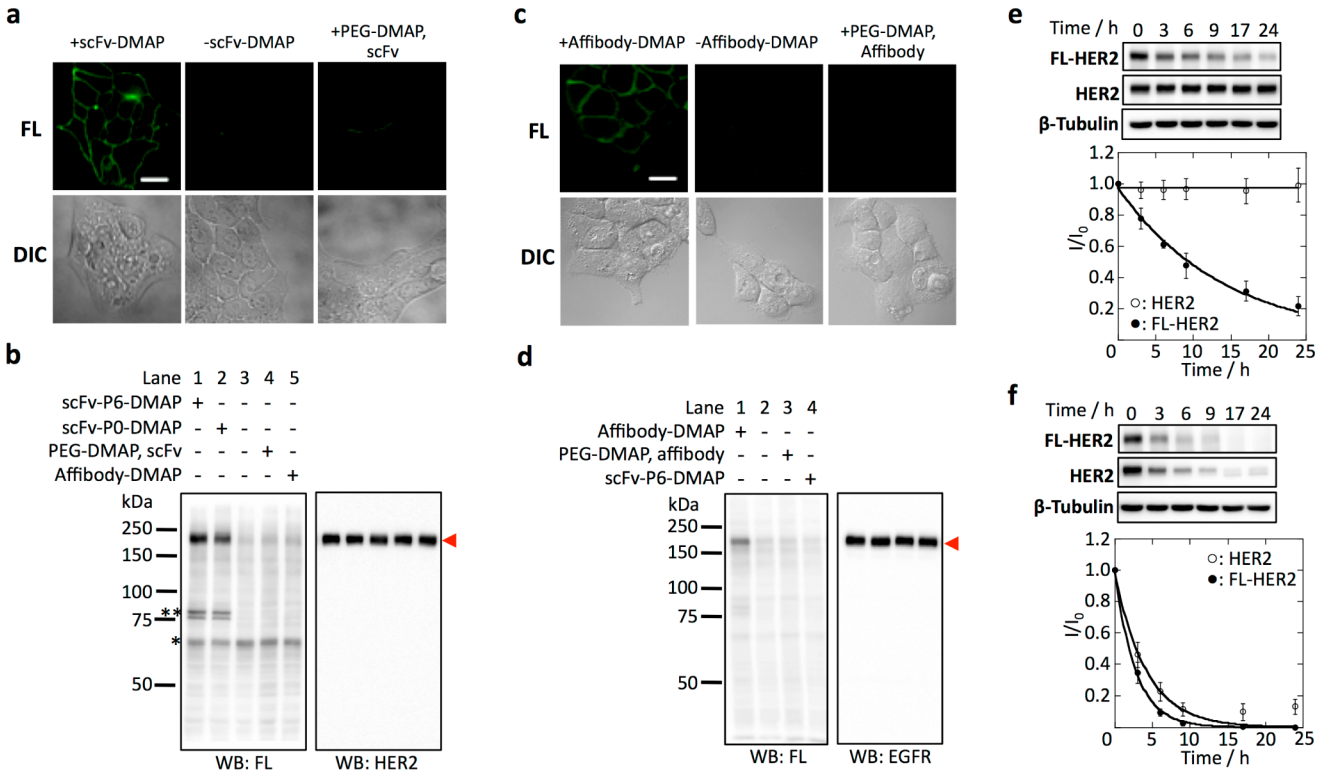

Figure 3. Efficient and selective labeling of endogenous HER2 and EGFR by DMAP-tethered antibodies on live cell and pulse-chase analysis of HER2 endogenously expressed on N87. (a,b) Fluorescence imaging (a) and Western blot analysis (b) of N87 labeled by DMAP-tethered anti-HER2 scFv catalysts in the presence of acyl donor **4**. FL-labeled BSA (*) originating from the reaction between the acyl donor and BSA in the culture medium, and an uncharacterized band observed in the presence of anti-HER2 scFv catalysts (**) are both indicated.[23] (c,d) Fluorescence imaging (c) and Western blot analysis (d) of A431 labeled by the DMAP-tethered anti-EGFR affibody catalyst in the presence of acyl donor **4**. All scale bar, 20 μm. (e,f) Time course of HER2 degradation in the absence (e) or presence of GA (f). Plots show the level of FL-HER2 (filled circles) and total HER2 (circles). I and I_0 are band intensities at the indicated time points and at 0 h, respectively. Error bars represent SD, n = 3.

RESULTS AND DISCUSSION

Preparation of DMAP-Tethered Antibodies. As a proof of concept, we chose anti-HER2 scFv for conjugation with three types of DMAP tethers bearing tri-DMAP groups joined via different proline (Pro)-based linker structures (no Pro: P0, Pro ×2: P2, Pro ×6: P6, see Figure 1b). These DMAP tethers were expected to exhibit different levels of accessibility toward distinct sets of amino acid residues on the target protein, thereby providing structural information regarding the mode of interaction between anti-HER2 scFv and HER2. Upon incubation of anti-HER2 scFV-cys with compounds **1**, **2**, and **3**, we confirmed that a new peak appeared at 27892 (+876), 28150 (+1134), or 28533 (+1517) m/z, which corresponds to the calculated mass of anti-HER2 scFv-cys modified with the respective DMAP tethers (Figure 1d). The conjugation reaction was also verified by up-shifts of the protein bands observed by SDS-PAGE analysis (Supporting Information Figure S1). The reactions proceeded nearly quantitatively, and the isolated protein yield was approximately 60−70%. Following the same scheme, we also modified anti-EGFR affibody with the DMAP catalysts. The progress of the reaction was monitored by MALDI-TOF-MS and SDS-PAGE, confirming the formation of DMAP-tethered affibody (Supporting Information Figure S2). The yields for the DMAP-tethered proteins were approximately 50%, similar to that of anti-HER2 scFv-DMAP conjugates.

Selective Labeling of HER2 on Live Cell Surfaces. We next examined the ability of the DMAP-tethered antibody catalysts to label cell-surface HER2 using HEK293T cells transfected with HER2 expression plasmids. In the presence of anti-HER2 scFv-DMAPs and fluorescein (FL)-appended acyl donor **4** (Figure 1c), a single band corresponding to FL-modified HER2 was observed in the Western blots using anti-FL antibody (lanes 1 and 2 in Figure 2a). The intensity of FL-modified HER2 increased for ∼2 h (Figure 2b), and the labeling yield was estimated to be 19.6 ± 2.6% on the basis of band intensity in the Western blots (Supporting Information Appendix 1).[17] It should be noted that this band was not observed for the reaction in the absence of DMAP-tethered anti-HER2 scFv catalyst, in the presence of PEG-DMAP and unconjugated anti-HER2 scFv-cys, or without transfection (lanes 3−5 in Figure 2a). These results indicated that the labeling reaction was selective and driven by the antigen−antibody interaction between HER2 and anti-HER2 scFv.

Compared with anti-HER2 scFv-P6-DMAP, the labeling reaction using scFv-P0-DMAP catalyst was less efficient (7.6 ± 3.1%, lane 2 in Figure 2a). This might be due to a different accessibility of the DMAP moieties to the nucleophilic "reactive" amino acids on HER2, suggesting that there is an optimum linker length required for efficient labeling, which is in good agreement with our previous report.[18]

We also investigated labeling efficiency as a function of temperature. The initial reaction rate for HER2 labeling increased at higher temperatures (∼2- and ∼7-fold increase at 17 and 37 °C, respectively, compared with 4 °C, see Supporting Information Figure S4a-c); however, prolonged reaction times at higher temperatures resulted in significant levels of

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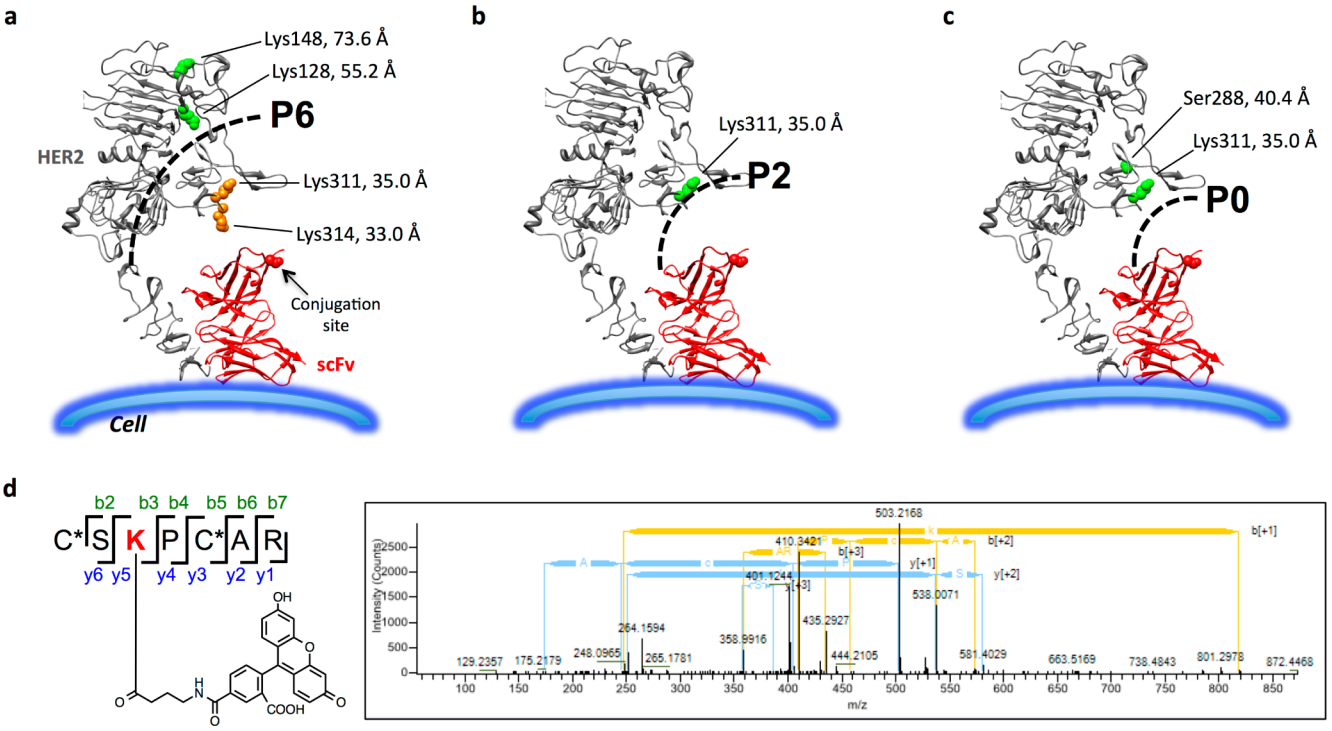

Figure 4. Labeling site mapping of HER2 on live cell surfaces. (a–c) Labeling sites of HER2 shown on the crystal structure of the extracellular domain of HER2 (gray) complexed with anti-HER2 scFv (red) (PDB code: 1N8Z). Predicted reaction range for scFv-P6-DMAP (a), -P2-DMAP (b), and -P0-DMAP (c) and the labeled amino acid(s) that are outside (green spheres) or inside (orange spheres) of the predicted reaction range are shown. Anti-HER2 scFv was modeled using the light chain (1–106) and heavy chain (1–121) of anti-HER2 Fab in the original structure. The reaction range was simulated from the DMAP-conjugation site of scFv (black arrow) using UCSF Chimera. (d) MS/MS analysis of the FL-modified peptide CSK(FL)PCAR detected from FL-labeled HER2. *Site(s) where carbamidomethylation was observed.

nonspecific products (Supporting Information Figure S4a lane 4). Thus, the shorter reaction times were optimal for target-specific labeling at the higher temperatures.

Together, these results demonstrate that the efficient and specific covalent labeling of HER2 on living cell surfaces can be achieved using anti-HER2 scFv-DMAP conjugates.

Labeling of Endogenous HER2 and EGFR on Live Cancer Cells. Exploiting the chemical reaction driven by the antigen–antibody interaction, we next sought to employ the antibody-DMAP conjugates to selectively label the cancer markers HER2 and EGFR endogenously expressed on tumor cells. Cultured human gastric cancer cells N87 were treated with acyl donor **4** in the presence of anti-HER2 scFv-DMAP for 90 min, during which time the cell morphology remained unchanged. As shown in Figure 3a, strong fluorescence was predominantly observed from the cell membrane by confocal laser-scanning microscopy (CLSM).[19] In contrast, no or minimal fluorescence was detected in the absence of anti-HER2 scFv-DMAP or in the presence of PEG-DMAP and unconjugated anti-HER2 scFv-cys (Figure 3a).

In addition, Western blot analysis of the N87 cells treated with anti-HER2 scFv-DMAP and acyl donor **4** revealed an intense band at ~180 kDa, which corresponds to FL-labeled HER2 (lanes 1 and 2 in Figure 3b). Consistent with the confocal laser-scanning microscopy images, no appreciable bands were detected in the absence of anti-HER2 scFv-DMAP or in the presence of PEG-DMAP and unconjugated anti-HER2 scFv-cys (lanes 3 and 4 in Figure 3b). Labeling of endogenous HER2 by anti-HER2 scFv-DMAP was also confirmed in another HER2-expressing cancer cell line, SK-BR3 (Supporting Information Figure S7). Clearly, DMAP-tethered anti-HER2 scFv is a powerful tool for the selective labeling of endogenous HER2 on living cells.

The anti-EGFR affibody-DMAP and acyl donor **4** were used for labeling endogenous EGFR expressed on A431 human epidermoid carcinoma cells. Similar to anti-HER2 scFv-DMAPs, intense fluorescence was observed predominantly in the cell membrane and only in the presence of affibody-DMAP (Figure 3c). Western blots showed a single FL-labeled protein band at ~170 kDa, which corresponded to the molecular weight of EGFR, and no significant labeling occurred in the absence of affibody-DMAP or in the presence of PEG-DMAP and unconjugated affibody (lanes 1–3 in Figure 3d). It should also be noted that anti-HER2 scFv-DMAP and anti-EGFR affibody-DMAP showed no cross-reactivity; that is, no band for HER2 labeled by anti-EGFR affibody-DMAP was observed for HER2-expressing N87 cells and EGFR labeling did not occur with anti-HER2 scFv-DMAP on EGFR-expressing A431 cells (lane 5 in Figure 3b, lane 4 in Figure 3d).[21] It is clear that the current approach is capable of covalently labeling selected cancer markers endogenously expressed on live cancer cells and that this selectivity can be controlled simply by switching the recognition module, i.e., the antibody.[22]

Cellular Dynamics of HER2 and EGFR on the Surface of Live Cancer Cells. Understanding the regulation of cell-surface receptor dynamics is essential for numerous cellular processes.[1−3] With our covalent labeling methods, we were able to investigate cellular trafficking and the lifetime of HER2 endogenously expressed in N87 cells. Cells were treated with anti-HER2 scFv-P6-DMAP and acyl donor **5**, washed with buffer, and incubated in medium at 37 °C. Cytosolic fluorescent granules appeared after 4–6 h, suggesting relatively slow

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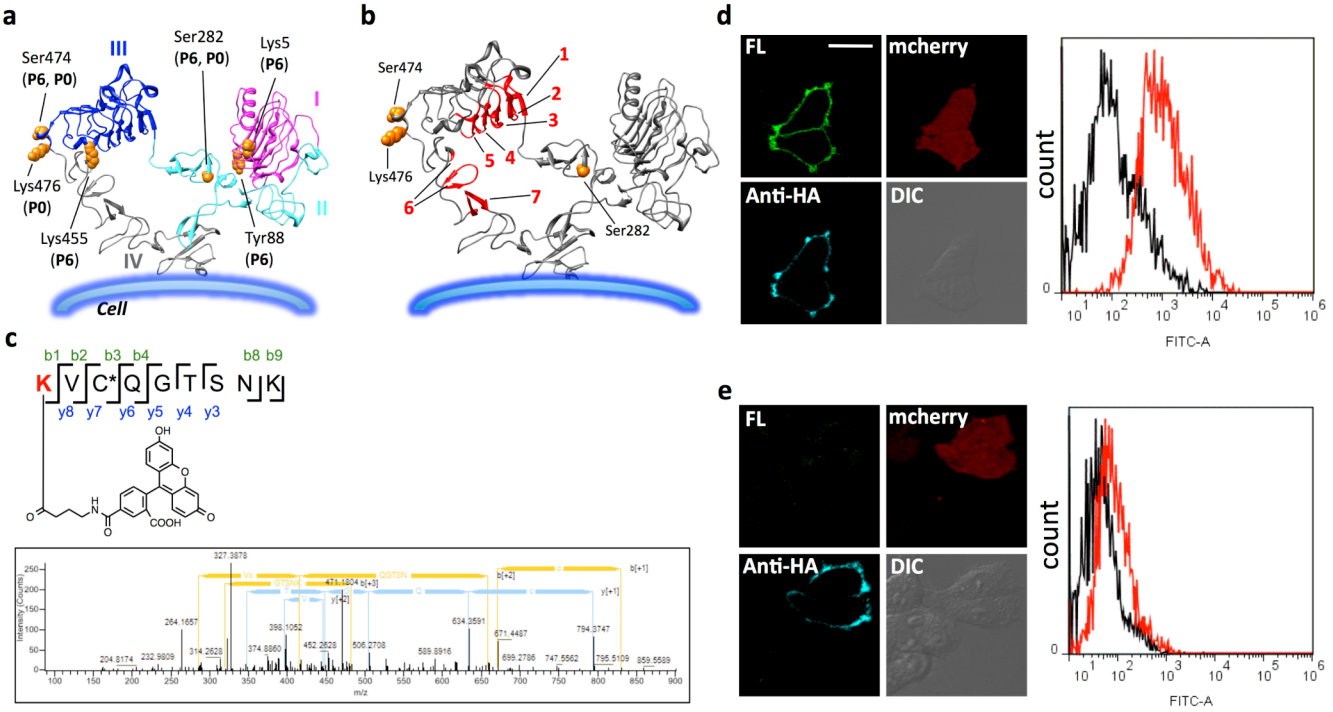

Figure 5. Epitope mapping of anti-EGFR affibody on A431 cells. (a) Labeling sites (orange spheres) of EGFR on the crystal structure of the extracellular domain of EGFR (PDB code: 1YY9). Nomenclatures (P6, P0) in parentheses denote the affibody-DMAP catalyst used to specifically label the corresponding amino acid residues. (b) Potential binding sites of anti-EGFR affibody (red) and the amino acids labeled by the affibody-P0-DMAP catalyst (orange spheres). The red numbers denote fragment numbers. (c) MS/MS analysis of the FL-modified peptide K(FL)VCQGTSNK detected from labeled EGFR. *Site(s) where carbamidomethylation was observed. (d,e) Assessment of affibody-FL binding to HEK293T expressing EGFR variants by CLSM and flow cytometry: (d) HA-fused wild-type EGFR and (e) HER2-swap. Affibody-FL (green), mcherry (red) and anti-HA immunofluorescence (cyan) are shown in left panel. For flow cytometry analysis, cells expressing mcherry at low (black) and high levels (red) were gated separately and shown their affibody-FL binding (see Supporting Information Figure S14). Scale bar, 20 μm.

internalization of HER2 (Figure S8a), which is in good agreement with previous reports.[24,25] Exposure of the labeled cells to benzoquinoid ansamycin geldanamycin (GA), however, induced rapid internalization of HER2. That is, fluorescent granules in the cytosol appeared within 2 h, and only cytosolic fluorescence was observed at the expense of membrane fluorescence after 6 h (Supporting Information Figure S8).

We next sought to determine the half-life of endogenous HER2. The N87 cells labeled by anti-HER2 scFv-P6-DMAP and acyl donor **4** were harvested at the indicated time points, and the amount of remaining FL-labeled HER2 was quantified by Western blot. Where no stimuli were applied, FL-labeled HER2 gradually decreased over 24 h with a half-life of 9.4 h (Figure 3e), while no significant change was observed for the total HER2 level, implying that HER2 degradation was counterbalanced by its biosynthesis. The determined half-life of HER2 was consistent with the value previously measured by radioactive pulse-chase experiments.[26,27] In contrast, GA treatment resulted in a rapid decrease of FL-labeled HER2 in N87 cells (with a half-life of 1.9 h, which was consistent with the imaging data, Figure 3f), suggesting that GA induced rapid degradation of membrane-bound HER2.[24,27] The total HER2 level also decreased at a slightly slower rate than did FL-labeled HER2 ($t_{1/2}$ = 2.8 h, Figure 3f). This can presumably be ascribed to the GA-induced degradation of HER2 outpacing HER2 biosynthesis and/or HER2 biosynthesis being downregulated by GA, as suggested by a recent report.[28] The current approach therefore allows the precise measurement of the half-life and trafficking of endogenous HER2 with minimal perturbation to cells.

Similarly for EGFR, EGF-stimulated internalization of labeled EGFR was observed on A431 cells (Supporting Information Figure S9b). In addition, careful inspection of the cocrystal structure of EGFR and EGF revealed that the labeled amino acids were located far from the ligand binding site (Supporting Information Figure S9a, see the section below for labeling site determination). Given these results, it is clear that the receptors investigated in this study retained their biological functions even after the labeling reaction.

Antibody Epitope Mapping on Live Cell Surfaces. We subsequently carried out labeling site mapping of HER2. As shown in Figure 4a–c, peptide mass-fingerprinting analysis revealed that five amino acids of HER2 were modified with FL by the three anti-HER2 scFv-DMAP catalysts (Figure 4a–d and S10). The distribution of labeling sites depended on the linker structure of the three catalysts. On the basis of the crystal structure of the extracellular domain of HER2 and anti-HER2 scFv (Figure 4a–c),[29] amino acids relatively proximal (<40 Å) to the DMAP-conjugation site of anti-HER2 scFv were labeled by scFv-P0-DMAP and scFv-P2-DMAP (Ser288 and Lys 311 in Figure 4b and c), whereas distal amino acids (<74 Å, Lys128 and Lys148) were labeled by scFv-P6-DMAP in addition to two proximal residues (Lys311 and Lys314) (Figure 4a). Such differences in labeling sites are likely attributable to the different linker lengths of the DMAP catalysts (~25 Å for P0-DMAP, ~30 Å for P2-DMAP, and ~46 Å for P6-DMAP; Supporting Information Appendix 2). These results indicate the interesting possibility that one can roughly assign antibody-binding sites on target proteins (i.e., epitopes) on the basis of the labeling

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site(s) and the linker structures employed, even without structural information on the antigen−antibody complex.[30]

To our surprise, most of the labeled amino acids (Lys128 and Lys148 for P6-DMAP, Lys311 for P2-DMAP, and Lys311 and Ser288 for P0-DMAP) located further than the "reaction range" predicted on the basis of the crystal structure (green spheres in Figure 4a−c). This may reflect the dynamic nature of HER2 and/or dynamic fluctuations of protein−protein interactions on living cells. Several structural studies have suggested that the extracellular region of HER2 adopts an extended conformation that resembles the ligand-bound form of EGFR.[29,31] Although these experiments were carried out at 4 °C, our results provide experimental evidence that HER2 exhibits some structural fluctuations, including a bent conformation, on cell surfaces as observed for other members of the ErbB family.[32,33]

Encouraged by this model study with HER2, we finally attempted antibody epitope mapping of anti-EGFR affibody, for which the binding site(s) have remained unknown due to the lack of a corresponding crystal structure. As shown in Figure 5a, six labeled amino acids were identified from endogenous EGFR expressed on A431 cells labeled by the two affibody-DMAP catalysts (Figure 5a, c, and Supporting Information Figure S11). With affibody-P6-DMAP, the five labeling sites (Lys5, Tyr88, Ser282, Ser474, and Lys455) were observed to spread across domains I−III (Figure 5a), whereas the three amino acids labeled by affibody-P0-DMAP (Ser282, Ser474, and Lys476) remained within domains II and III (Figure 5a). On the basis of the labeling sites and the dynamic "reaction range" of P0-DMAP determined by the HER2 model study (<40 Å), we were able to predict the binding sites of anti-EGFR affibody on EGFR exposed on live cell surfaces. The possible affibody binding areas were first simulated for each determined labeling site (Supporting Information Figure S12a−c), and the region where these areas overlapped were selected as the potential binding site(s) of the affibody. In the current model, the affibody was expected to bind on the side face of domain III and/or IV (V505−V512 or E521-Q533), but not on domain I or II (red-colored region in Figure 5b).

To validate our prediction, we constructed EGFR variants with mutations in the predicted binding region and tested their binding ability to affibodies on living cells. First we prepared two mutants: (1) "HER2-swap" where the entire predicted binding region (domain III and IV) of EGFR was swapped with the corresponding region of HER2, and (2) "charge-altered" where all the amino acids with charge in the binding region were replaced with an alanine residue (see Figure 5b and Supporting Information Figure S13 and Table S1). Using HEK293T cells transfected with the relevant plasmids, we examined the binding ability of these EGFR variants to fluorescein-conjugated affibody (affibody-FL) on live cells by CLSM imaging and FACS measurements. A strong fluorescent signal was observed from cells expressing HA-fused wild-type EGFR (Figure 5d and Supporting Information Figure S14a), whereas no or minimal fluorescence was detected from those expressing HER2-swap or charge-altered proteins (Figure 5e and Supporting Information Figure S14b). These results indicated that the extensive mutations in the predicted binding region of EGFR resulted in the loss of affibody binding ability, thereby validating the use of our labeling technique for antibody epitope mapping.

To further narrow down the binding region, we additionally prepared seven mutants where the charged amino acids were replaced with alanine in the individual fragments (from fragment 1 to 7) of the predicted binding region of EGFR (see Figure 5b and Supporting Information Figure S13 and Table S1) and confirmed membrane localization of these by immunostaining using anti-HA antibody (Supporting Information Figure S14). Our binding assay showed that the mutations in fragments 1−4 and 6 lost binding ability to the affibody, but not to the fragments 5 and 7 (Supporting Information Figure S14 and Table S1), which strongly suggests that the affibody binds to the side face of domain III of EGFR. The results obtained by the mutation experiments are remarkably consistent with the prediction offered by our labeling method, highlighting the utility of our antibody-DMAP conjugates for structural characterization of protein−protein interactions on live cells.

■ SUMMARY

We developed a novel catalyst-antibody conjugate as a new chemical tool for the efficient, specific labeling and analysis of selected proteins such as HER2 and EGFR on living cells by rational coupling of the antibody and DMAP catalyst. Because of the high selectivity and efficiency of this labeling technique, we were able to monitor cellular dynamics and the lifetime of HER2 endogenously expressed on cancer cells. Further, characterization of the labeling sites of HER2 and EGFR enabled antibody epitope mapping on living cells and the identification of potential binding sites of anti-EGFR affibody, which were previously unknown. The covalent labeling method also provided experimental evidence that HER2 exhibits a more dynamic structure than expected from crystallographic analyses. This method represents a powerful design strategy for catalyst-antibody conjugates, and is, in principle, applicable to other antibodies and their derivatives as well as proteins in general. We believe that the current approach will provide a general tool for investigating the dynamics, fluctuations, and molecular interactions of an important class of cell-surface receptors on live cell surfaces.

■ MATERIALS AND METHODS

Physical Measurements. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz Varian Mercury and JEOL JNM-ECA600 FT spectrometer. Chemical shifts were referenced to residual solvent peaks or tetramethylsilane (δ = 0 ppm). UV−vis absorption spectra were acquired on a Shimazu UV-2550 spectrophotometer. Matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Autoflex III (Bruker Daltonics, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) as a matrix. High resolution mass spectra were measured on an Exactive (Thermo Scientific, CA, USA) equipped with electron spray ionization (ESI). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi Chromaster system equipped with a diode array and fluorescence detectors, and an YMC-Pack Triat C18 or ODS-A column. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B).

Biochemical Experiments. SDS-PAGE and Western blotting were performed using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence and chemical luminescent signals were detected with a ChemiDoc XRS system equipped with a 520DF30 filter (ChemiDoc, Bio-Rad laboratory) and Imagequant LAS 4000 (GE Healthcare), respectively.

Construction, Expression and Purification of Anti-HER2 scFv S242C. A plasmid encoding anti-HER2 single chain Fv (scFv) (4d5) was a kind gift from CANON Inc. (Tokyo, Japan). The anti-HER2 scFv gene was subcloned into the pET28a(+) vector (Merck Millipore, MA, USA) and the S242C mutant was made by mutating cysteine into serine at position 242 residue using following forward and reverse

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primers (cys codon underlined): 5′-tggtcaccgtctgctcggcggccgcac-3′ and 5′-gtgcggccgccgagcagacggtgacca-3′, respectively. Mutations were confirmed by DNA sequencing service provided by Sigma-Aldrich Japan (Tokyo, Japan). Plasmid DNA of the scFv was transformed into Shuffle T7 *Escherichia coli* (New England Biolabs, MA, USA) for protein expression. A single colony of freshly transformed cells was cultured overnight in 10 mL of LB medium containing Kanamycin. The culture was used to inoculate 1 L of LB medium and it was incubated for ∼3 h at 30 °C with shaking speed at 180 rpm. When the OD_{600} of the culture reached ∼0.5, IPTG was added to produce a final concentration of 1.0 mM to induce overexpression of the scFv gene. The induced culture was incubated for another ∼18 h at 16 °C with the shaking speed at 180 rpm, and the cells were subsequently harvested by centrifugation at 8000*g* for 25 min. The pelleted bacterial cells were suspended in PBS(−) containing 0.5% Lysozyme and 0.01% DNase and sonicated to disrupt the cells. The lysate was centrifuged and the supernatant was subjected to affinity chromatography using TALON metal affinity resin (Takara Bio Inc., Shiga, Japan). His-tagged scFv-cys was eluted by PBS(−) containing 300 mM imidazole and 500 mM NaCl and was subjected to gel filtration chromatography on HiLoad 16/600 Superdex 75 prep grade (GE healthcare, Little Chalfont, UK) with a flow rate of 1.0 mL/min using an ÄKTA purifier system equilibrated with PBS(−). The fractions were monitored at 280 nm, collected using Frac-920, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). If necessary, the protein solution was concentrated by Amicon Ultra-15 10-kDa cutoff centrifugal filter device (Millipore Corp., MA, USA) and stored in freezer at −80 °C for long-term storage.

Preparation of scFv-DMAP Conjugates. The concentration of scFv-cys was calculated on the basis of the molar extinction coefficient at 280 nm of 50 100 M^{-1} cm^{-1} for anti-HER2. Purified protein solution (∼30 μM) was incubated with maleimide compound **1**−**3** (45 μM) in PBS(−) at room temperature for 30 min. The conjugation reaction was monitored by MALDI-TOF MS to confirm the conversion to corresponding scFv-DMAP conjugates. The protein solution was then subjected to gel filtration on Superdex 75 10/300 GL (GE healthcare) to remove the remaining labeling reagents. The protein fractions were monitored at 280 nm and analyzed by SDS-PAGE.

Preparation of Anti-EGFR Affibody-DMAP Conjugate. Anti-EGFR affibody was purchased from Affibody AB (Solna, Sweden) and used without further purification. The concentration of affibody was calculated on the basis of the molar extinction coefficient at 280 nm of 37 500 M^{-1} cm^{-1}. The DMAP conjugation, followed by gel filtration purification was performed by the same procedure as that for scFvs.

HER2 Labeling by scFv-DMAP on HEK293T. HEK293T cells were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotic-antimycotic (Gibco, Carlsbad, CA) and incubated in a 5% CO_2 humidified chamber at 37 °C. Transfection was carried out by standard procedures described anywhere. In brief, HEK293T cells (1.0×10^6 cells) were incubated on 10 cm dishes treated with polystyrene (BD Falcon, Franklin Lakes, NJ) at 37 °C. After 24 h, OPTI-MEM I (Gibco, Carlsbad, CA) containing 12 μg of the plasmid encoding HER2-FLAG and 12 μL of Lipofectamine 2000 transfection reagent (Life Technology, CA, USA) was added to the medium and the cells were incubated for 5 h at 37 °C. The medium was exchanged with fresh growth medium and the cells were further incubated for 48 h prior to the labeling reaction. Before labeling, the medium was removed and the cells were washed twice with HBS (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 8.0). To the cells were added labeling mixtures containing 1 μM scFv-DMAP and 8 μM acyl donor **4** in HBS and incubated at 4 °C for indicated time. For Western blotting analysis, cells were washed three times with ice-cold PBS, lysed with RIPA buffer and mixed with an equal volume of 2× SDS-PAGE loading buffer containing 200 mM DTT. The samples were applied to SDS-PAGE and electrotransferred onto immune-blot PVDF membranes (Biorad, Hercules, CA), followed by blocking with 5% nonfat dry milk in TBS containing 0.05% Tween (Sigma-Aldrich, St. Louis, MO). The membranes were stained with rabbit antifluorescein antibody (abcam, ab19491), anti-HER2 antibody (cell signaling, 2242L) followed by an HRP-conjugated goat antirabbit IgG (santa cruz, SC-2004) or goat antimouse IgG (santa cruz, SC-2005). The membranes were developed with Chemi-Lumi One L (Nacalai tesque, Kyoto, Japan).

On-Cell Labeling of Endogenous HER2/EGFR. HER2-expressing human gastotic cancer cell N87 and EGFR-expressing human epidermoid carcinoma cell A431 were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotic-antimycotic (Gibco, Carlsbad, CA) and incubated in a 5% CO_2 humidified chamber at 37 °C. Prior to labeling experiments, N87 and A431 cells (8×10^5 cells) were incubated on 35 mm dishes treated with polystyrene (BD Falcon, Franklin Lakes, NJ) at 37 °C. After 48 h, the media was removed, and the cells were washed twice with HBS. To the cells were added labeling mixtures containing 1 μM scFv-DMAP or affibody-DMAP and 8 μM acyl donor **4** in HBS and incubated at 4 °C for 90 min. For imaging experiments, the cells were washed with ice-cold HBS buffer six times. Cell imaging was performed with a confocal laser scanning microscope (CLSM, FV1000 IX81, Olympus) equipped with a 60×, NA = 1.40 oil objective. Fluorescence images were acquired using the 488 nm excitation derived from an Ar laser. Western blotting analysis was performed as mentioned above, except that anti-EGFR antibody (cell signaling, 2232S) was used for the detection of EGFR.

Characterization of Antibody Dissociation by Flow Cytometry. N87 and A431 cells ($6-8 \times 10^5$ cells) were incubated on 35 mm dishes at 37 °C in 5% humidified CO_2. After 48 h, the media was removed, and the cells were washed with PBS(−), trypsinized and suspended in PBS(−) buffer. The cells were then exposed to 1 μM antibody-fluorescein conjugates and incubated at 4 °C for 1 min. After washing with PBS(−) six times or acid buffer (100 mM Glycine, 150 mM NaCl, pH 3.0 containing 0.1% Tween-20) twice followed by PBS(−) twice, the cells were resuspended in stain buffer (BD Pharmingen stain buffer, BD PharMingen). Flow cytometry was performed on a LSR Fortessa (BD Biosciences) with a blue laser (488 nm) and FITC filter (530/30), and data analysis was carried out by FlowJo software.

Internalization and degradation of endogenous HER2. HER2-expressing N87 was cultured as described above. After cells were washed twice with PBS, to the cells were added labeling mixtures containing 1 μM scFv-DMAP and 8 μM acyl donor **4** or **5** in HBS, and the cells were incubated at 17 °C for 30 min. The cells were washed with HBS buffer six times and incubated in DMEM medium containing 25 mM HEPES (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotic-antimycotic at 37 °C in the presence or absence of 1 μM geldanamycin (TCI, Tokyo, Japan). At indicated time points, the cells were harvested and lysed as mentioned above, followed by SDS-PAGE and Western blotting analysis. Fluorescence images were acquired as mentioned above.

Identification of Labeling Site of HER2 and EGFR by Peptide Fingerprint Analyses. To identify labeling site, labeled HER2 or EGFR was purified by immunoprecipitation with antifluorescein, followed by silver staining. Immunoprecipitation was carried out by a standard procedure provided by GE healthcare with slight modifications. In brief, cell pellet was suspended in 10 mM Tris-HCl (pH 7.2) and the cells were disrupted by gentle sonication. The lysate was ultracentrifuged at 100000*g* for 60 min at 4 °C. The pellet containing membrane proteins was dissolved in RIPA buffer containing 1% SDS, followed by sonication (10 bursts × 2) and addition of 10 volume of NP-40 buffer (PBS(−), 0.1% NP-40). Each sample was pretreated with nProtein A sepharose 4 Fast flow (GE healthcare) for 1 h. The sample was then incubated with antifluorescein (abcam, ab19491) at 4 °C overnight with continuous rotation, followed by addition of nPtotein A sepharose 4 Fast flow and further incubation at 4 °C for 1 h. The sepharose was washed 3 times with RIPA. Protein was eluted by addition of 2× SDS-PAGE sample buffer containing 250 mM DTT and boiling in heatblock for 5 min, and resolved by SDS-PAGE. The protein band corresponding to HER2 was excised from the silver stained gel. The excised gels were destained and subjected to in-gel digestion using homemade Trypsin/

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LysC or Trypsin/LysC/Chymotrypsin protease cocktail (Thermo Fisher Scientific). All samples were analyzed by nanoflow reverse phase liquid chromatography followed by tandem MS, using a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific). For LTQ-Orbitrap XL, a capillary reverse phase HPLC−MS/MS system composed of an Agilent 1100 series gradient pump equipped with Valco C2 valves with 150-μm ports, and LTQ-Orbitrap XL hybrid mass spectrometer equipped with an XYZ nanoelectrospray ionization (NSI) source (AMR). Samples were automatically injected using PAL system (CTC analytics, Zwingen, Switzerland) into a peptide L-trap column OSD (5 μm, AMR) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% trifluoroacetic acid and 2% acetonitrile (solvent C), the peptides were loaded into a nano HPLC capillary column (C18-packed with the gel particle size of 3 μm, 0.1 × 150 mm, Nikkyo Technos, Tokyo Japan) by switching the valve. The eluents used were A, 100% water containing 0.1% formic acid, and B, 80% acetonitrile containing 0.1% formic acid. The column was developed with the concentration gradient of acetonitrile as follows: from 5% B to 45% B in 60 min, 45% B to 95% B in 1 min, sustaining 95% B for 10 min, from 95% B to 5% B in 1 min, and finally re-equilibrating with 5% B for 8 min. Xcalibur 2.1 system (Thermo Fisher Scientific) was used to record peptide spectra over the mass range of m/z 350−1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 150−2000. Repeatedly, MS spectra were recorded followed by three data-dependent collision induced dissociation (CID) MS/MS spectra generated from three highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted, and peak lists were generated by Proteome Discoverer 1.3.0.339 (Thermo Fisher Scientific). Searches were performed by using the SEQUEST (Thermo Fisher Scientific) against latest uniprot database for HER2 (ERBB2_HUMAN, P04626) and EGFR (EGFR_HUMAN, P00533). Searching parameters were set as follows: enzyme selected as used with three maximum missing cleavage sites, a mass tolerance of 10 ppm for peptide tolerance, 0.5 Da for MS/MS tolerance, dynamic of carbamidomethyl (C) and oxidation (M) and chemical modification (K, S, T, Y). Peptide identification and modification information returned from SEQUEST were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS.

Construction of EGFR Variants. A plasmid encoding HA-EGFR was a kind gift from Dr. Shinya Tsukiji (Nagaoka University of Technology). The EGFR variants were constructed by overlap extension PCR using following primers: for charge-altered EGFR variant, EGFR_*Kpn*I(+) 5′-ggtggcggtacccgtcgggctctgg-3′ and EGFR_1(−) 5′-ccaatacgtattcggttacacactttgcggc-3′, EGFR_1(+) 5′-gtgtgtaaccgaatacgtattggtgaatttaaag-3′ and EGFR_2(−) 5′-ggaggtgcagtttgcgaaggctgcaatattcgtagcatttatgg-3′, EGFR_2(+) 5′-cttcgcaaactgcacctccatcagtggcgctctccacatcctgcc-3′ and EGFR_3(−) 5′-cggttgccagaatagccagtgcctgtggatccagaggaggag-3′, EGFR_3(+) 5′-ctgggtattctggcaaccgtagcggcaatcacagggtttttgctg-3′ and EGFR_4(−) 5′-gattgctaggttcgcaaaggcagcgaggtccgtcctgttttcagg-3′, EGFR_4(+) 5′-cctttgcgaacctagcaatcatagccggcaggaccaagcaacatg-3′ and EGFR_5(−) 5′-cacttatcgccgcgagggaggctaatcccaaggatgttatgttc-3′, EGFR_5(+) 5′-gggattagcctccctcgcggcgataagtgatggagatgtg-3′ and EGFR_6(−) 5′-ctgacattccggcaagagacgcagtccgcgggctccgggccccagcagccctcgggggga-3′, EGFR_6(+) 5′-ctcttgccggaatgtcagcgcaggcgcggcatgcgtggacaagtgcaagcttc-3′ and EGFR_7(−) 5′-gttcgccacaaacgccgctggcgcaccctccagaagcttgcac-3′, EGFR_7(+) 5′-gcggcgtttgtggcgaactctgcgtgcatacagtgccacccagag-3′ and EGFR_BstEII(−) 5′-cccaaacggtcaccccgtagctcc-3′. The mutated fragment was digested by *Kpn*I and BstEII and was inserted into the same digestion site of HA-EGFR. As for HER2-swap, HER2 sequence relevant to the predicted affibody binding region of EGFR was cloned using following primers: EGFR_*Kpn*I(+) 5′-ggtggcggtacccgtcgggctctgg-3′ and EGFR_(−) 5′-ccatgcccagaccatagcacactttgcggcaaggcccttcgc-3′, HER2(+) 5′-gccgcaaagtgtgctatggtctgggcatgg-3′ and HER2(−) 5′-gggtggcacggcaaacagtgcctgg-3′. The mutated fragment was digested by *Kpn*I and BstEII and was inserted into the same digestion site of HA-EGFR. Mutations were confirmed by DNA sequencing service provided by Sigma-Aldrich Japan (Tokyo, Japan).

Affiody Binding Analysis by CLSM and Flow Cytometry. HEK293T cells were cotransfected with plasmids encoding the EGFR variants as well as mcherry as an expression marker. After 24 h, the cells were dissociated by pipetting and reseeded on glass-bottom dishes pretreated with poly-L-lysine for CLSM experiments and 35 mm dishes for flow cytometry and further incubated for 24 h. For flow cytometry, the cells were washed with PBS(−) twice and exposed to 1 μM affibody-fluorescein conjugates and incubated at 4 °C for 1 min. After washing with PBS(−) twice, the cells were resuspended in stain buffer. Flow cytometry was performed on a LSR Fortessa (BD Biosciences) with a blue laser (488 nm) and FITC filter (530/30), yellow-green laser (561 nm) and PE filter (610/20). Data analysis was carried out by FlowJo software. For CLSM experiments, the cells were treated with PBS(−) containing 1 μM affibody-fluorescein conjugates for 1 min. After washing with PBS(−) twice, the cells were subjected to immunostaining using anti-HA antibody (abcam, ab9110) and antirabbit IgG Alexa Fluor633 (Invitrogen, A21070). Cell imaging was performed with a confocal laser scanning microscope (CLSM, FV1000 IX81, Olympus) equipped with a 60×, NA = 1.40 oil objective. Fluorescence images were acquired using the 488 nm excitation derived from an Ar laser, and 547 and 633 nm derived from a HeNe laser.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures including chemical synthesis of DMAP catalysts and acyl donors, characterization of antibody-DMAP conjugates, general property of labeling reactions, MS/MS analysis of FL-modified HER2 and EGFR, and assessment of affibody binding to EGFR binding are detailed. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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