Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A Noncanonical Amino Acid-based Relay System for Site-specific Protein Labeling

Yuda Chen[a], Axel Loredo [a], Aviva Gordon [a], Juan Tang [a], Chenfei Yu [a], Janett Ordonez[a], and Han Xiao[a, b,*]

Materials and methods

LB agar and 2YT were ordered from BD Difco™. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Anatrace. 4-12% Bis-Tris gels for SDS-PAGE were purchased from Invitrogen. The QuickChange Lightning Multi Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Cat. 210515). Oligonucleotide primers were purchased from Integrated DNA Technologies and Eurofins Genomics (Supplementary Table S1 lists the oligonucleotides used in this report). Plasmid DNA preparation was carried out with the GenCatch^{TM} Plus Plasmid DNA Miniprep Kit and GenCatch^{TM} Advanced Gel Extraction Kit. BugBuster^{TM} Protein Extraction Reagent was purchased from Novagen (Cat. 70584). Protease inhibitor Cocktail was purchased from Biotool. Pierce^{TM} universal nuclease was purchased from Thermo Scientific (Cat. 88700). Ni-NTA Agarose was obtained from Qiagen (Cat. 30230). Hoechst 33342 was purchased from Life Technologies^{TM} (Cat. H3570). DiOC18(3) was obtained from Marker Gene (Cat. M1197). M9-glucose minimal medium used for protein expression contains M9 salt (6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl), heavy metal solution (1 µg/L $CuSO_4{\cdot}5H_2O$, 4 µg/L $MnCl_2{\cdot}4H_2O$, 4 µg/L $ZnCl_2$, 1.2 µg/L $FeSO_4{\cdot}5H_2O$), 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 µg/mL Thiamine, 300 µM Leucine 4 µM D-Biotin, Glucose (4 g/L).

E. coli BL21(DE3) cells were kindly provided by Dr. Peter G. Schultz's lab (The Scripps Research Institute). Breast cancer cell lines SKBR3, MDA-MB-468 were obtained from American Type Culture Collection (ATCC).

Unless otherwise mentioned, all solvents and chemicals for synthesis were purchased from Alfa Aesar and Fisher Chemical and used as received without further purification, unless otherwise specified.

Plasmid construction.

The *Mjp*AFRS/tRNA^{Tyr} gene cassette was generated by PCR using primers YC0002 and YC0008, and inserted into the pUltra vector amplified using YC0009 and YC0010 to generate pUltra-*p*AF. pBad-anti-HER2-scFv-113* was generated by site-directed mutagenesis using primer YC0014 and pBad-anti-HER2-scFv as a template following the QuikChange Site-Directed Mutagenesis Kit instructions (Agilent Technologies). Anti-HER2-scFv113* gene cassette was generated by PCR

using primers YC0040, YC0041 and inserted into pET28a vector using NcoI and XhoI restriction sites to provide pET28a-anti-HER2-scFv*.

Protein Expression and Purification.

E. coli BL21(DE3) cells, co-transformed with pET28a-anti-HER2-scFv* and pUltra-*p*AF plasmids, were grown in M9-glucose minimal medium supplemented with or without 1 mM *p*AF at 37°C. To express proteins containing biosynthesized *p*AF, an additional biosynthetic plasmid (pLASC-lppPW) was transformed into *E. coli* BL21(DE3) cells. The protein expression was carried out in M9-glucose minimal medium. When the OD600 of the cell culture reached to 0.6, protein expression was induced by the addition of IPTG to a final concentration of 1 mM, and cells were grown for an additional 20 h at 30 °C. Cells were harvested by centrifugation at 4,750 × g for 10 min. The cell pellets were suspended in lysis buffer (30 mM Tris-HCl buffer with 20 % sucrose, 1 mM EDTA, 0.2 mg/mL lysozyme, and 0.1‰ benzonase, pH 8.0) and lysed at 37 °C. The resulting cell lysate was clarified by centrifugation at 14,000 × g for 30 min, and protein was purified on Ni-NTA resin (Qiagen) following the manufacturer's instructions.

Expression and Fluorescence Measurement of sfGFP

After sfGFP expression with the same method described above, 0.25 mL cells were harvested by centrifugation at 4,750 × g for 10 min and then suspended with 5 ml PBS (pH 7.4). Cell Fluorescence of the cells was measured using excitation/emission wavelengths of 395/509 nm.

Reaction conditions

Anti-HER2-*p*AF reaction with 2-amino-4-methyl-phenol

700 µM 2-amino-4methylphenol was combined with 100 µM anti-HER2-*p*AF in pH 6.5 phosphate buffer. With 6 mM sodium periodate, the reaction proceeded for 5 minutes at room temperature. The reaction was quenched by adding tris(2-carboxyethyl) phosphine (TCEP), followed by removal of excess 2-amino-4-methylphenol and $NaIO_4$ using the Amicon 5,000 molecular-weight-cutoff protein concentrator.

Anti-HER2-*p*AF reaction with Rhodamine B labeled o-aminophenol

To a pH 6.5 PBS solution containing 30 uM anti-HER2-*p*AF, Rhodamine B labeled 2-amino-4-methylphenol was added to achieve the final concentration of 10 mM. Sodium periodate was added to the solution later to achieve the final concentration of 1 mM. After reaction at room

temperature for 50 minutes, the reaction was quenched by adding TCEP. Excess 2-amino-fluorescent 4-methylphenol and $NaIO_4$ were removed using Zeba™ 7,000 molecular-weight-cutoff spin desalting columns.

Fluorescence Microscopy.

Confocal fluorescent images of living cells were performed using Nikon A1R-si Laser Scanning Confocal Microscope (Japan), equipped with lasers of 405/488/561/638 nm. $DiOC_{18}(3)$ and Hoechst 33342 were prepared as 2 mM DMSO stock solution and 10 mg/mL water solution, respectively. The stock solution was diluted to the working concentration in complete medium (5 μM and 1 μg/mL, respectively). SK-BR-3 cells and MDA-MB-468 cells were incubated in complete medium (RPMI 1640 Medium or Dulbecco's modified Eagle's Medium, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37°C in atmosphere containing 5% CO_2.

SK-BR-3 cells and MDA-MB-468 cells were grown to about 80% confluency in 8-well confocal imaging chamber plates. Cells were incubated with 30 nM Rhodamine B-labeled anti-HER2-*p*AF for 30 min and then fixed by 4 % paraformaldehyde for 15 min. Cells were washed with PBS (pH 7.4) for three times, followed by incubating with DiOC18(3) for 20 min and Hoechst 33342 for 5 min, respectively. After washing with PBS (pH 7.4) for three times, confocal imaging was carried out. Images were taken under conditions as follows: 60× immersion lens with a resolution of 1024×1024 and a speed of 0.25 frame per second; 30% laser power for Hoechst 33342, 405 nm excitation wavelength and 425 to 475 nm detector slit; 50% laser power for DiOC18(3), 488 nm excitation wavelength and 500 to 530 nm detector slit; 15% laser power for Rhodamine B, 561 nm excitation wavelength and 582 to 617 nm detector slit. Differential interference contrast (DIC) and fluorescent images were processed and analyzed using ImageJ.

oligonucleotide	Sequence (5'-3')

YC0002	TCACAaAGGAGGTGCGGCCGCATGGACGAATTTGAAATGATAAAGAGAAACACA TC
YC0008	GACCGTTTAAACGCGGCCGCTTATAATCTCTTTCTAATTGGCTCTAAAATCTTTATA AGT
YC0009	GCGGCCGCGTTTAAACGGTCTCCAGC
YC0010	GCGGCCGCACCTCCTTTGTGAAATTGTTATCCGC
YC0014	GGATCGACTAGTGGCTAGGGGTCCGGTGGGGG
YC0040	AACTTTAAGAAGGAGATATACCATGAAAAAGAATATCGCATTTCTTCTTGCTAGC ATGTT
YC0041	AGTGGTGGTGGTGGTGGTGCTCGAGTTACCCGTGATGATGGTGATGGTGGCCG

Table S1. DNA oligomers

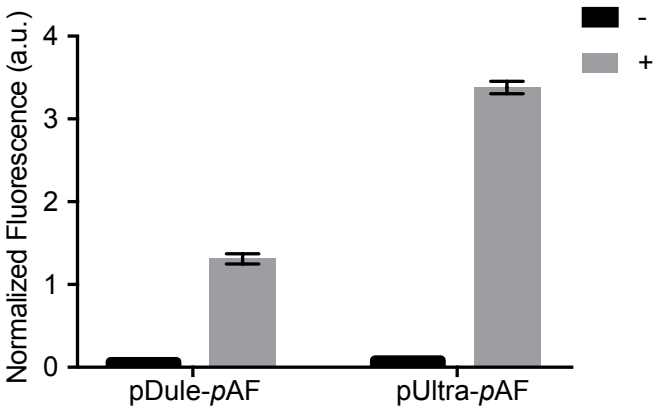

Figure S1: Comparison of the suppression efficiencies of pUltra-*p*AF and pDule-*p*AF measured by normalized fluorescence of full-length GFP expressed in the presence (+) or absence (-) of 1 mM *p*AF.

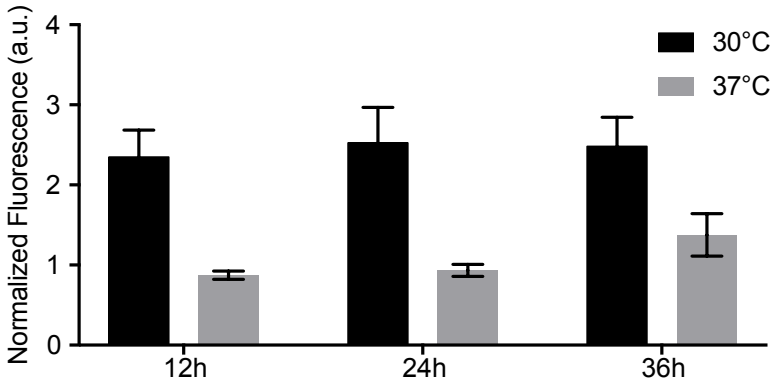

Figure S2: Expression level of sfGFP with biosynthetic *p*AF at different expression times and temperatures.

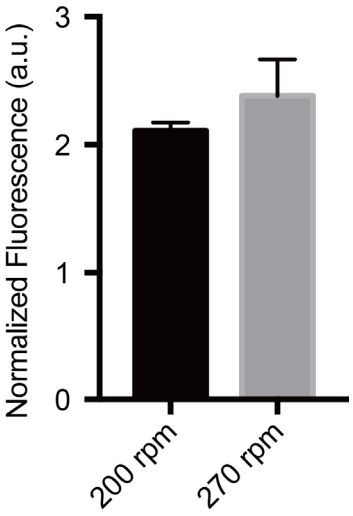

Figure S3: Expression level of sfGFP with biosynthetic *p*AF at different shaking speed.

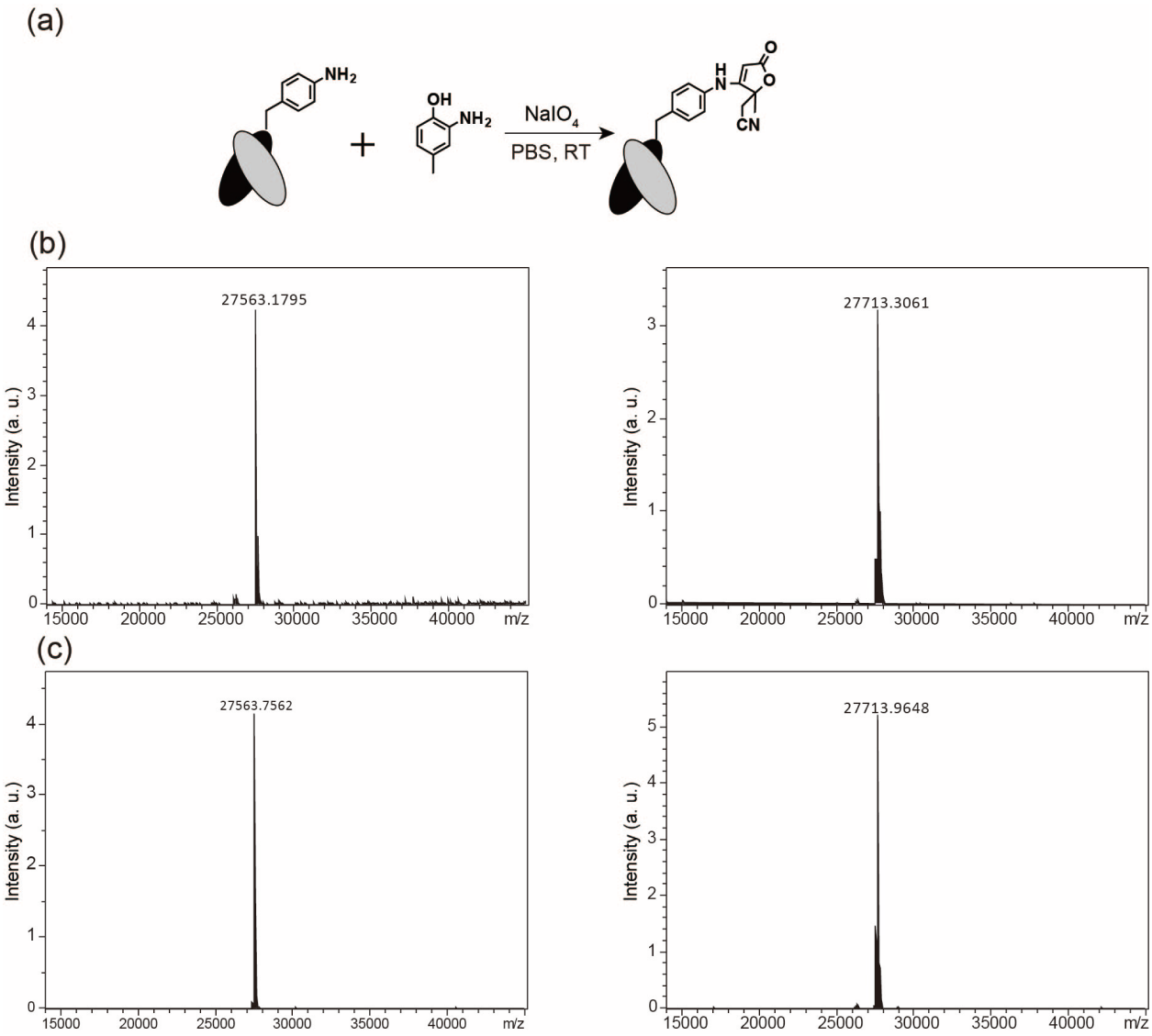

Figure S4: Reactivity of anti-HER2-*p*AF.
(a) Biorthogonal reaction schemes for the 2-amino-4-methyl phenol and anti-HER2-*p*AF. (b) Mass spectra of the reaction product with anti-HER2-*p*AF (left) from feeding and the 2-amino-4-methylphenol (right). (c) Mass spectra of the reaction product anti-HER2-*p*AF (left) from biosynthesis and the 2-amino-4-methylphenol (right).