Supplemental information for:

Antigen-responsive fluorescent antibody probes generated by selective N-terminal modification of IgGs

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Supplemental figures and tables S2-S12

Materials and methods S13-S32

Supplemental references S33

Supplemental figures

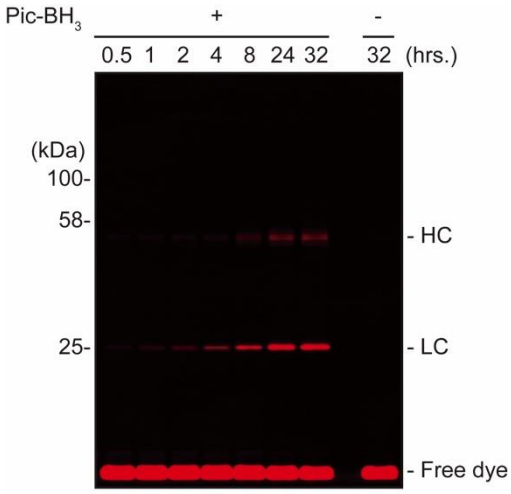
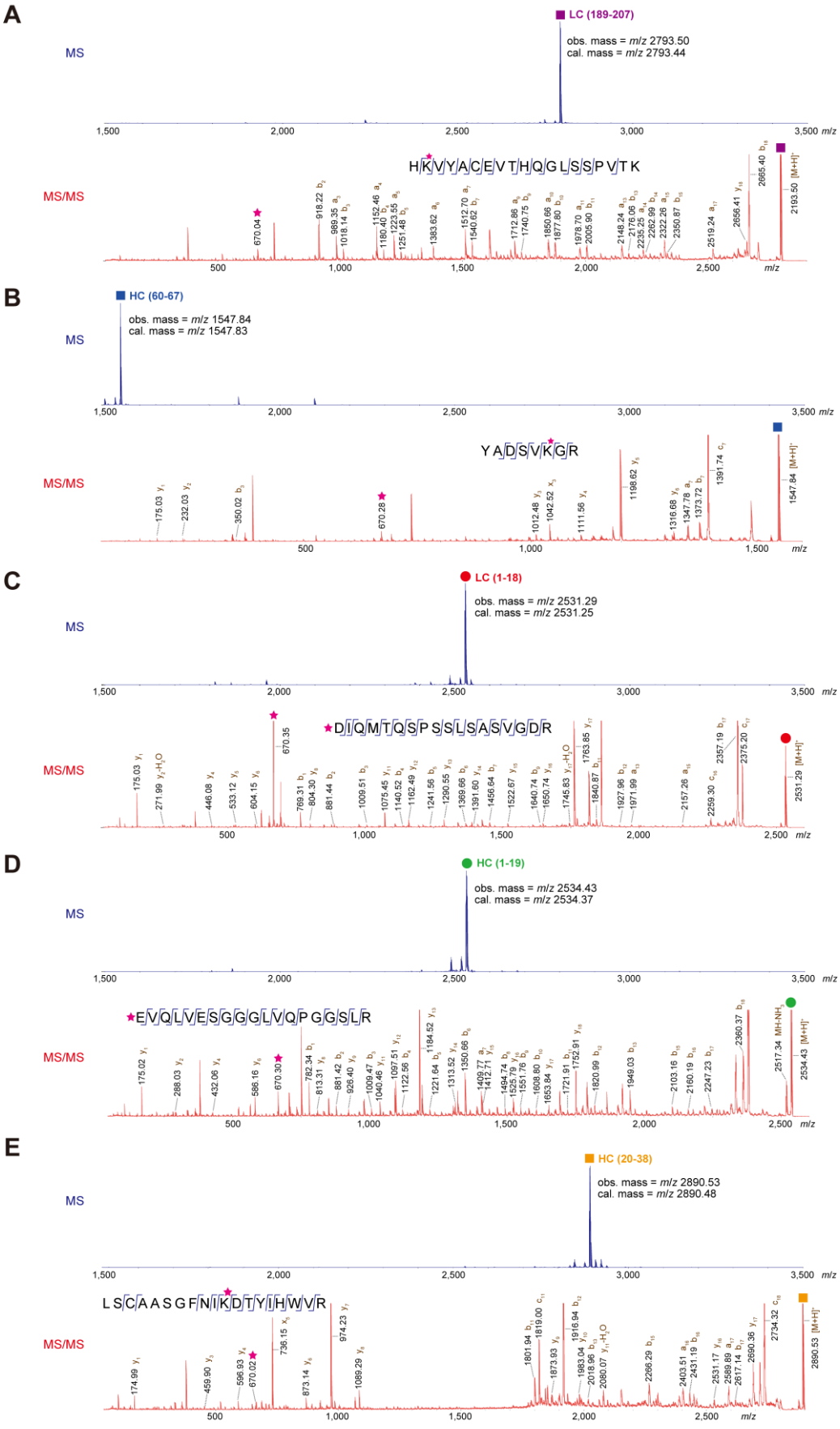


Figure S1. Trace of fluorescent labeling reaction by in-gel fluorescence imaging of SDS-PAGE. Anti-FLAG-tag IgG was labeled with TAMRA-X-C7-CHO (80 μM).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obs. mass = m/z 2793.50
cal. mass = m/z 2793.44
MS
MS/MS
H K V Y A C E V T H Q G L S S P V T K
B
HC (60-67)
obs. mass = m/z 1547.84
cal. mass = m/z 1547.83
MS
MS/MS
Y A D S V K G R
C
LC (1-18)
obs. mass = m/z 2531.29
cal. mass = m/z 2531.25
MS
MS/MS
D I Q M T Q S P S S L S A S V G D R
D
HC (1-19)
obs. mass = m/z 2534.43
cal. mass = m/z 2534.37
MS
MS/MS
E V Q L V E S G G G L V Q P G G S L R
E
HC (20-38)
obs. mass = m/z 2890.53
cal. mass = m/z 2890.48
MS
MS/MS
L S C A A S G F N I K D T Y I H W V R

Figure S2. MS and MS/MS spectra of (A) LC 189-207, (B) HC 60-67, (C) LC 1-18, (D) HC 1-19, and (E) HC 20-38 fragments of TAMRA-labeled trastuzumab. The magenta star represents the TAMRA-labeled amino-groups.

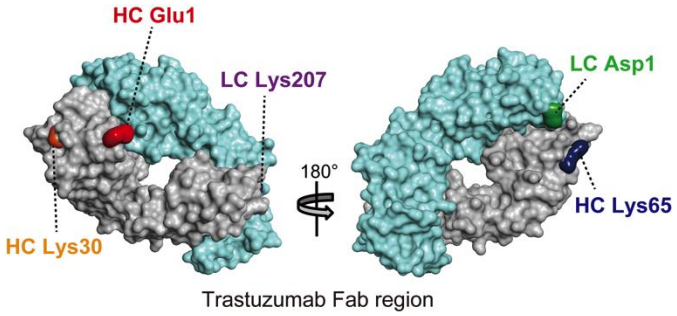


Figure S3. Crystal structure of trastuzumab antigen-binding fragment (Fab) region. Cyan and gray colors represent light chain (LC) and heavy chain (HC), respectively. TAMRA-labeled amino groups are highlighted in the different colors.

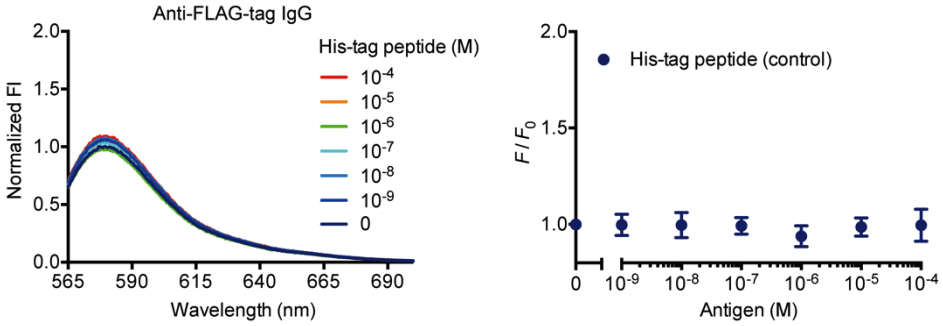


Figure S4. Fluorescence spectra (the left panel) and antigen-titration curve (the right panel) of TAMRA-labeled anti-FLAG-tag IgG upon addition of His-tag peptide as a mock antigen. Each data points represent the mean value ± S.D. of three independent experiments.

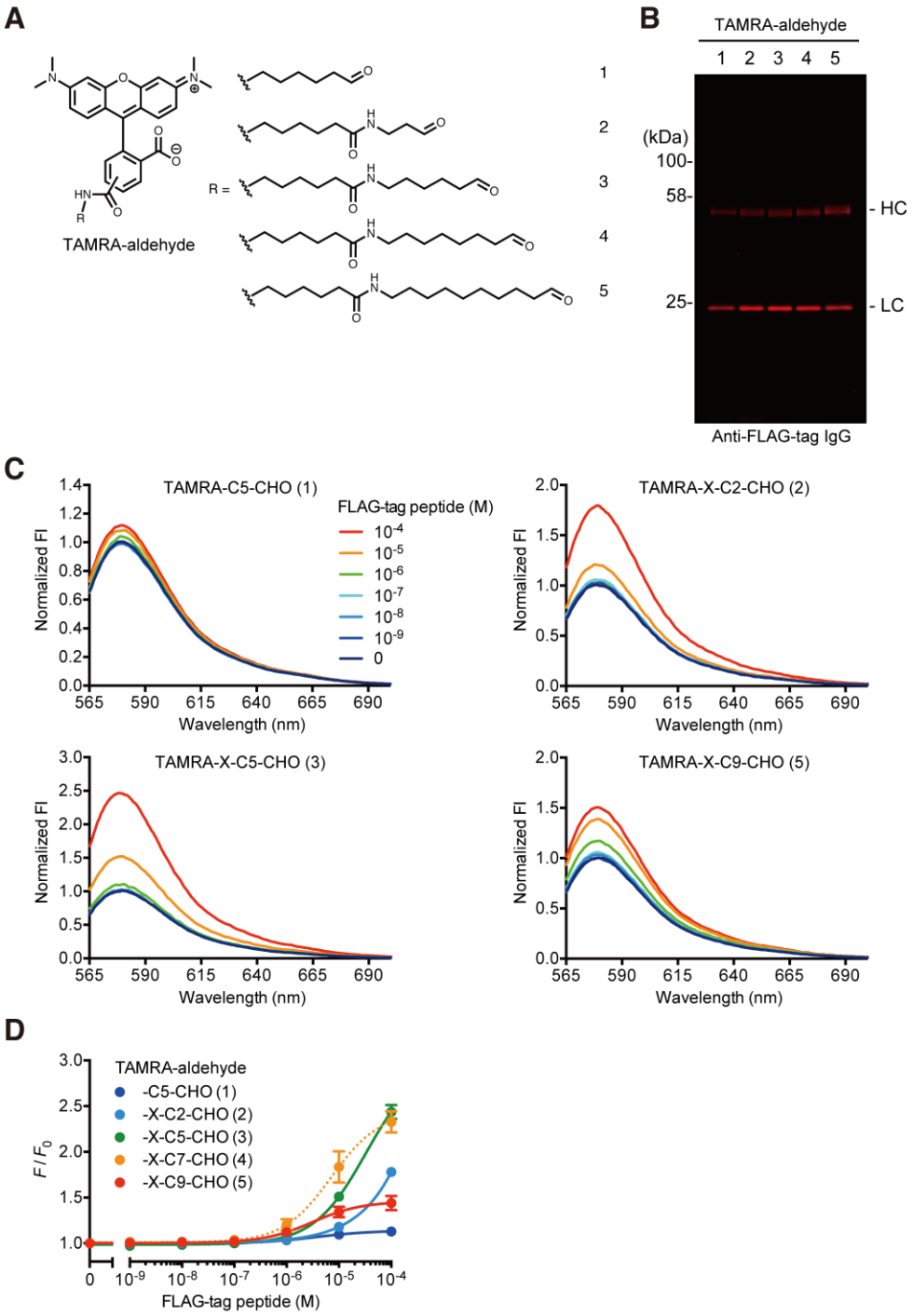


Figure S5. Optimization of linker length of TAMRA-aldehyde. (A) Chemical structures of TAMRA-aldehydes possessing different length of alkyl linkers. 1: TAMRA-C5-CHO, 2: TAMRA-X-C2-CHO, 3: TAMRA-X-C5-CHO, 4: TAMRA-X-C7-CHO, and 5: TAMRA-X-C9-CHO. (B) In-gel fluorescence imaging of SDS-PAGE of TAMRA-labeled anti-FLAG-tag IgGs after gel-filtration. (C) Fluorescence spectra of TAMRA-labeled anti-FLAG-tag IgGs in the presence of

varying concentrations of FLAG-tag peptide. Fluorescence spectra of anti-FLAG-tag IgG labeled with TAMRA-X-C7-CHO were shown in Fig. 2B. (D) Antigen-titration curves of TAMRA-labeled anti-FLAG-tag IgGs. Each data points represent the mean value ± S.D. of at least three independent experiments.

Table S1: Fluorescence responses of anti-FLAG-tag IgGs labeled with TAMRA-aldehyde possessing different length of alkyl linkers.

Entry	TAMRA-aldehyde	Fluorescent labeling ratio (mol TAMRA / IgG)	$F_{0.1\ \mathrm{mM\ FLAG}} / F_0$	Dissociation constant (M)
1	-C5-CHO	2.2 ± 0.4	1.1 ± 0.01	$K_D = (3.5 \pm 1.3) \times 10^{-6}$
2	-X-C2-CHO	2.9 ± 0.2	1.8 ± 0.01	N.D.
3	-X-C5-CHO	2.7 ± 0.1	2.4 ± 0.1	$K_D = (3.3 \pm 1.5) \times 10^{-5}$
4	-X-C7-CHO	2.6 ± 0.1	2.3 ± 0.1	$K_D = (7.2 \pm 1.3) \times 10^{-6}$
5	-X-C9-CHO	3.0 ± 0.4	1.4 ± 0.1	$K_D = (2.0 \pm 1.5) \times 10^{-6}$

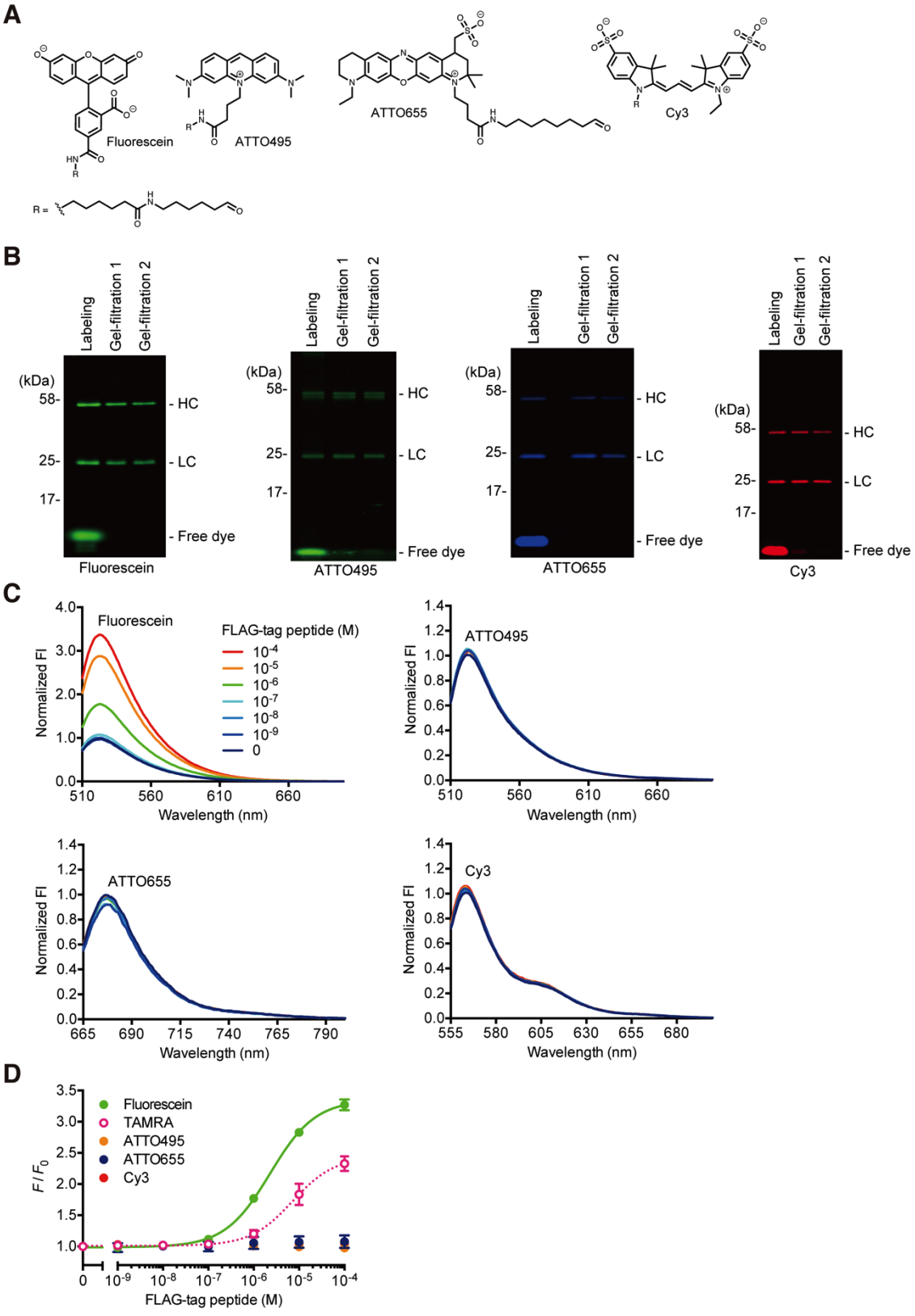


Figure S6. Fluorescence responses of anti-FLAG-tag IgGs labeled with different fluorescent dye-aldehydes. (A) Chemical structures of fluorescent dye-aldehydes. (B)

In-gel fluorescence imaging of SDS-PAGE of the fluorescently-labeled IgGs. (C) Fluorescence spectra and (D) antigen-titration curves of the fluorescently-labeled anti-FLAG-tag IgGs. Excitation wavelengths were 495 nm for fluorescein and ATTO495, 540 nm for Cy3, and 650 nm for ATTO655. Each data points represent the mean value ± S.D. of at least three independent experiments.

Table S2: Fluorescence responses of anti-FLAG-tag IgGs labeled with different fluorescent dye-aldehydes

Entry	Dye-aldehyde	Fluorescent labeling ratio (mol TAMRA / IgG)	$F_{0.1\ mM\ FLAG}$ / F_0	Dissociation constant (M)
1	Fluorescein	2.5 ± 0.5	3.3 ± 0.1	$K_D = (2.3 \pm 0.2) \times 10^{-6}$
2	ATTO495	1.1 ± 0.2	1.0 ± 0.1	N.D.
3	ATTO655	2.0 ± 0.1	1.1 ± 0.1	N.D.
4	Cy3	1.0 ± 0.1	1.0 ± 0.1	N.D.

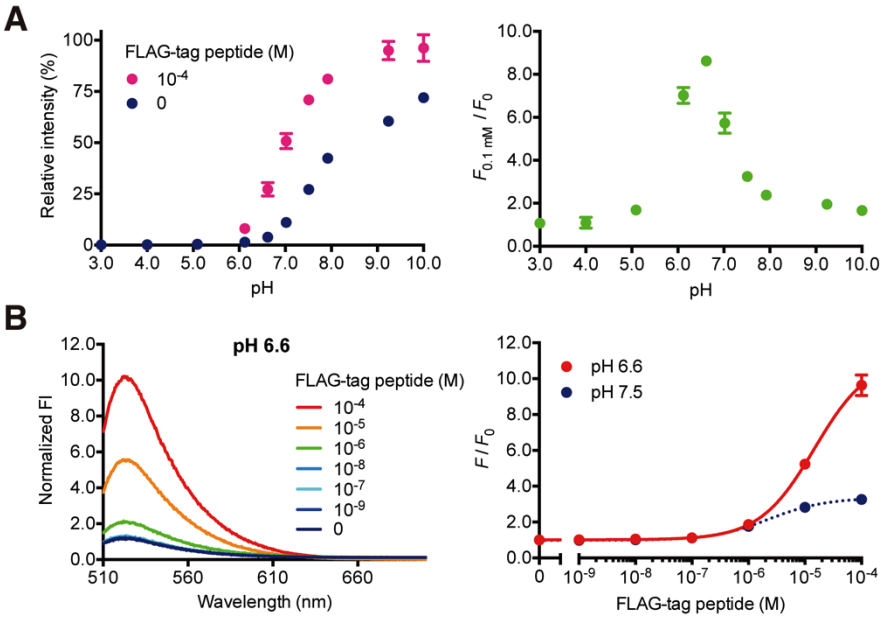


Figure S7. Fluorescence responses of fluorescein-labeled anti-FLAG-tag IgGs in the different pH conditions. (A) Relative fluorescence intensities of the antibody in the

presence or absence of FLAG-tag peptide (the left panel). Fluorescence responses in the different pH conditions (the right panel). (B) Fluorescence spectra (the left panel) and antigen-titration curve (the right panel) of fluorescein-labeled anti-FLAG-tag IgG in the pH 6.6 condition. For the comparison, the plots of neutral pH condition (pH 7.5) were also included (the same data to the Fig. S6D). The dissociation constant in the pH 6.6 condition was calculated as $K_D = (1.5 \pm 0.3) \times 10^{-5}$ M. Fluorescein was excited at 495 nm. Each data points represent the mean value ± S.D. of three independent experiments.

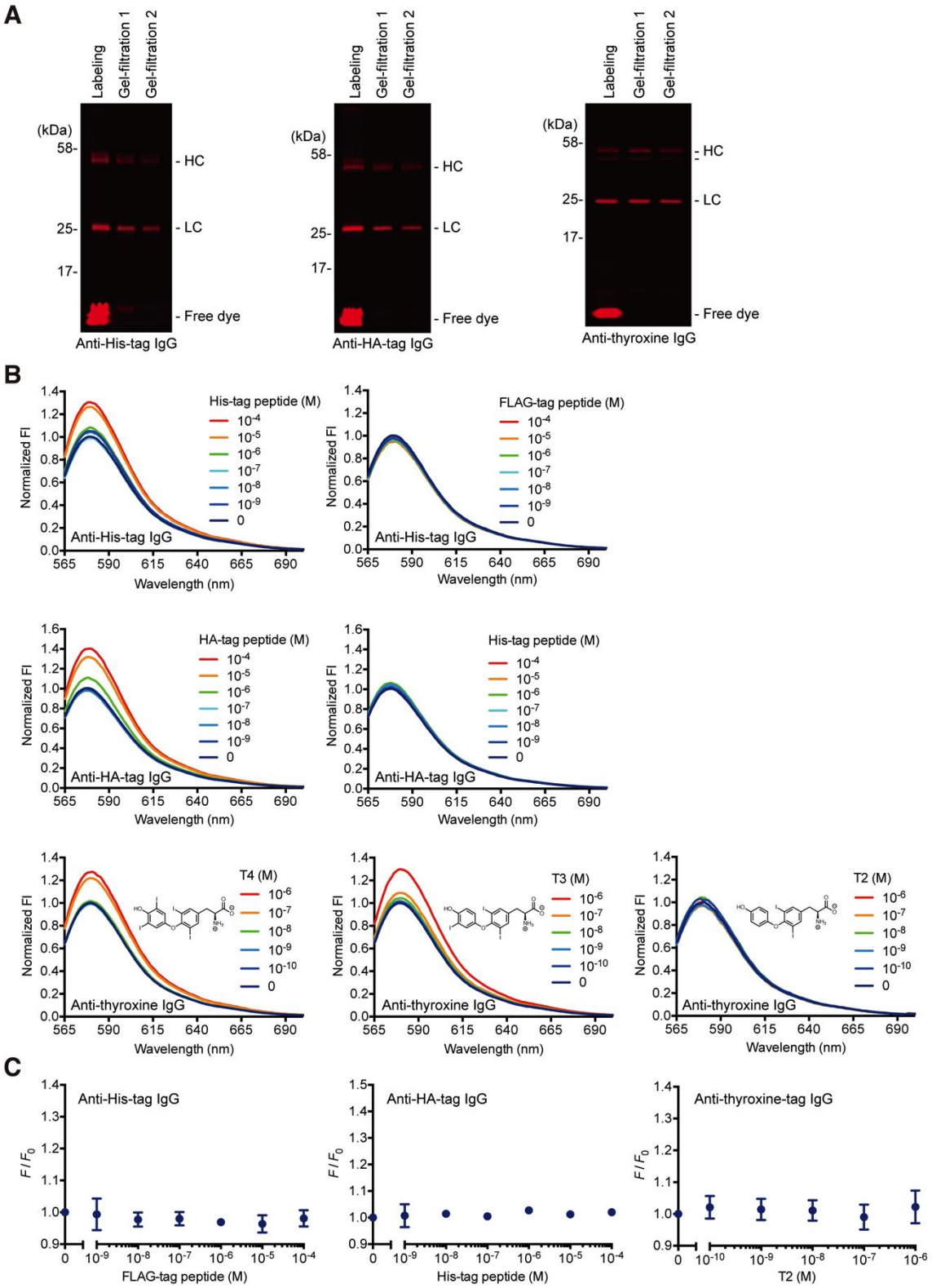
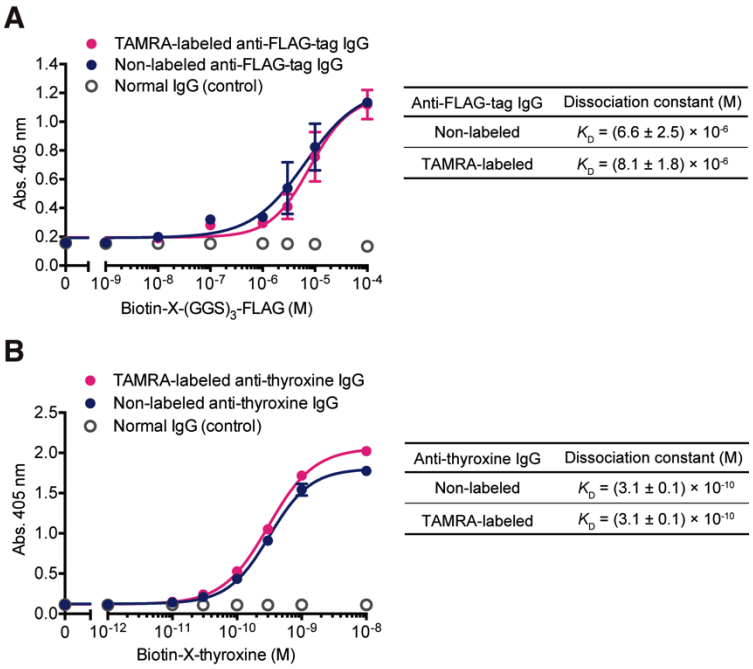


Figure S8. Fluorescence responses of anti-His-tag, anti-HA-tag, and anti-thyroxine IgGs labeled with TAMRA-X-C7-CHO. (A) In-gel fluorescence imaging of SDS-PAGE of TAMRA-labeled IgGs. (B) Fluorescence spectra of TAMRA-labeled

IgGs in the presence of the corresponding antigens and mock antigens. T4, T3, and T2 indicate thyroxine, triiodothyronine, and diiodothyronine, respectively. (C) Antigen-titration curves of TAMRA-labeled IgGs upon addition of mock antigens. Each data points represent the mean value ± S.D. of three independent experiments.



Anti-FLAG-tag IgG	Dissociation constant (M)
Non-labeled	K_D = (6.6 ± 2.5) × 10^{-6}
TAMRA-labeled	K_D = (8.1 ± 1.8) × 10^{-6}

Anti-thyroxine IgG	Dissociation constant (M)
Non-labeled	K_D = (3.1 ± 0.1) × 10^{-10}
TAMRA-labeled	K_D = (3.1 ± 0.1) × 10^{-10}

Figure S9. Comparison of binding affinities of non-labeled and TAMRA-labeled IgGs against the corresponding antigen by sandwich ELISA for (A) anti-FLAG-tag IgG and (B) anti-thyroxine IgG. Each data points represent the mean value ± S.D. of three independent experiments. Non-labeled human IgG (normal IgG) was used as a negative control.

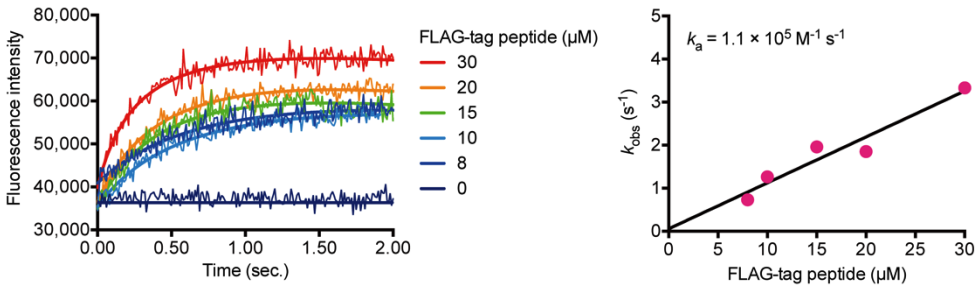


Figure S10. Time-dependent fluorescence responses of TAMRA-labeled anti-FLAG-tag IgG (the left panel) and observed reaction rate constants (k_{obs}) in the antigen binding (the right panel).

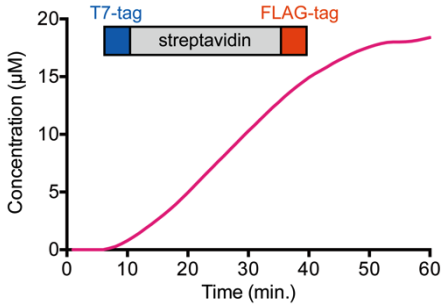


Figure S11. Quantitative real-time detection of the expression of FLAG-tagged streptavidin in a cell-free translation system. The concentration of FLAG-tagged streptavidin was calculated from a standard curve (data not shown).

Materials and methods

General information

Antibodies and antigens were obtained from the following companies. Antibodies: trastuzumab (Lot. 14K051E; Chugai Pharmaceutical), anti-FLAG-tag (clone M2, Lot. SLBR7936V; cat. No. F1804, Sigma), anti-thyroxine (clone 1H1, Lot. 15/05-T6-H1; cat. No. 2T-6, HyTest), anti-HA-tag (clone 5D8, Lot. 013; cat. No. M132-3, MBL), anti-His-tag (clone 9C11, Lot. CTN4018; cat. No. 011-23091, Wako), and normal human IgG (cat. No. 143-09501, Wako). All antibodies were supplied as a BSA-free solution. Antigens: FLAG-tag peptide (cat. 6002, APExBIO, and cat. No. 044-30951, Wako), *L*-thyroxine (T4; CAS No. 51-48-9, cat. No. T2376, Sigma), 3,3',5-triiodo-*L*-thyronine (T3; CAS No. 6893-02-3, cat. No. T0453, TCI), 3,5-diiodo-*L*-thyronine (T2; CAS No. 1041-01-6, cat. No. D1119, TCI), HA-tag peptide (cat. No. 3320-205, MBL), and His-tag peptide (cat. No. 083-09253, Wako).

Fluorescent dyes and synthetic reagents were obtained from the following companies. Fluorescent dyes: 6-(tetramethylrhodamine-5-(and-6)-carboxamido) hexanoic acid succinimidyl ester (TAMRA-X-SE; cat. No. T6105, Thermo Scientific), 5-carboxytetramethylrhodamine succinimidyl ester (TAMRA-SE; cat. No. C2211, Thermo Scientific), 6-(fluorescein-5-carboxamido) hexanoic acid succinimidyl ester (FAM-X-SE; cat. No. AS-81009, ANASPEC), ATTO 495 NHS ester (cat. No. AD495-31, ATTO-TEC), ATTO 655 NHS ester (cat. No. AT655-31, ATTO-TEC), and Cy3 NHS Ester Mono-reactive (cat. No. PA13101, GE healthcare). Synthetic reagents: 3-amino-1-propanol (CAS No. 156-87-6, cat. No. A0438, TCI), 6-amino-1-hexanol (CAS No. 4048-33-3, cat. No. A1027, TCI), 8-amino-1-octanol (CAS No. 19008-71-0, cat. No. A1522, TCI), 10-amino-1-decanol (CAS No. 23160-46-5, cat. No. A1523, TCI), Boc-*ε*-aminocaproic acid succinimidyl ester (Boc-*ε*Ahx-OSu; CAS No. 51513-80-5, cat. No. A-1155, Bachem), *N*-succinimidyl 6-biotinamidohexanoate (Biotin-Ahx-NHS ester; CAS No. 156-87-6, cat. No. A0438, TCI), Dess-Martin reagent (CAS No. 87413-09-0, cat. No. 350-39641, Wako), and borane-2-methylpyridine complex (CAS No. 3999-38-0, cat. No. B3018, TCI).

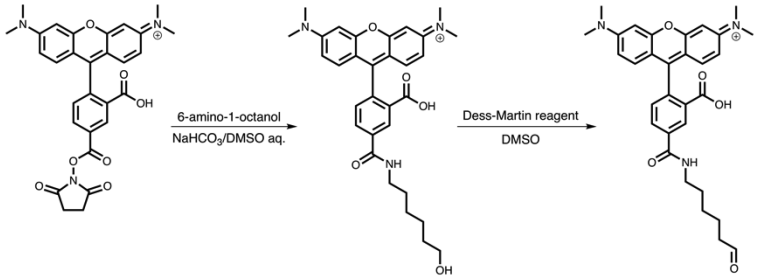
Mass spectrometric analysis was performed using a MALDI-TOF/TOF mass spectrometer (ultrafleXtreme; Bruker), which was operated in the reflector positive ion mode and externally calibrated. Matrix solution (saturated α-cyano-4-hydroxylcinnamic acid in 0.05% aqueous TFA/50% acetonitrile) was spiked with the equal volume of sample, and spotted onto a MALDI target plate. MS data were analyzed with Compass Data Analysis software v. 4.4 (Bruker). Reaction traces and purifications of synthetic molecules and peptides were performed using a Shimadzu RP-HPLC system with any condition of the following (Table S3).

Table S3: RP-HPLC conditions

Method	Column	Flow rate (mL/min)	Solvent and gradient	Detector
A	XBridge C18 column (2.5 µm, 4.6 × 20 mm; Waters)	1.5	0-100% methanol in 0.38% aqueous formic acid during 10 min.	UV
B			0-100% acetonitrile in 0.1% aqueous TFA during 10 min.	
C	XBridge Prep C18 column (5 µm, 10 × 50 mm; Waters)	3.0	0-100% acetonitrile in 0.1% aqueous TFA during 15 min.	
D	XBridge BEH C18 column (3.5 µm, 4.6 × 150 mm; Waters)	0.6	3-steps: (1) 0-20% acetonitrile in 0.1% aqueous TFA during 10 min. (2) 20-50% acetonitrile in 0.1% aqueous TFA during 60 min. (3) 50-100% acetonitrile in 0.1% aqueous TFA during 10 min.	PDA, Fl (Ex. 550 nm, Em. 580 nm)

Synthesis of fluorescent dye-aldehyde

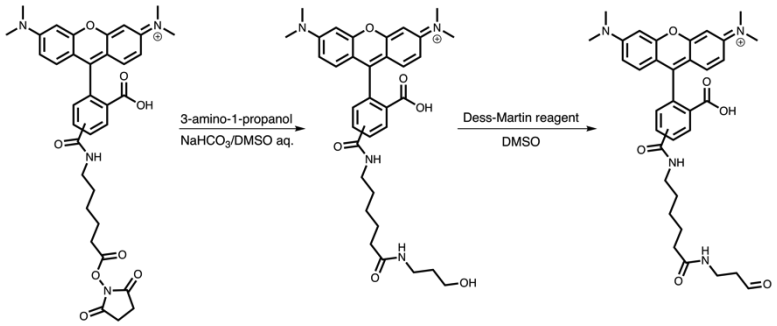
TAMRA-C5-CHO



To a mixture of 50 mM DMSO solution of 5-TAMRA-SE (30 μL, 1.50 μmol) and 100 mM DMSO solution of 6-amino-1-hexanol (75 μL, 7.50 μmol), 100 mM aqueous $NaHCO_3$ (15 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 6.7 min.) to afford TAMRA-C6-OH (1.5 μmol; 99% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 530.26 $[M+H]^+$, observed *m*/*z* value = 530.24). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.1 min.), and estimated to be 99%.

To a solution of 1 mM DMSO solution of TAMRA-C6-OH (200 μL, 0.20 μmol), 500 mM DMSO solution of Dess-Martin reagent (200 μL, 100 μmol) was added. After incubation with shaking at 37 ºC for 1.5 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3, *time was extended to 30 min.; RT = 12.2 min.) to afford TAMRA-C5-CHO (0.15 μmol; 75% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 528.25 $[M+H]^+$, observed *m*/*z* value = 528.22). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.3 min.), and estimated to be 92%.

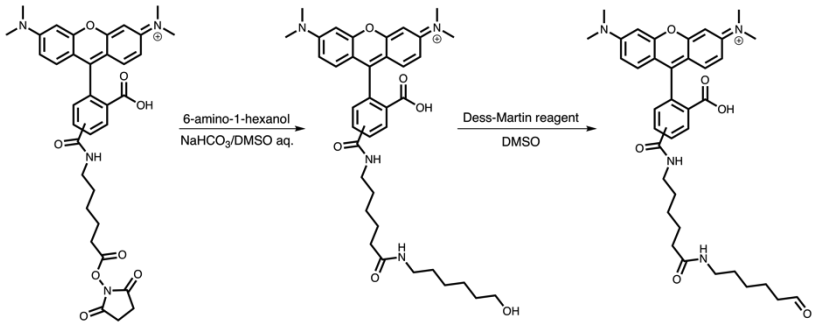
TAMRA-X-C2-CHO



To a mixture of DMSO (4 μL), 100 mM DMSO solution of 5(6)-TAMRA-X-SE (4 μL, 0.40 μmol) and 100 mM DMSO solution of 3-amino-1-propanol (40 μL, 4 μmol), 100 mM aqueous $NaHCO_3$ (8 μL) was added. After incubation on ice for 10 min., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 6.5 min.) to afford TAMRA-X-C3-OH (0.39 μmol; 98% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 601.30 $[M+H]^+$, observed *m*/*z* value = 601.31). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 6.5 min.), and estimated to be 97%.

To a mixture of DMSO (100 μL) and 2 mM DMSO solution of TAMRA-X-C3-OH (100 μL, 0.20 μmol), 100 mM DMSO solution of Dess-Martin reagent (200 μL, 20 μmol) was added. After incubation with shaking at 37 ºC for 1.5 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 6.6 min.) to afford TAMRA-X-C2-CHO (0.16 μmol; 80% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 599.29 $[M+H]^+$, observed *m*/*z* value = 599.29). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 6.6 min.), and estimated to be 95%.

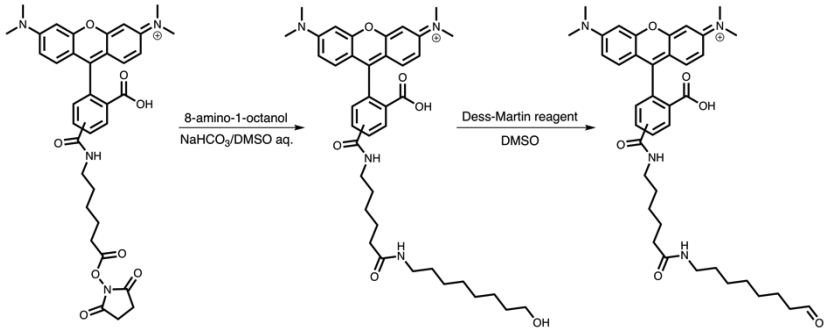
TAMRA-X-C5-CHO



To a mixture of DMSO (4 μL), 100 mM DMSO solution of 5(6)-TAMRA-X-SE (4 μL, 0.40 μmol) and 100 mM DMSO solution of 6-amino-1-hexanol (40 μL, 4 μmol), 100 mM aqueous $NaHCO_3$ (8 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C* in the Table S3, *gradient and time were changed to 25-75% and 30 min., respectively; RT = 4.8 min.) to afford TAMRA-X-C6-OH (0.15 μmol; 37% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 643.35 $[M+H]^+$, observed *m*/*z* value = 643.29). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 6.9 min.), and estimated to be 97%.

To a mixture of DMSO (55 μL) and 2 mM DMSO solution of TAMRA-X-C6-OH (55 μL, 0.11 μmol), 250 mM DMSO solution of Dess-Martin reagent (110 μL, 27.5 μmol) was added. After incubation with shaking at 37 ºC for 1.5 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.2 min.) to afford TAMRA-X-C5-CHO (0.065 μmol; 59% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 641.33 $[M+H]^+$, observed *m*/*z* value = 641.28). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 7.2 min.), and estimated to be 94%.

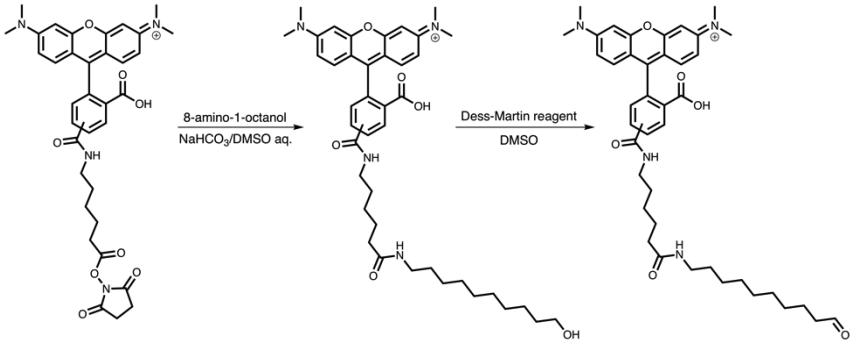
TAMRA-X-C7-CHO



5(6)-TAMRA-X-C7-CHO was synthesized in the previous study.[1] To a mixture of DMSO (15 μL), 100 mM DMSO solution of 5(6)-TAMRA-X-SE (15 μL, 1.5 μmol), and 100 mM DMSO solution of 8-Amino-1-octanol (150 μL, 15 μmol), 100 mM aqueous $NaHCO_3$ (30 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.4 min.) to afford TAMRA-X-C8-OH (1.05 μmol; 70% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 671.38 $[M+H]^+$, observed *m*/*z* value = 671.36). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.4 min.), and estimated to be 99%.

To a mixture of DMSO (250 μL) and 2 mM DMSO solution of TAMRA-X-C8-OH (250 μL, 0.50 μmol), 500 mM DMSO solution of Dess-Martin reagent (500 μL, 250 μmol) was added. After incubation with shaking at 37 ºC for 1 hr., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.8 min.) to afford TAMRA-X-C7-CHO (0.33 μmol; 65% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 669.36 $[M+H]^+$, observed *m*/*z* value = 669.34). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.6 min.), and estimated to be 94%.

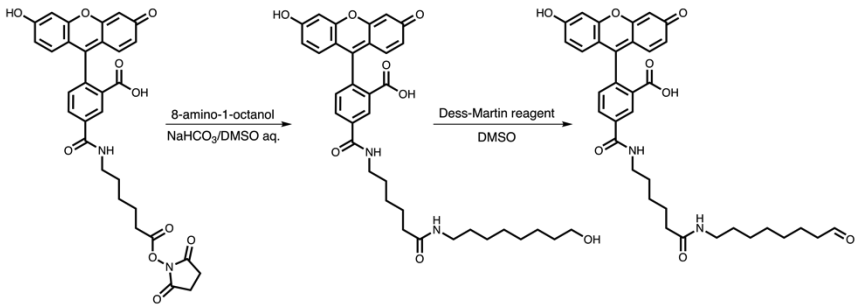
TAMRA-X-C9-CHO



To a mixture of DMSO (4 μL), 100 mM DMSO solution of 5(6)-TAMRA-X-SE (4 μL, 0.40 μmol) and 100 mM DMSO solution of 10-amino-1-decanol (40 μL, 4 μmol), 100 mM aqueous $NaHCO_3$ (8 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 8.2 min.) to afford TAMRA-X-C10-OH (0.11 μmol; 28% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 699.41 $[M+H]^+$, observed *m*/*z* value = 699.37). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 8.2 min.), and estimated to be 94%.

To a mixture of DMSO (55 μL) and 2 mM DMSO solution of TAMRA-X-C10-OH (55 μL, 0.11 μmol), 250 mM DMSO solution of Dess-Martin reagent (110 μL, 27.5 μmol) was added. After incubation with shaking at 37 ºC for 1.5 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 8.7 min.) to afford TAMRA-X-C9-CHO (0.066 μmol; 60% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 697.40 $[M+H]^+$, observed *m*/*z* value = 697.36). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 8.7 min.), and estimated to be 98%.

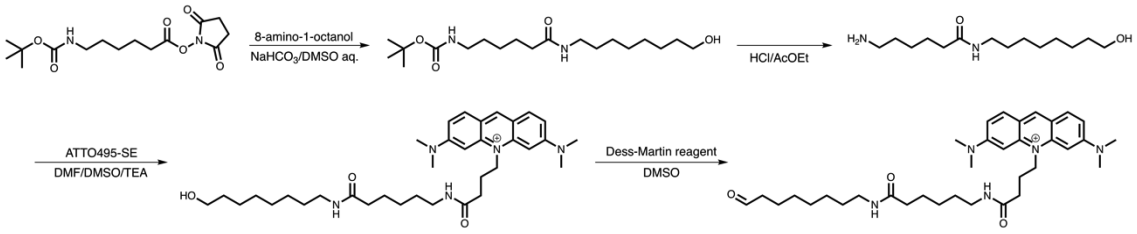
Fluorescein-X-C7-CHO



To a mixture of 100 mM DMSO solution of 5-FAM-X-SE (16 μL, 1.60 μmol) and 100 mM DMSO solution of 8-amino-1-octanol (150 μL, 15 μmol), 100 mM aqueous $NaHCO_3$ (30 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.3 min.) to afford FAM-X-C8-OH (0.60 μmol; 38% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 617.28 $[M+H]^+$, observed *m*/*z* value = 617.38). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.3 min.), and estimated to be 98%.

To a mixture of DMSO (420 μL) and 4 mM DMSO solution of FAM-X-C8-OH (140 μL, 0.56 μmol), 15 mM DMSO solution of Dess-Martin reagent (560 μL, 8.4 μmol) was added. After incubation with shaking at 37 ºC for 3.5 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.7 min.) to afford Fluorescein-X-C7-CHO (0.13 μmol; 24% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 615.26 $[M+H]^+$, observed *m*/*z* value = 615.38). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.6 min.), and estimated to be 96%.

ATTO495-X-C7-CHO



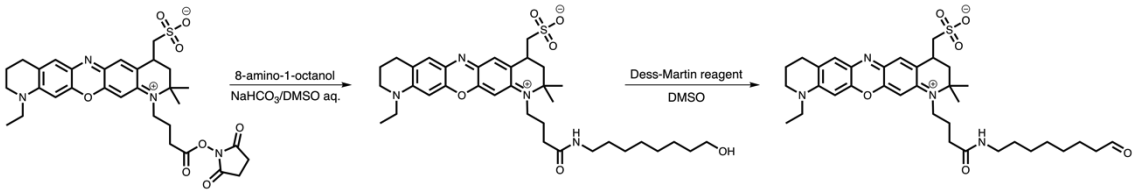
To a mixture of 100 mM DMSO solution of Boc-ε-aminocaproic acid succinimidyl ester (30 μL, 3 μmol) and 100 mM DMSO solution of 8-amino-1-octanol (150 μL, 15 μmol), 100 mM aqueous $NaHCO_3$ (40 μL) was added. After incubation on ice for a few minutes, the crude reaction product was mixed with 4% aqueous $NaHCO_3$ (200 μL) and extracted with ethyl acetate. The organic layer was acidified with 5% aqueous $KHSO_4$ (300 μL), and the crude reaction product was extracted with ethyl acetate. The organic layer was dried over magnesium sulfate. The solvent was removed by evaporation to obtain crude Boc-ε-amino-X-C8-OH. 4 M hydrochloride in ethyl acetate solution (200 μL) was added to the crude sample, and the reaction solution was incubated at room temperature for 30 min with shaking. The solvent was removed by evaporation, and then the pellet was dissolved in ethyl acetate and *n*-hexane. The solvent was removed by evaporation to obtain ε-amino-X-C8-OH as a crude product. The product was dissolved in DMSO (30 μL) for the next synthetic process.

To a mixture of DMF (10 μL), 25 mM DMSO solution of ATTO495-SE (20 μL, 0.5 μmol), and DMSO solution of ε-amino-X-C8-OH (10 μL), 1 M DMF solution of triethylamine (22.5 μL) was added. After incubation with shaking at room temperature for 15 min., 0.1% aqueous TFA and acetonitrile were added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 8.1 min.) to afford ATTO495-X-C8-OH (0.049 μmol; 10% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 592.42 $[M+H]^+$, observed *m*/*z* value = 592.36). The purity was characterized by analytical RP-HPLC (method C in the Table S3; RT = 8.1 min.), and estimated to be 92%.

To a solution of 1 mM DMSO solution of ATTO495-X-C8-OH (46 μL, 0.046 μmol), 250 mM DMSO solution of Dess-Martin reagent (46 μL, 11.5 μmol) was added. After incubation with shaking at 37 ºC for 30 min., 0.1% aqueous TFA was added to quench

the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 8.5 min.) to afford ATTO495-X-C7-CHO (0.020 μmol; 43% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 590.41 $[M+H]^+$, observed *m*/*z* value = 590.35). The purity was characterized by RP-HPLC (method C in the Table S3; RT = 8.5 min.), and estimated to be 97%.

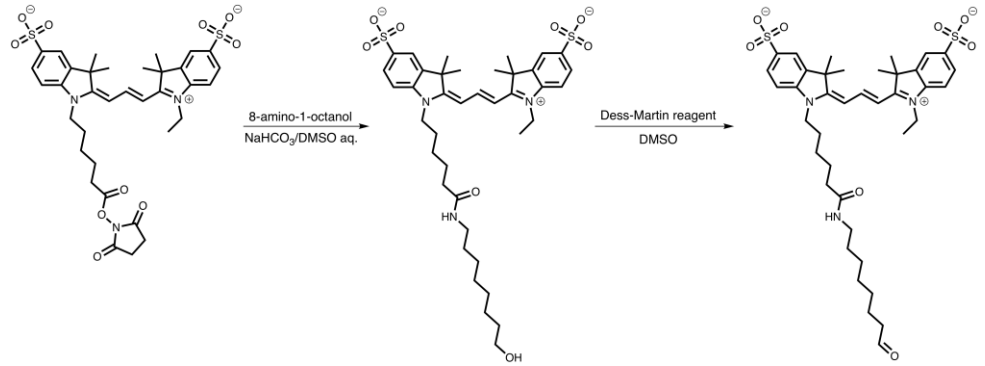
ATTO655-C7-CHO



To a mixture of 50 mM DMSO solution of ATTO655-SE (5 μL, 0.25 μmol) and 100 mM DMSO solution of 8-amino-1-octanol (25 μL, 2.5 μmol), 100 mM aqueous $NaHCO_3$ (5 μL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 6.7 min.) to afford ATTO655-C8-OH (0.34 μmol). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 655.35 $[M+H]^+$, observed *m*/*z* value = 655.39). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.0 min.), and estimated to be 95%.

To a mixture of DMSO (240 μL) and 4 mM DMSO solution of ATTO655-C8-OH (80 μL, 0.32 μmol), 100 mM DMSO solution of Dess-Martin reagent (320 μL, 32 μmol) was added. After incubation with shaking at 37 ºC for 1 hr., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 7.0 min.) to afford ATTO655-C7-CHO (0.30 μmol; 93% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 653.34 $[M+H]^+$, observed *m*/*z* value = 653.40). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 4.2 min.), and estimated to be 98%.

Cy3-C7-CHO



To a mixture of 50 mM DMSO solution of Cy3-SE (10 µL, 0.50 µmol) and 100 mM DMSO solution of 8-amino-1-octanol (50 µL, 5 µmol), 100 mM aqueous $NaHCO_3$ (10 µL) was added. After incubation on ice for a few minutes, 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 5.5 min.) to afford Cy3-C8-OH (0.08 µmol; 17% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 758.35 $[M+H]^+$, observed *m*/*z* value = 758.37). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 3.5 min.), and estimated to be 93%.

To a mixture of DMSO (57 µL) and 4 mM DMSO solution of Cy3-C8-OH (19 µL, 0.076 µmol), 100 mM DMSO solution of Dess-Martin reagent (76 µL, 7.6 µmol) was added. After incubation with shaking at 37 ºC for 2 hrs., 0.1% aqueous TFA was added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 5.7 min.) to afford Cy3-C7-CHO (0.064 µmol; 84% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 756.33 $[M+H]^+$, observed *m*/*z* value = 756.37). The purity was characterized by analytical RP-HPLC (method B in the Table S3; RT = 3.6 min.), and estimated to be 90%.

Fluorescent labeling of antibody

Prior to the labeling, antibody storage buffer was replaced with a labeling buffer (50 mM citrate-Na, pH 4.8, 100 mM KCl, 0.1% polyethylene glycol 8000, and 0.05% polyoxyethylene(23) lauryl ether) using an ultrafiltration device (Nanosep, 30 kMWCO; cat. No. OD030C34, PALL). It should be noted that this buffer exchange step is very important to eliminate unfavorable components of antibody storage buffer (*e.g.*, Tris, glycerol, and NaN_3 etc.). Typically, 300 μL of the labeling buffer and 9 μg of IgG antibody (approximately 60 pmol) were added onto the devise, and then the devise was centrifuged at 8,000 *g* for 8-10 min. at room temperature.

Reagents were mixed in order in the 1.5 mL Protein LoBind Tube (cat. No. 0030108116, Eppendorf), which was put on ice. First, purified antibody (9 μg) was mixed with the labeling buffer so that the volume was reached to 21.37 μL. Second, 1.13 μL of 1.92 mM dye-aldehyde was added. Dye-aldehyde was prepared in advance with 50% DMSO aq. Finally, 4.5 μL of 24 mM picoline borane was added. From 1,067 mM DMSO stock solution of the picoline borane, 24 mM picoline borane solution was prepared with ultrapure water just before the mixing. The diluted 24 mM picoline borane solution was strongly vortexed for a short time before use. The reductive alkylation reaction was carried out at 4 ºC for 24 hrs. in the 27 μL scale. The final concentrations of antibody and picoline borane were 2.2 μM and 4 mM, respectively, and those of fluorescent dye-aldehydes were summarized in Table S4. It should be noted that the concentration of dye-aldehydes must be optimized to maintain the labeling ratio, possibly because the linkers and dyes might sterically affect the labeling reaction.

Table S4: Concentrations of fluorescent dye-aldehyde used for the labeling reaction

Fluorescent dye-aldehyde	Final concentration (μM)
TAMRA-C5-CHO	240
TAMRA-X-C2-CHO	160
TAMRA-X-C5-CHO	80
TAMRA-X-C7-CHO	80
TAMRA-X-C9-CHO	80

Fluorescein-X-C7-CHO	80
ATTO495-X-C7-CHO	160
ATTO655-C7-CHO	160
Cy3-C7-CHO	160

After 24 hours incubation, unconjugated dye was removed with a Sephadex G50-loaded gel filtration spin column (EconoSpin; cat. No. EP-31401, Gene Design) for two times. The spin column was centrifuged at 2 krpm at room temperature for 2 min. The gel was equilibrated with an equilibration buffer (20 mM phosphate-Na, pH 7.5, 100 mM NaCl, 0.1% polyethylene glycol 8000, and 0.05% polyoxyethylene(23) lauryl ether) (300 μL × 5 times). Fluorescent labeling degree was determined by absorption measurement followed by a calculation using the following parameters (Table S5).

Table S5: Molecular excitation coefficients and correction factors

Molecule	Molar excitation coefficient (M^{-1} cm^{-1})	Correction factor: $CF_{280\ nm}$
Trastuzumab	ε = 225,000 [2]	-
IgG antibodies excepting trastuzumab	ε = 204,790	-
TAMRA	ε = 92,000 [3]	0.30
Fluorescein	ε = 78,000 (at pH 9) [3]	0.30
ATTO495	ε = 80,000 [3]	0.39
ATTO655	ε = 125,000 [3]	0.08
Cy3	ε = 150,000 [3]	0.08

SDS-PAGE and in-gel fluorescence imaging

Antibody solutions were dissolved in an SDS sample buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 5 mM

ethylenediaminetetraacetic acid, and 100 mM dithiothreitol). The solutions were incubated at 95 °C for 5 min. before the electrophoresis. The samples were resolved by a home-made 15% polyacrylamide gel or a ready-made 12.5% one (SuperSep Ace; cat. 199-14971, Wako). After the electrophoresis, fluorescently-labeled IgGs were visualized by in-gel fluorescence imaging using an fluoroimager (FMBIO-III; Hitachi Software Engineering) as reported previously.[4] TAMRA and Cy3 were excited at 532 nm and visualized at 580 nm. Fluorescein and ATTO495 were excited at 532 nm and visualized at 505 nm. ATTO655 and prestained protein maker were excited at 635 nm and visualized at 670 nm.

Trace of fluorescent labeling reaction

To quench the reaction and avoid side-reactions during the sample preparation, an aromatic amine was used as a quencher of aldehyde because aldehyde preferentially reacts with an aromatic amine.[1] 4-Aminobenzyl alcohol (CAS No. 623-04-1, cat. No. A1096, TCI) was added to the collected sample at a final concentration of 3 mM.

MS analysis of trypsinized trastuzumab

As described in the above section (fluorescent labeling of antibody), trastuzumab was labeled with TAMRA-X-C7-CHO. The fluorescently-labeled IgG was purified by gel-filtration purification, and then the buffer was removed by acetone precipitation. After rinse with cold 70% aqueous acetone, the precipitate was mixed with 30 μL of a digestion buffer (50 mM ammonium bicarbonate, pH 8) supplemented with 20 mM dithiothreitol and 0.05% RapiGest SF (cat. No. 186001860, Waters). The mixture was incubated at 95 ºC for 10 min to reduce disulfide bonds and denature the protein. After cooling to room temperature, cysteine thiol-groups were alkylated with 35 mM iodoacetamide at room temperature for 30 min. Unreacted iodoacetamide was quenched by the addition of 15 mM dithiothreitol. Digestion was carried out with modified trypsin/lysyl endopeptidase (cat. No. V5071, Promega) at 37 ºC overnight. After the incubation, TFA was added at a final concentration of 1% to quench the reaction. Peptides were analyzed and purified with RP-HPLC system in the method D (see the

Table S2). The purified products were lyophilized, dissolved in 0.1% aqueous TFA/50% acetonitrile, and then subjected to MS and MS/MS analyses. Amino acid sequence of trastuzumab and the identified TAMRA-labeled peptides (Fig. S2) were shown in the below. Underlined characters indicate the identified peptides. Asterisks (*) and slashes (/) indicate TAMRA-labeled amino groups and cleavage-sites by trypsin/lysyl endopeptidase, respectively.

>Trastuzumab_light chain

*DIQMTQSPSSLSASVGDR/VTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRF
SGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKS
GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK
/HK*VYACEVTHQGLSSPVTK/SFNRGEC

>Trastuzumab_heavy chain

*EVQLVESGGGLVQPGGSLR/LSCAASGFNIK*DTYIHWVR/QAPGKGLEWVARIYPTNGYTR/
YADSVK*GR/FTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVF
LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVS
LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK

Fluorescence spectral measurement

Fluorescently-labeled IgG was diluted with a measurement buffer (20 mM phosphate-Na, pH 7.5, 100 mM NaCl, 0.1% polyethylene glycol 8000, and 0.005% polyoxyethylene(23) lauryl ether) to 1:100 ratio. After addition of antigen at desired concentrations, the mixture was incubated at 25 °C for several minutes. The mixture was added into a 5 × 5 mm quarts cell, and fluorescence spectrum was acquired using a fluorescent spectrophotometer (Fluorolog-III; Horiba) at 25 °C as described previously.[5] Excitation and emission bandwidths were set to 5.0 nm. Excitation wavelength and recorded spectrum range were summarized in the below (Table S6).

Table S6: Excitation and emission wavelengths used in this study

Fluorescent dye	Excitation (nm)	Recorded range (nm)	Emission maximum (nm)
TAMRA	550	565-700	579
Fluorescein	495	510-700	524
ATTO495	495	510-700	522
IC3	540	555-700	562
ATTO655	650	665-800	678

Fluorescence intensity change ratio (F/F_0) was calculated from fluorescence intensities at emission maximum (Table S2). Data plots were generated using GraphPad Prism software 6.0 (GraphPad Software). Dissociation constant value (K_D) was calculated by a non-linear curve fitting.

pH-dependent fluorescence response of fluorescein-labeled anti-FLAG-tag antibody

pH-dependent fluorescence response of fluorescein-labeled anti-FLAG-tag IgG was examined using the following measurement buffers: 20 mM citrate-Na for pH 3.0, pH 4.0, and pH 5.1; 20 mM phosphate-Na for pH 6.1, pH 6.6, and pH 7.9; 20 mM carbonate-Na for pH 9.2 and pH 10.0. All the buffers contained 100 mM NaCl, 0.1% polyethylene glycol 8000, and 0.005% polyoxyethylene(23) lauryl ether.

Preparation of mRNA

pGSF vector encoding T7-streptavidin-FLAG was constructed by site-directed mutagenesis from pGSH vector[4] using mutagenic primers (forward: 5'-GCTGTTCAGCAAGACTACAAGGACGACGATGACAAGTAATAAAAGCTTGAG-3', reverse: 5'-CTCAAGCTTTTATTACTTGTCATCGTCGTCCTTGTAGTCTTGCTGAACAGC-3'). DNA fragment from T7 promoter to T7 terminator was amplified from the pGSF

vector by polymerase chain reaction using primers (forward: 5'-CCCGCGCGTTGGCCGATTCA-3', reverse: 5'-ATGCTAGTTATTGCTCAGCGG-3'). Messenger RNA was prepared by *in vitro* transcription using T7 RNA polymerase (cat. No. TRL-201, TOYOBO). Amino acid sequence of T7-streptavidin-FLAG was shown in the below (italic: T7-tag; underlined: FLAG-tag).

>T7-streptavidin-FLAG

*MASMTGGQQMG*TEFHMDPSKDSKAQVSAAEAGITGTWYNQLGSTFIVTAGADGALTGTYE SAVGNAESRYVLTGRYDSAPATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEA RINTQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAASIDAAKKAGVNNGNPLDAVQQDYK DDDDK

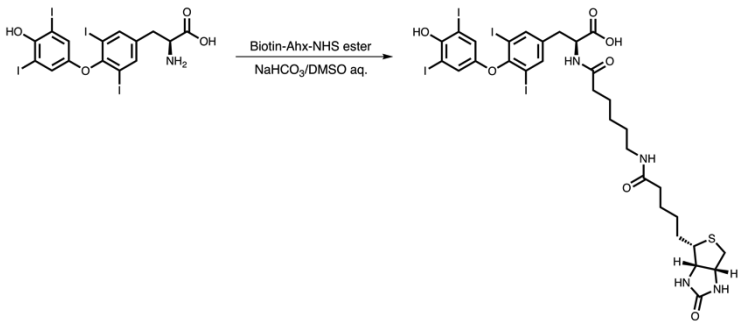
Cell-free translation and real-time monitoring of the translation reaction

Cell-free translation was performed as described previously[4] with minor modifications. In brief, the translation reaction was carried out in the 20 μL scale. The final concentrations of reagents in the translation mixture were as follows: 55 mM HEPES-KOH (pH 7.5), 210 mM *L*-glutamic acid potassium salt, 6.9 mM ammonium acetate, 12 mM magnesium acetate, 1.2 mM adenosine triphosphate, 0.28 mM guanosine triphosphate, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9 % polyethylene glycol 8000, 35 μg/mL folic acid, 0.1 mM 19 kinds of *L*-amino acids excepting arginine, 0.01 mM *L*-arginine, 2 mM oxidized glutathione, 0.8 μg/μL mRNA, 20% *E. coli* S30 extract (cat. No. L1030, Promega), and 50 nM TAMRA-labeled anti-FLAG tag antibody. These reagents excepting mRNA were mixed in a 1.5 mL microcentrifuge tube on ice. Just after addition of mRNA, 20 μL of the mixture was transferred onto a microplate (cat. No. 30132700, Eppendorf). Then, the microplate was sealed with a sealing film (cat. No. 0030132947, Eppendorf), and immediately incubated at 37 °C using a real-time PCR machine (Mx3005P; Stratagene) equipped with appropriate filters (545 nm for excitation and 568 nm for emission). Fluorescence was monitored at an interval of one minute.

Western blot analysis

Western blot analysis was performed as reported previously.[4] Proteins were resolved by 15% SDS-PAGE, and then transferred onto a polyvinylidene difuoride membrane (Immun-Blot PDVF Membrane; Bio-Rad) with a semidry blotter (Trans-Blot Semi-Dry Electrophoretic Transfer Cell; Bio-Rad). After blocking with 5% BSA-supplemented TBS-T buffer, the blot was incubated with anti-FLAG-tag mouse monoclonal antibody (1:5,000 dilution), followed by incubation with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:10,000 dilution; cat. No. S3721, Promega). After several washes, the blot was incubated with Western blue stabilized substrate (cat. No. S3841, Promega). The colored blot was scanned with a conventional scanner.

Synthesis of biotinylated-thyroxine



To a mixture of 50 mM DMSO solution of *L*-thyroxine (20 μL, 1.0 μmol) and 100 mM one of biotin-Ahx-NHS ester (20 μL, 2.0 μmol), 100 mM aqueous $NaHCO_3$ (40 μL) was added on ice. After incubation at room temperature for a few minutes, 0.1% aqueous TFA and acetonitrile were added to quench the reaction. The desired product was purified with a preparative scale RP-HPLC (method C in the Table S3; RT = 8.9 min.) to afford biotin-X-thyroxine (0.42 μmol; 42% yield). The product was identified by MALDI-TOF-MS (calculated *m*/*z* value = 1116.85 $[M+H]^+$, observed *m*/*z* value = 1116.89). The purity was characterized by analytical RP-HPLC (method A in the Table S3; RT = 7.9 min.), and estimated to be 90%.

ELISA

The biotinylated FLAG-tag peptide (biotin-Ahx-GGSGGSGGS-DYKDDDDK, 96% purity) was synthesized and characterized by GenScript. Protein G-coated 96-well plate (cat. No. 15131, Thermo Fisher Scientific) was rinsed with PBS-B buffer (PBS supplemented with 0.05% polyoxyethylene lauryl ether). IgG antibody was diluted with blocking buffer (PBS-B supplemented with 0.5% BSA), and 100 μL of the diluted solution (1 ng/μL) was applied to the well. For immobilization of IgG, the plate was incubated for 1 hr. at room temperature with shaking. After three times washing with the PBS-B buffer, 100 μL of biotinylated-antigen was added, and the plate was incubated for 1 hr. with shaking. The plate was washed three times, and then antigen-bound IgG was incubated with alkaline phosphatase-conjugated streptavidin (1:1,000 dilution, cat. No. V5591, Promega) for 30 min. After three times washing, 100 μL of *p*-nitrophenyl phosphate solution (cat. No. 37621, Thermo Fisher Scientific) was added. The plate was incubated for 10 min. at room temperature with shaking, and then absorbance at 405 nm was measured using a microplate reader (Multiskan GO with incubator; Thermo Fisher Scientific). Dissociation constant (K_D) value was determined as described in the fluorescence spectral measurement section.

It is worth noting that biotin-Ahx-DYKDDDDK, which is lacking a peptide spacer did not work in the sandwich ELISA (data not shown). This was presumably due to a steric hindrance between anti-FLAG-tag antibody and streptavidin.

Protein structural models

Structural models of mouse IgG_1 monoclonal antibody (used in the graphical abstract, Scheme 1, and Fig. 4A, PDB ID: 1IGY)[6], streptavidin (used in the Fig. 4A, PDB ID: 1STP)[7], and trastuzumab Fab region (used in the Fig. S3, PDB ID: 1N8Z)[8] were generated by Discovery Studio Visualizer software (BIOVIA).

Abbreviations

Abs: absorption

BOC: *tert*-butoxycarbonyl group

BSA: bovine serum albumin

DMF: *N*, *N*-dimethyl formamide

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

Fl: fluorescence

HA: hemagglutinin

HC: heavy chain

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IgG: immunoglobulin

LC: light chain

MALDI: matrix assisted laser desorption ionization

MS: mass spectrometry

NHS: *N*-Hydroxysuccinimide

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PDA: photodiode array

RhG: rhodamine green

Pic-BH_3: picoline borane

RP-HPLC: reverse-phase high-performance liquid chromatography

RT: retention time

S.D.: standard deviations

SDS: sodium dodecyl sulfate

SE: *N*-succinimidyl ester

TAMRA: tetramethylrhodamine

TBS: tris-buffered saline

TFA: trifluoroacetic acid

TOF: time-of-flight

Tris: tris(hydroxymethyl)aminomethane

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