Supporting Information for:

Ligand-directed Acyl Imidazole Chemistry for Labeling of Membrane-bound Proteins on Live Cells

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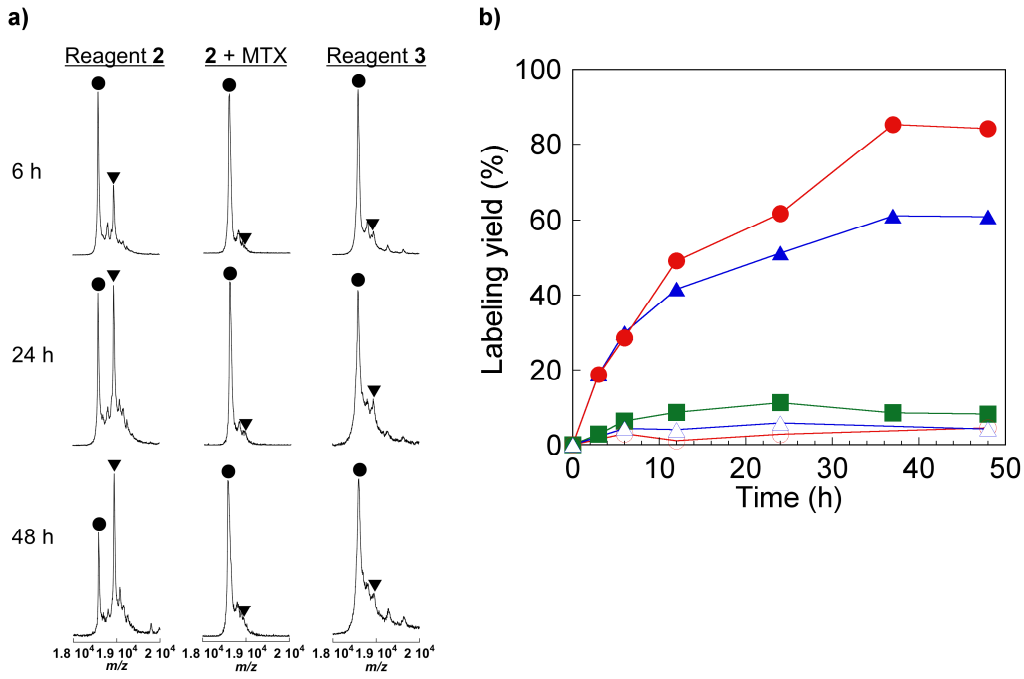


Figure S1. Analysis of the DHFR labeling *in vitro* by LDAI chemistry. (**a**) MALDI-TOF MS analysis of the labeling reaction of DHFR (25 μM) with 50 μM of LDAI reagent **2** (left), **2** in the presence of 500 μM MTX (center), or LDT reagent **3** (right) in 100 mM phosphate (pH 6.0) at 37 ˚C. The peaks of unlabeled DHFR and singly labeled DHFR are marked with ● and ▼, respectively. (**b**) Time-trace plot of the labeling reaction with **1**(●), **2**(▲), **3**(■), **1** in the presence of MTX(○), or **2** in the presence of MTX(△).

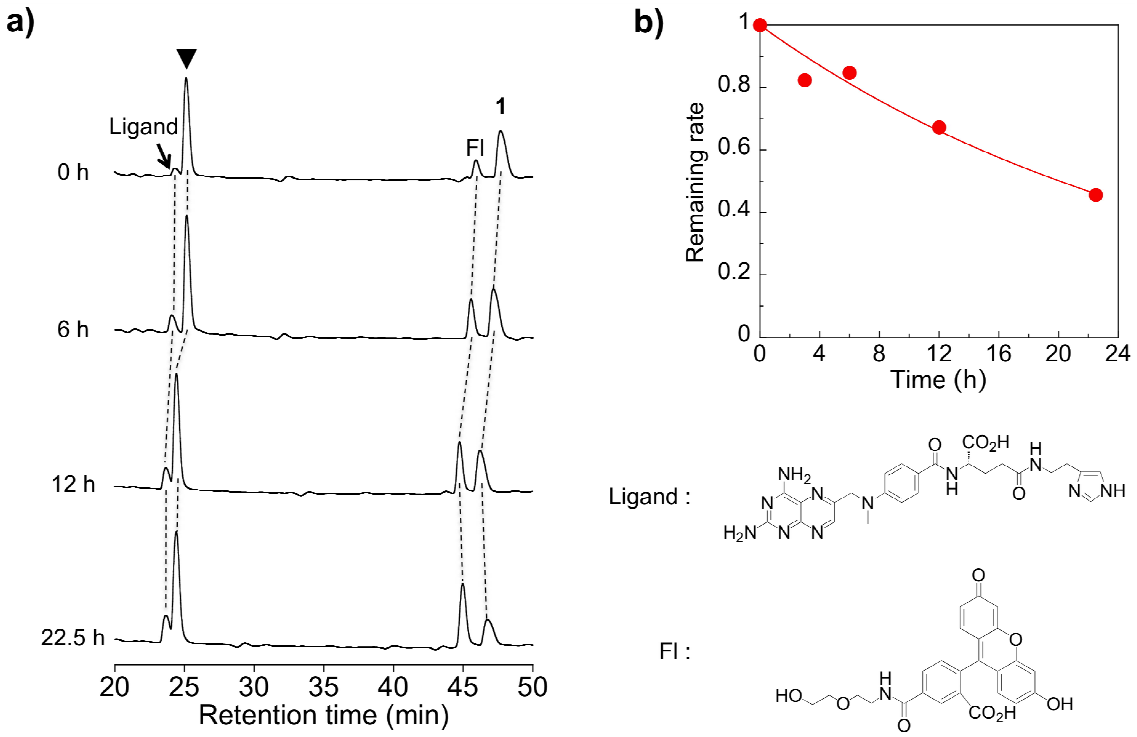


Figure S2. Evaluation of the hydrolysis stability of LDAI reagent under the aqueous condition. A solution of LDAI reagent **1** (10 μM) in aqueous buffer solution (100 mM phosphate, pH 6.0) was incubated at 37 ˚C. (**a**) HPLC analysis of the hydrolysis of **1** (column; 5C18-AR-II (COSMOSIL®), 250 mm × 4.6 mm, mobile phase; MeOH (no TFA) : H_2O (containing 0.1% TFA) = 0 : 100 → 50 : 50 (linear gradient over 40 min), flow rate; 1.0 mL/min, detection; UV (220 nm)). The peak marked with (▼) corresponds to trimethoprim (20 μM) as an internal standard. The structures of "Ligand" and "Fl" generated by the hydrolysis of **1** are shown in the lower right side. (**b**) Time-trace plot of the remaining rate of **1** under the aqueous buffer (100 mM phosphate, pH = 6.0) at 37 ˚C (●).

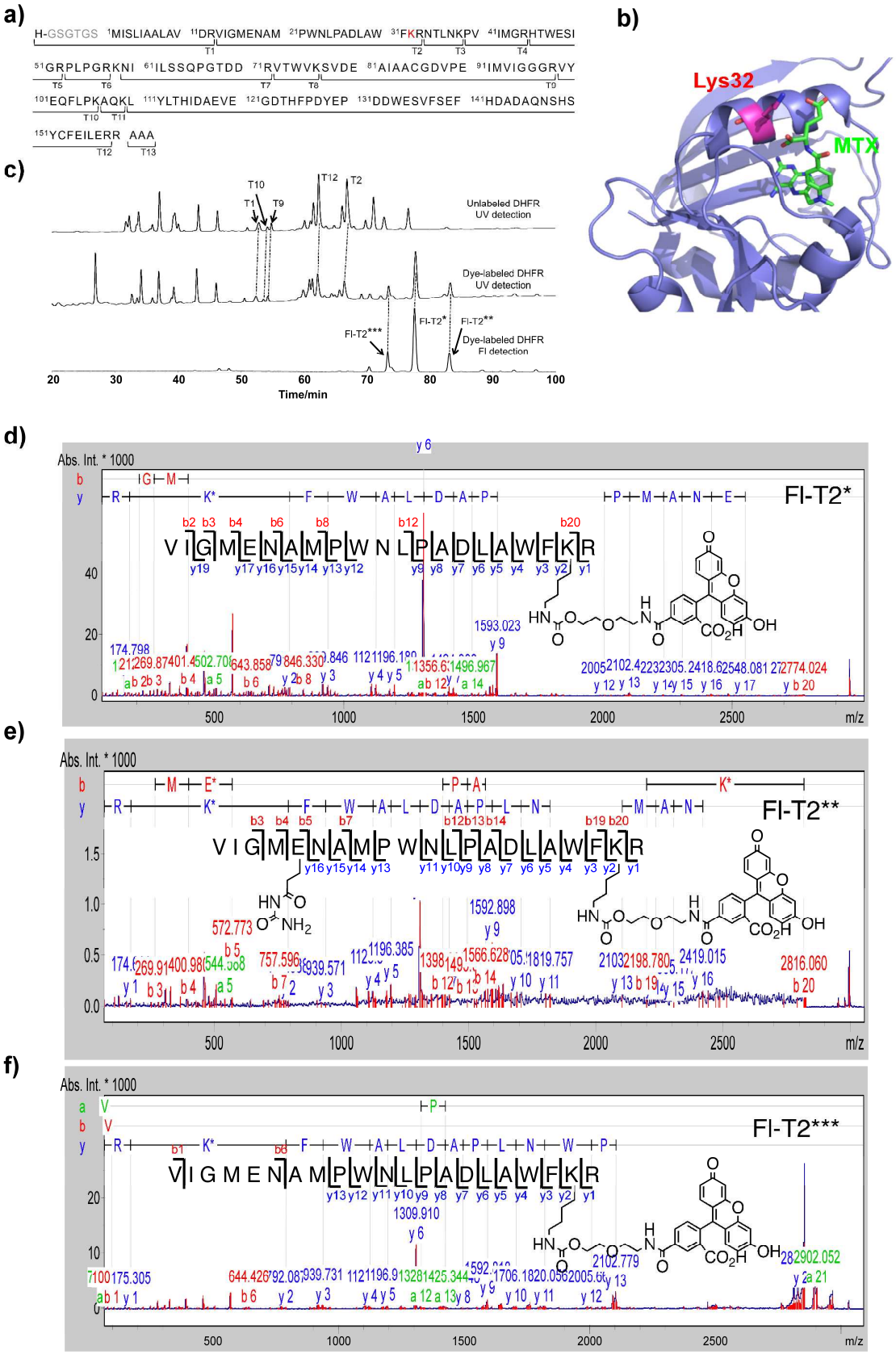

a)
H-GSGTGS 1MISLIAALAV 11DRVIGMENAM 21PWNLPADLAW 31FKRNTLNKPV 41IMGRHTWESI
T1 T2 T3 T4
51GRPLPGRKNI 61ILSSQPGTDD 71RVTWVKSVDE 81AIAACGDVPE 91IMVIGGGRVY
T5 T6 T7 T8 T9
101EQFLPKAQKL 111YLTHIDAEVE 121GDTHFPDYEP 131DDWESVFSEF 141HDADAQNSHS
T10 T11
151YCFEILERR AAA
T12 T13
b)
Lys32
MTX
c)
T10
T1
T9
T12
T2
Unlabeled DHFR
UV detection
Dye-labeled DHFR
UV detection
Fl-T2***
Fl-T2*
Fl-T2**
Dye-labeled DHFR
Fl detection
20 30 40 50 60 70 80 90 100
Time/min
d)
Abs. Int. * 1000
Fl-T2*
e)
Abs. Int. * 1000
Fl-T2**
f)
Abs. Int. * 1000
Fl-T2***
500 1000 1500 2000 2500
m/z

Figure S3. Mass spectral analysis of the labeling site of DHFR with LDAI reagent **1**. (**a**) The primary sequence of DHFR and the predictable fragments generated by trypsin digestion. The extra amino acids shown in gray (GSGTGS) are derived from the expression vector. The amino acid labeled with **1** is shown in red. (**b**) Crystal structure of DHFR protein complexed with MTX (PDB ID: 3DAU). The amino acid (Lys32) modified by **1** is highlighted by a colored stick model. (**c**) HPLC analysis of the digested fragments of DHFR. The top figure shows the chromatogram of the fragments derived from unlabeled native DHFR (UV detection at 220 nm). The middle and bottom figures show the chromatogram of the fragments derived from the labeled DHFR detected with UV absorption (220nm) and fluorescence emission (λ_{em} = 530 nm, λ_{ex} = 495 nm). MALDI-TOF MS (CHCA): peak Fl-T2*; calcd. for $[M+H]^+$ = 2948.34, obsd. = 2950.31, peak Fl-T2**; obsd. 2992.42, peak Fl-T2***; obsd. 3025.53. (**d**) MALDI-TOF MS/MS analysis of the Fl-T2* fragment labeled at Lys32. Peak Fl-T2** was assigned to be a T2 fragment modified at Lys32 and Glu17 with a fluorescein dye and urea, respectively (**e**). The amino acid sequence of peak Fl-T2*** was also assigned to possess the dye-labeled Lys32 by MS/MS analysis (**f**).

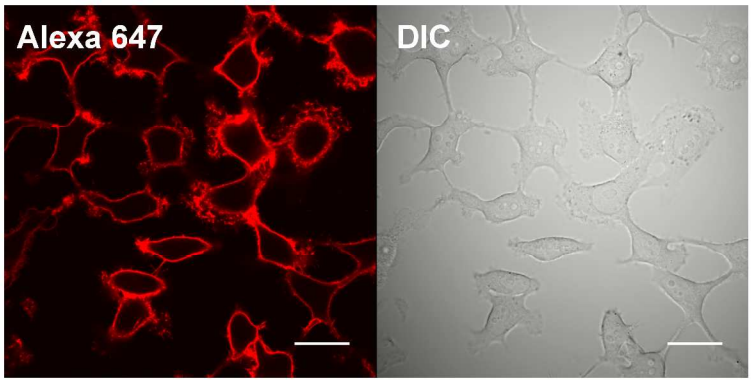


Figure S4. Competitive washing experiment of the labeled FR on KB cells by solution containing folic acid (FA). KB cells were treated with LDAI reagent **2** (1 μM, 24 h at 37 °C) followed by washing with 25 μM of FA. Fluorescence image was obtained after treatment with the fluorescent SAv647. Scale bars; 30 μm.

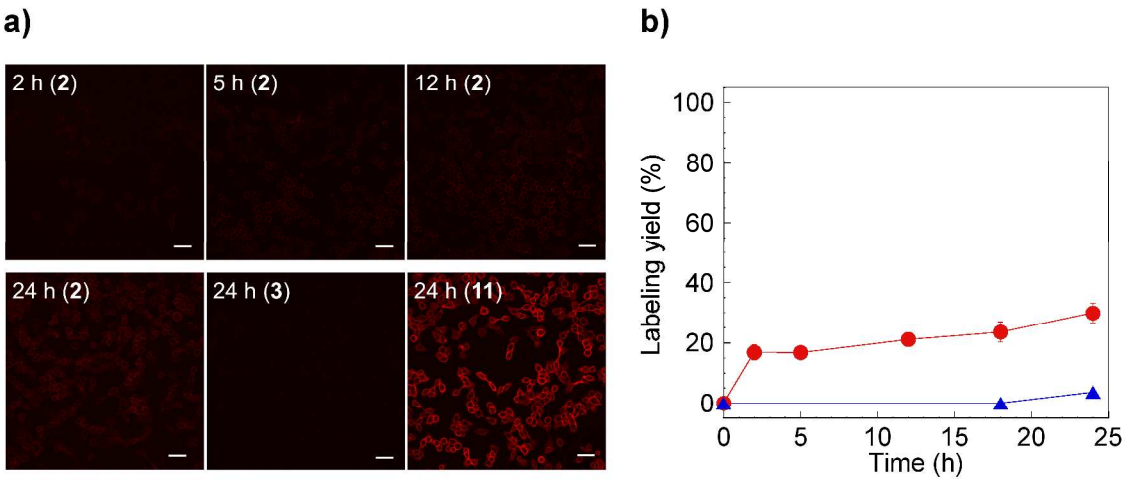


Figure S5. Evaluation of the labeling efficiency of FR on live cells. (**a**) Fluorescence images of KB cells upon labeling with LDAI reagent **2**, LDT reagent **3** or biotin-modified folic acid **11** (1 μM) in RPMI 1640 (folate free, 10% FBS) at 37 ˚C for 24 h. Images were obtained after treatment with the fluorescent SAv647. Scale bars, 50 μm. (**b**) Time-trace plot of the FR labeling with **2** (●) or **3** (▲).

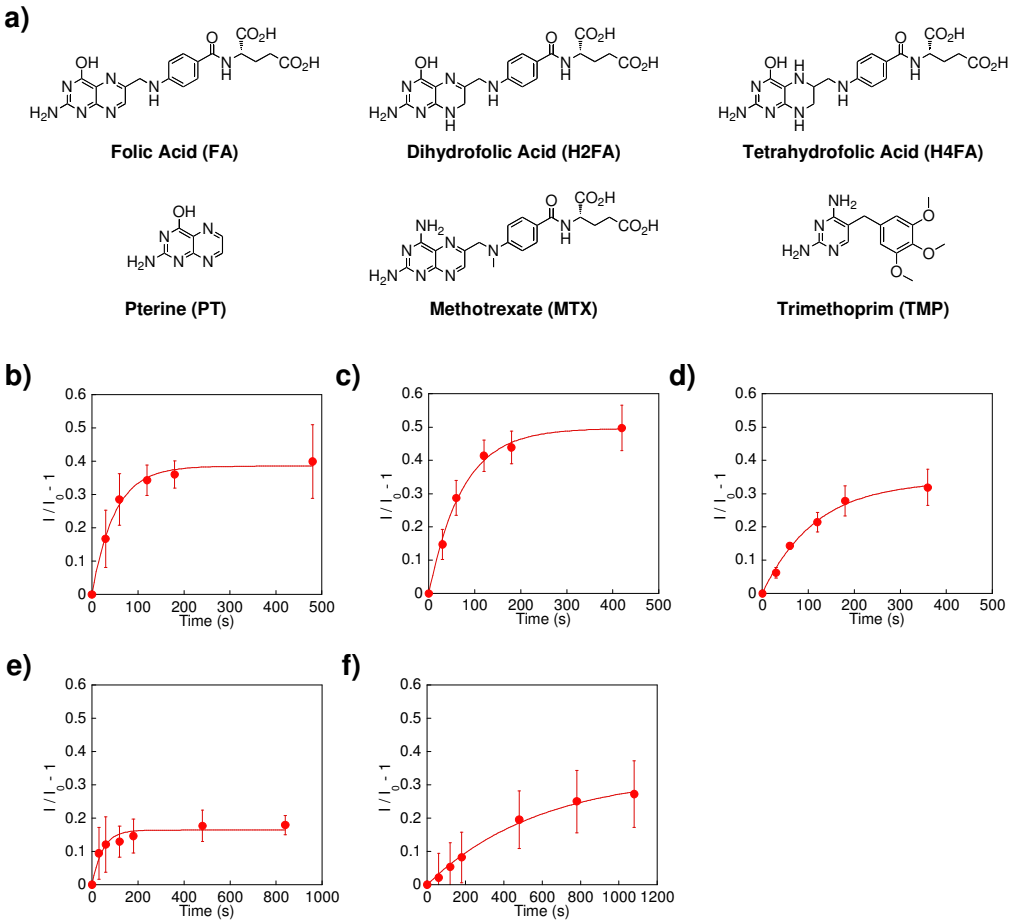


Figure S6. Time trace plots of fluorescence intensity change of Fl-FR on live cells induced by various FR ligands (N = 3). (**a**) Chemical structures of FR ligands used in this study. (**b**) 600 nM of FA, (**c**) 600 nM of H2FA, (**d**) 600 nM of H4FA, (**e**) 5 μM of PT, and (**f**) 5 μM of MTX. The k_{obs} of TMP was not determined due to slow fluorescence intensity change.

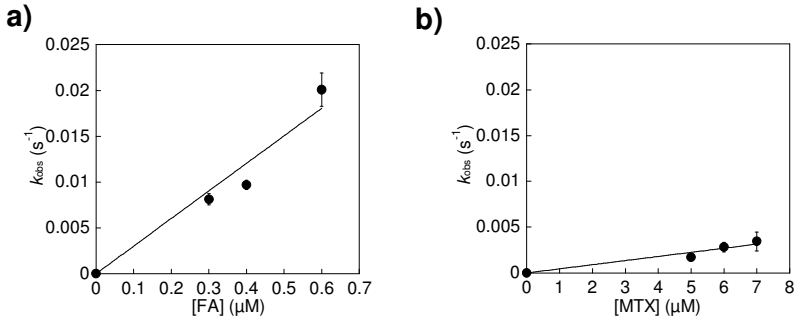


Figure S7. Ligand concentration dependence of k_{obs} evaluated by the time-lapse fluorescence detection using CLSM (N = 3). Each k_{on} value for various ligands ((**a**) FA, (**b**) MTX) was determined based on the equation (3) in the Experimental Materials. These kinetics values for H2FA, H4FA, and PT were also evaluated in the same way.

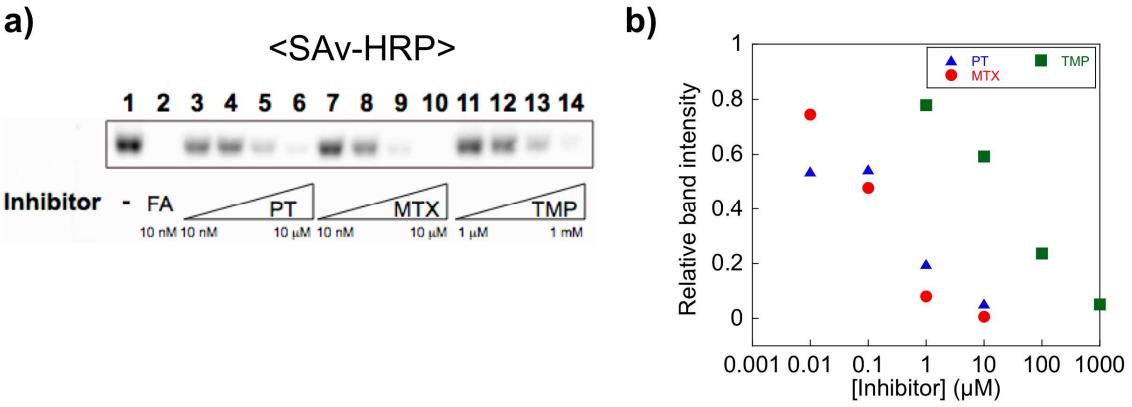


Figure S8. Blotting analysis of competitive labeling reaction with the FR ligands for evaluation of their binding affinities. KB cells (1 x 10^5 cells) were mixed with MTX-based LDAI reagent **2** (1 μM) in folate-free medium (10% FBS) in the presence of each ligand (FA, PT, MTX, TMP) as a competitive binding inhibitor, and incubated at 37 ˚C for 24 h. Blotting analysis using SAv-HRP (**a**) and inhibitor concentration dependence of the relative band intensity based on the lane 1 (**b**) clearly showed that FA has the highest inhibition activity, and PT has almost the same inhibition activity as MTX, and TMP has a lowest inhibition activity. These results indicate their relative strength in binding affinity toward FR.

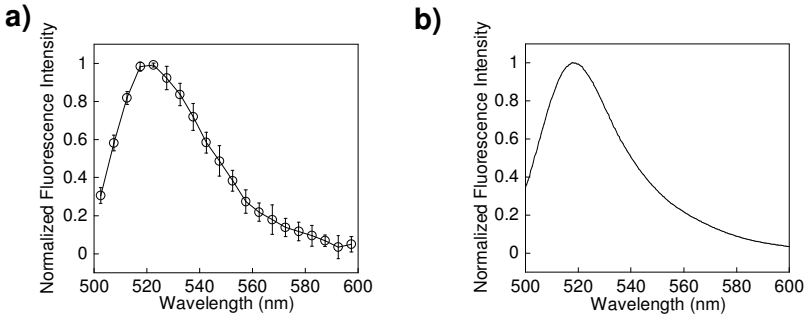


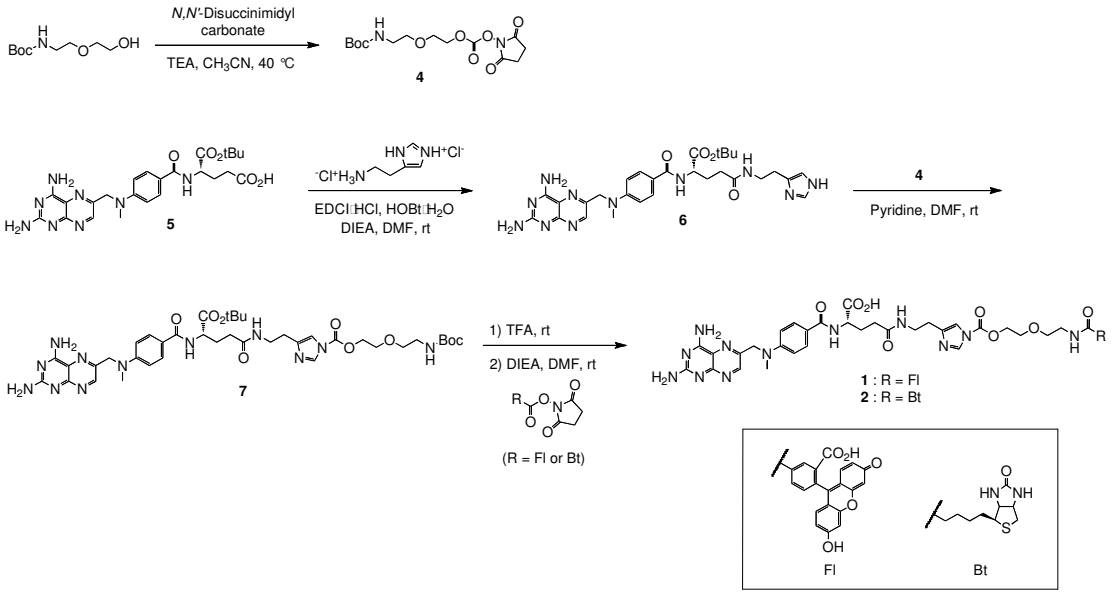
Figure S9. Normalized fluorescence spectra of LDAI reagent **1** and FR labeled by **1** on live cells. KB cells (1 x 10^5 cells) were mixed with LDAI reagent **1** (1 μM) in folate-free medium (10% FBS), and incubated at 37 ˚C for 24 h. The cells were washed 3 times with medium and subjected to fluorescence imaging (**a**) in 500-600 nm using CLSM to evaluate fluorescence intensities on cell membrane (N = 5). (**b**) Fluorescence spectrum of LDAI reagent **1** (1 μM) in PBS.

General materials and methods for organic synthesis

All chemical reagents and solvents were obtained from commercial suppliers (Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Acros Organics, Sasaki Chemical, or Watanabe Chemical Industries) and used without further purification.

Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated aluminium sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was conducted by flash column chromatography on silica gel 60 N (neutral, 40–50 μm, Kanto Chemical). ^{1}H NMR spectra were recorded in deuterated solvents on a Varian Mercury 400 (400 MHz) spectrometer and calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, dd = double doublet, br s = broad singlet. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was measured by an Autoflex II instrument (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid as the matrix. High-resolution electrospray ionization quadrupole fourier transform mass spectroscopy (HR-ESI Qq-LTMS) were measured by a JEOL JMS-HX110A mass spectrometer and Bruker apex-ultra (7T) mass spectrometer, respectively.

Synthesis of compound 1 and 2



Scheme S1. Synthetic scheme of compound **1** and **2**

Compound **4**

A solution of *N*-(*tert*-butoxycarbonyl)-2-(2-aminoethoxy)ethanol (2.00 g, 9.47 mmol), *N,N'*-disuccinimidyl carbonate (5.00 g, 19.5 mmol), and triethylamine (1.96 g, 19.5 mmol) in CH_3CN (70 mL) was stirred for 1 h at 40 ˚C. After removal of the solvent by evaporation, the residue was dissolved in AcOEt. The organic layer was washed with *sat.* $NaHCO_3$ and dried over Na_2SO_4 followed by concentration in vacuo. The residue was purified by flash column chromatography on SiO_2 ($CHCl_3$: MeOH = 30 : 1) to give **4** (2.36 g, 70% yield) as a pale-brown oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.47 (t, *J* = 4.8 Hz, 2H), 3.74 (t, *J* = 4.8 Hz, 2H), 3.56 (t, *J* = 4.8 Hz, 2H), 3.33 (m, 2H), 2.85 (s, 4H), 1.45 (s, 9H). HR-ESI MS *m/e* calcd for $[M+Na]^+$ 369.1268, found 369.1266.

Compound **6**

A solution of **5**[S1] (150 mg, 294 μmol), histamine•2HCl (54 mg, 294 μmol), EDCI•HCl (84 mg, 441 μmol), HOBt•H_2O (68 mg, 441 μmol), and DIEA (154 mL, 882 μmol) in dry DMF (10 mL) was stirred for 12 h at rt. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CH_2Cl_2 : MeOH = 5 : 1 → 3 : 1) to give **6** (163 mg, 92%) as a yellow amorphous powder. ^{1}H-NMR (400 MHz, CD_3OD) δ 8.55 (s, 1H), 7.74 (d, *J* = 9.2 Hz, 2H), 7.73 (s, 1H), 6.86 (m, 3H), 4.40 (dd, *J* = 9.6 Hz and 4.8 Hz, 1H), 3.60

(q, *J* = 6.8 Hz, 2H), 3.24 (s, 3H), 2.73 (t, *J* = 7.6 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 2.17 (quin, *J* = 6.8 Hz, 1H), 2.02 (quin, *J* = 6.8 Hz, 1H), 1.46 (s, 9H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 604.3103, found 604.3105.

Compound **7**

A solution of **6** (161 mg, 267 μmol), **4** (139 mg, 401 μmol), and pyridine (65 μL, 801 μmol) in dry DMF (5 mL) was stirred for 19 h at rt. After removal of the solvent in vacuo, the residue was dissolved in $CHCl_3$. The organic layer was washed with water and brine followed by drying over Na_2SO_4. After removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (CH_2Cl_2 : MeOH = 15 : 1 → 8 : 1) to give **7** (101 mg, 45%) as a yellow amorphous powder. ^{1}H-NMR (400 MHz, $CHCl_3$) δ 8.58 (s, 1H), 8.01 (s, 1H), 7.69 (d, *J* = 8.8 Hz, 2H), 7.11 (s, 1H), 6.94 (br s, 1H), 6.69 (d, *J* = 8.4 Hz, 2H), 5.85 (br s, 1H), 5.31 (br s, 1H), 4.69 (s, 2H), 4.57-4.62 (m, 1H), 4.49 (t, *J* = 4.4 Hz, 2H), 3.77 (t, *J* = 4.4 Hz, 2H), 3.57 (t, *J* = 5.2 Hz, 2H), 3.41-3.51 (m, 2H), 3.32 (q, *J* = 5.2 Hz, 2H), 3.15 (s, 3H), 2.63 (br s, 2H), 2.29-2.39 (m, 2H), 2.10-2.25 (m, 2H), 1.46 (s, 9H), 1.43 (s, 9H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 835.4209, found 835.4209.

Compound **1**

A solution of **7** (10 mg, 12 μmol) in TFA (1 mL) was stirred for 2 h at rt. After removal of the solvent by evaporation, the residual TFA was further removed azeotropically with toluene (x2) to give deprotected **7**.

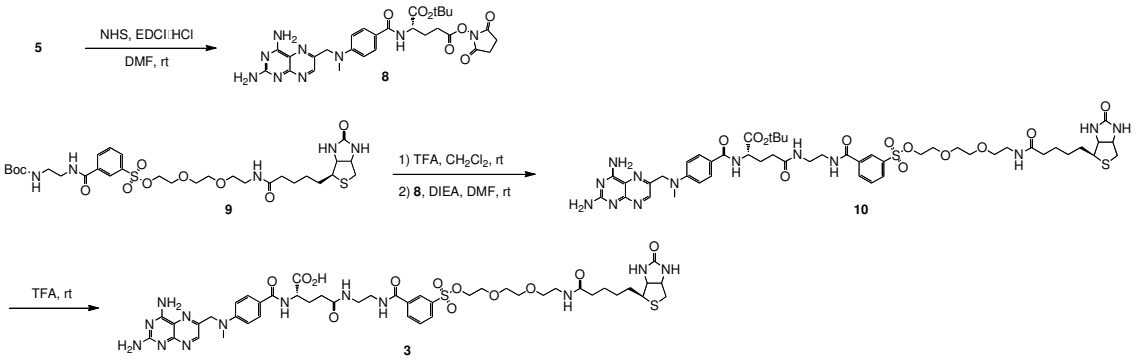
Deprotected **7**, 5-carboxyfluorescein succinimidyl ester (5 mg, 10.6 μmol), and DIEA (15 μL, 84 μmol) in dry DMF (3 mL) was stirred for 14 h at rt. After removal of the solvent by evaporation, the reside was purified by RP-HPLC (column; YMC-pack ODS-A, 250 x 25 mm, mobile phase; CH_3CN : H_2O = 5 : 95 → 30 : 70 (linear gradient over 50 min), flow rate; 10 mL/min, detection; UV (220 nm)) to yield **1** (0.7 mg, 5.6% in 2 steps) as a yellow powder. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.88 (t, *J* = 5.6 Hz, 1H), 8.54 (s, 1H), 8.42 (s, 1H), 8.20 (d, *J* = 9.2 Hz, 1H), 8.08 (s, 1H), 7.88 (t, *J* = 5.6 Hz, 1H), 7.70 (d, *J* = 8.8 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 1H), 7.30 (s, 1H), 6.80 (d, *J* = 8.8 Hz, 2H), 6.67 (s, 2H), 6.57 (d, *J* = 8.4 Hz, 2H), 6.53 (d, *J* = 8.8 Hz, 2H), 4.76 (s, 2H), 4.47 (br s, 2H), 4.26 (br s, 1H), 3.77 (br s, 2H), 3.62 (t, *J* = 6.0 Hz, 2H), 3.48 (t, *J* = 4.4 Hz, 2H), 3.18 (s, 3H), 2.14 (t, *J* = 7.6 Hz, 2H), 2.00-2.07 (m, 1H), 1.84-1.94 (m, 1H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 1037.3537, found 1037.3522.

Compound **2**

A solution of deprotected **7** (prepared from 23 mg (27.6 μmol) of **7**), biotin succinimidyl ester (11 mg, 30.4 μmol), and DIEA (24 μL, 138 μmol) in dry DMF (2 mL) was stirred for 12 h at rt.

After removal of the solvent in vacuo, the residue was purified by RP-HPLC (column; YMC-pack ODS-A, 250 x 25 mm, mobile phase; CH_3CN : 10 mM NH_4OAc = 5 : 95 → 30 : 70 (linear gradient over 40 min), flow rate; 10 mL/min, detection; UV (220 nm)) to give **2** (3 mg, 12% in 2 steps) as a yellow powder. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.50 (s, 1H), 8.09-8.11 (m, 2H), 7.91 (t, *J* = 5.6 Hz, 1H), 7.84 (t, *J* = 5.6 Hz, 1H), 7.66 (d, *J* = 9.2 Hz, 2H), 7.26 (s, 1H), 6.76 (d, *J* = 9.2 Hz, 2H), 6.41 (s, 1H), 6.37 (s, 1H), 4.73 (s, 2H), 4.39 (t, *J* = 4.4 Hz, 2H), 4.25 (dd, *J* = 7.6, 5.2 Hz, 1H), 4.19 (dt, *J* = 8.4, 6.0 Hz, 1H), 4.07 (t, *J* = 6.0 Hz, 1H), 3.66 (t, *J* = 4.4 Hz, 2H), 3.40 (t, *J* = 5.6 Hz, 2H), 3.22 (dd, *J* = 12.8, 7.2 Hz, 2H), 3.15 (s, 3H), 2.99-3.04 (m, 2H), 2.75 (dd, *J* = 12.4, 5.2 Hz, 1H), 2.54 (t, *J* = 5.2 Hz, 2H), 2.11 (t, *J* = 7.2 Hz, 2H), 1.97-2.01 (m, 3H), 1.81-1.89 (m, 1H), 1.49-1.58 (m, 2H), 1.35-1.46 (m, 2H), 1.18-1.27 (m, 2H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 905.3835, found 905.3822.

Synthesis of compound 3



Scheme S2. Synthetic scheme of compound **3**

Compound **8**

A solution of **5** (51 mg, 99 μmol), *N*-hydroxysuccinimide (12 mg, 104 μmol), and EDCI•HCl (38 mg, 197 μmol) in dry DMF (10 mL) was stirred for 6 h at rt. After removal of the solvent by evaporation, the residue was diluted with AcOEt. The organic layer was washed with *sat.* $NaHCO_3$ and brine followed by drying over Na_2SO_4. The solvent was removed in vacuo to give **8** (21 mg, 35%) as a yellow powder. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H), 7.72 (d, *J* = 8.8 Hz, 2H), 6.76 (d, *J* = 9.2 Hz, 2H), 4.76 (m, 3H), 3.19 (s, 3H), 2.81 (s, 4H), 2.71-2.74 (m, 2H), 2.41 (m, 1H), 2.17 (m, 1H), 1.49 (s, 9H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 608.2576, found 608.2567.

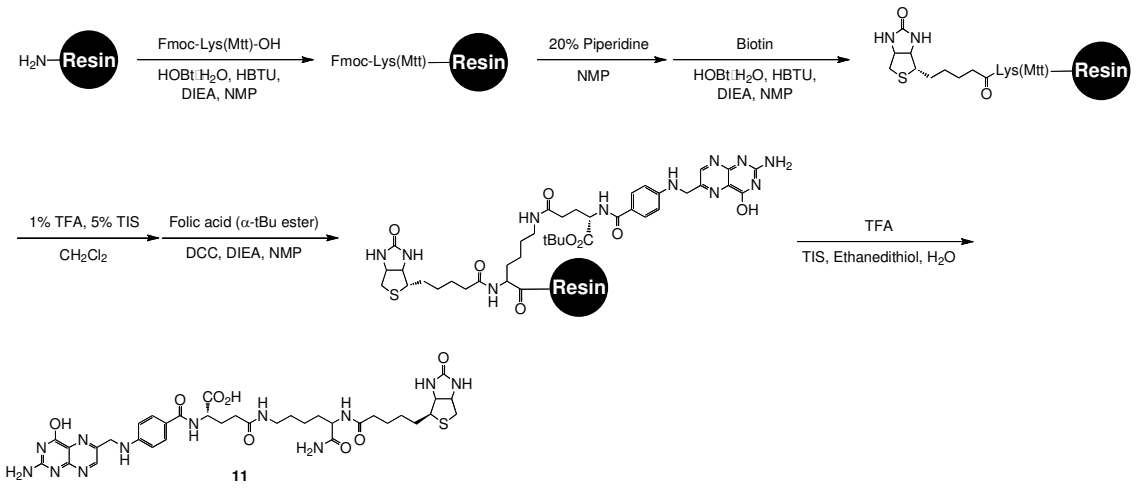
Compound **3**

A solution of **9**[S2] (38 mg, 58 μmol) in CH_2Cl_2 (3 mL) / TFA (3 mL) was stirred for 1.5 h at rt. After removal of the solvent by evaporation, the residual TFA was further removed azeotropically with toluene (x2) to give deprotected **9**.

Deprotected **9**, **8** (35 mg, 58 μmol), and DIEA (51 μL, 290 μmol) in dry DMF (3 mL) was stirred for 4 h at rt. After removal of the solvent in vacuo, the residue was purified by flash column chromatography on SiO_2 (CH_2Cl_2 : MeOH = 10 : 1 → 4 : 1) to give **10** (47 mg, 74% in 2 steps) as a yellow oil without further purification. HR-ESI MS *m/e* calcd for $[M+H]^+$ 1094.4546, found 1094.4554.

A solution of **10** (10 mg, 9 μmol) in TFA (2 mL) was stirred for 2 h at rt. After removal of the solvent by evaporation, the reside was purified by RP-HPLC (column; YMC-pack ODS-A, 250 x 25 mm, mobile phase; CH_3CN (containing 0.1% TFA) : H_2O (containing 0.1% TFA) = 5 : 95 → 35 : 65 (linear gradient over 50 min), flow rate; 10 mL/min, detection; UV (220 nm)) to give **3** (5 mg, 53%) as a yellow powder. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.21 (s, 2H), 8.98 (s, 2H), 8.81 (t, *J* = 5.6 Hz, 1H), 8.64 (s, 1H), 8.24-8.26 (m, 2H), 8.15 (d, *J* = 8.0 Hz, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.77 (t, *J* = 5.6 Hz, 1H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.67 (d, *J* = 9.2 Hz, 2H), 6.75 (d, *J* = 8.8 Hz, 2H), 6.36 (m 2H), 4.81 (s, 2H), 4.23-4.28 (m, 2H), 4.11 (t, *J* = 4.0 Hz, 2H), 4.06 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.51 (t, *J* = 4.4 Hz, 2H), 3.04-3.28 (m, 15H), 2.99-3.04 (m, 2H), 2.74 (dd, *J* = 12.4, 5.2 Hz, 1H), 2.15 (t, *J* = 7.2 Hz, 2H), 1.97-2.06 (m, 3H), 1.82-1.91 (m, 1H), 1.49-1.58 (m, 2H), 1.33-1.44 (m, 2H), 1.19-1.29 (m, 2H). HR-ESI MS *m/e* calcd for $[M+H]^+$ 1038.3920, found 1038.3922.

Synthesis of compound 11



Scheme S3. Synthetic scheme of compound **11**

For the synthesis of **11**, the solid-phase synthesis was carried out using Rink Amide resin (Novabiochem). The coupling reaction of Fmoc-Lys(Mtt)-OH and biotin was performed with a mixture of the corresponding carboxylic acid (4 eq.), HBTU (4 eq.), HOBt•H_2O (4 eq.), and DIEA (8 eq.) in *N*-methylpyrrolidone (NMP). Fmoc deprotection was performed with 20% piperidine in NMP, and 4-methyltrityl (Mtt) deprotection was performed in CH_2Cl_2 containing 1% TFA and 5% triisopropylsilane (TIS). The coupling of folic acid was performed with a mixture of the folic acid (α-*t*Bu ester) (4 eq.), *N,N'*-dicyclohexylcarbodiimide (DCC, 4 eq.), and DIEA (8 eq.) in NMP. All coupling and deprotection steps were monitored by Kaiser test. The cleavage from the resin was performed with TFA containing 1% TIS, 2.5% ethanedithiol and 2.5% H_2O. The crude peptide product was purified by RP-HPLC to give **11** (1.2 mg) as a yellow solid. HR-ESI MS *m/e* calcd for $[M+H]^+$ 795.3355, found 795.3342.

General materials and methods for biochemical/biological experiments

Unless otherwise noted, all proteins/enzymes and biochemical reagents were obtained from commercial suppliers (Sigma, Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Pierce Biotechnology, or Calbiochem) and used without further purification. UV-visible spectra were recorded on a Shimadzu UV-2550 spectrophotometer. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Chemiluminescent signals were detected with an LAS 4000 imaging system (Fuji Film). Fluorescence gel images were acquired using a Bio-Rad ChemiDoc XRS system with a 480BP70 filter, and analyzed with Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Analytical reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with LaChrom L-7400 UV and L-7485 fluorescence detectors. MALDI-TOF MS and MALDI quadrupole ion trap TOF MS/MS (MALDI-QIT-TOF MS/MS) analyses were performed on an Autoflex II instrument (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid as the matrix. Cell imaging was performed with a confocal laser scanning microscope (CLSM, Olympus, FV1000, IX81) equipped with a 20×, 60×, or 100× objective lens. Fluorescence images were acquired using 488 nm line of an argon laser for excitation of fluorescein (emission, 500-600 nm) and 633 nm line of a HeNe Red laser for excitation of streptavidin modified with HiLyte647 (SAv647) (emission, 645-745 nm).

Labeling of DHFR

The recombinant DHFR was prepared as described previously[S3]. The purified DHFR (25 μM) was mixed with LDAI reagent (**1**, **2** or **3**, 50 μM) in the absence or presence of MTX (500 μM) in phosphate buffer (100 mM, pH 6.0) and incubated at 37 ºC. The aliquots at different time points were taken, and the labeling yields were evaluated by MALDI-TOF MS (sinapinic acid (SA) used as the matrix).

Peptide mapping of the labeled DHFR

A solution of DHFR (40 μM) and **1** (50 μM) in phosphate buffer (100 mM, pH 6.0) was incubated for 48 h at 37 ºC. The labeled DHFR was purified by size-exclusion chromatography (TOYOPEARL HW-40F) and dialyzed against HEPES buffer (50 mM, pH 7.2) using a Spectra/Por® dialysis membrane (MWCO: 10,000). The labeling rate of CAI (54%) was determined by MALDI-TOF MS. For protein digestion, the solution of the labeled DHFR (10 μM) was treated with trypsin (trypsin/substrate ratio = 1/20 (w/w)) at 37 ºC for 9 h in the presence of urea (4 M). Native (unlabeled) DHFR was also subjected to trypsin digestion under the same conditions. The digested sample was subjected to RP-HPLC (column; YMC-pack ODS-A, 250 mm × 4.6 mm, mobile phase; CH_3CN (containing 0.1% TFA) : H_2O (containing 0.1% TFA) = 10 : 90 → 60 : 40 (linear gradient over 100 min), flow rate; 1.0 mL/min, detection; UV (220 nm) and fluorescence (excitation at 495 nm, emission at 530 nm)). All of the purified digested fragments were analyzed by MALDI-TOF MS. For the peptide mapping, the labeled fragments were further analyzed by MALDI-QIT-TOF MS/MS.

Biotin labeling of endogenous FR on KB cells

KB cells (1×10^5 cells) were treated with **2** or **3** (1 μM) at 37 °C in a folate-free RPMI 1640 medium (10% fetal bovine serum (FBS)). As a control experiment, the labeling was conducted in the presence of FA (5 μM). The cells were washed 3 times with the medium, treated with SAv647 (AnaSpec Inc.), and subjected to fluorescence imaging using CLSM. The FR-labeling yield was quantitatively evaluated based on the equation (1):

$$\text{Labeling yield (\%)} = \frac{I_{\text{label}}}{I_{\text{total}}} \times 100 \qquad (1)$$

, where I_{label} is the fluorescence intensity of SAv647 detectable on cell surfaces after being labeled with **2** or **3** and I_{total} is the fluorescence intensity of SAv647 detectable in the quantitative labeling of FR with 1 μM of biotin-modified folic acid **11**[S4].

For western blotting, the cells were lysed using RIPA buffer (pH 7.6, 25 mM Tris·HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholic acid) containing 1% protease inhibitor cocktail set III (Calbiochem®). The lysed sample was centrifuged, and the supernatant was mixed with the same volume of 2× sampling buffer (pH 6.8, 125 mM Tris·HCl, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 100 mM dithiothreitol) and incubated for 1 h at room temperature. The samples were subjected to SDS-PAGE (12.5%) and then electrotransferred onto an Immun-Blot® PVDF membrane (Bio-Rad). The biotinylated FR was detected by chemiluminescence analysis using SAv-HRP and ECL Plus Western Blotting Detection Reagents (GE Healthcare). The immunodetection of FR was performed with a mouse anti-FR antibody[S5] (Abcam, ×500) and anti-mouse IgG-HRP conjugate (GE Healthcare, ×10000).

Functionalization of folate receptor on the surface of living cells

KB cells (1×10^5 cells) endogenously expressing FR were treated with LDAI reagent **1** (1 μM) for 24 h at 37 °C for construction of Fl-labeled FR. The association rate constant (k_{on}, $M^{-1}s^{-1}$) of the ligands with FR was measured by time-lapse fluorescence detection using CLSM, in which the labeled cells in a folate-free RPMI medium (100 μL) were quickly mixed with a ligand solution of the same medium (900 μL) at once, and the fluorescence intensity change was monitored by CLSM (N = 3). The apparent association rate constant (k_{obs}, s^{-1}) values were calculated based on the equation (2):

$$R = R_{max}\{1 - \exp(-k_{obs}t)\} \quad (2)$$

, where R is the relative fluorescence intensity change (I/I_0 - 1) observable on cell surfaces and t is the time after the ligand injection. The experimental plots well fitted to the fitting curve (Figure 4c, S5). The k_{on} values were determined based on the equation (3):

$$k_{obs} = k_{on}[\text{Ligand}] \quad (3)$$

The association constant (K_a, M^{-1}) of the ligands for FR was evaluated by fluorescence titration, in which the fluorescence intensity on cell surfaces was measured upon addition of the ligands (N = 3). K_a (M^{-1}) was calculated by the Hill plot[S6] using the equation (4):

$$\frac{r}{1 - r} = K_a[\text{Ligand}] \quad (4)$$

, where r is the rate of the fluorescence intensity change ($(I - I_0)/(I_{max} - I_0)$) observable on cell surfaces.

References

S1) Nagy, A.; Szoke, B.; Schally, A. V. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6373-6376.

S2) Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T.; Hamachi, I. *Nat. Chem. Biol.* **2009**, 5, 341-343.

S3) Takaoka, Y.; Kiminami, K.; Mizusawa, K.; Matsuo, K.; Narazaki, M.; Matsuda, T.; Hamachi, I. *J. Am. Chem. Soc.* **2011**, 133, 11725-11731.

S4) It was reported that the dissociation constant of FA with FR is less than 1 nM[31]. In addition, the fluorescence microscopy experiment showed that increase of the fluorescence signal on the cell surface was saturated by up to 0.5 μM of biotin-modified folic acid **11** used. These data suggest that almost all FRs expressed on cell surface were labeled with 1 μM of **11**.

S5) Gravier, J.; Schneider, R.; Frochot, C.; Bastogne, T.; Schmitt, F.; Didelon, J.; Guillemin, F.; Barberi-Heyob, M. *J. Med. Chem.* **2008**, 51, 3867-3877.

S6) Hill, A.V. *J. Physiol.* **1910**, 40, iv-vii.