Genetic targeting of a small fluorescent zinc indicator to cell surface for monitoring zinc secretion

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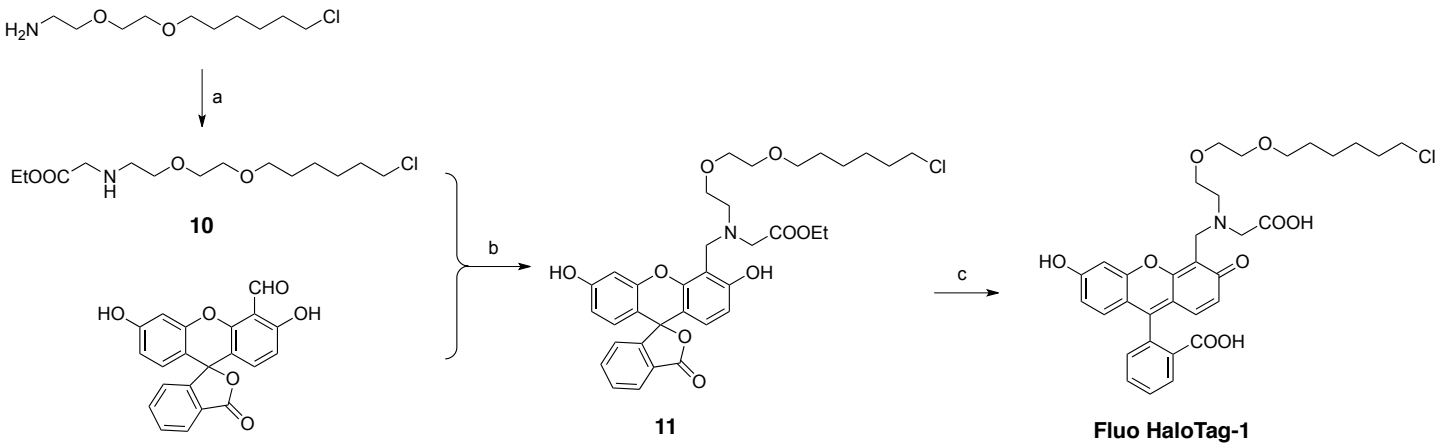
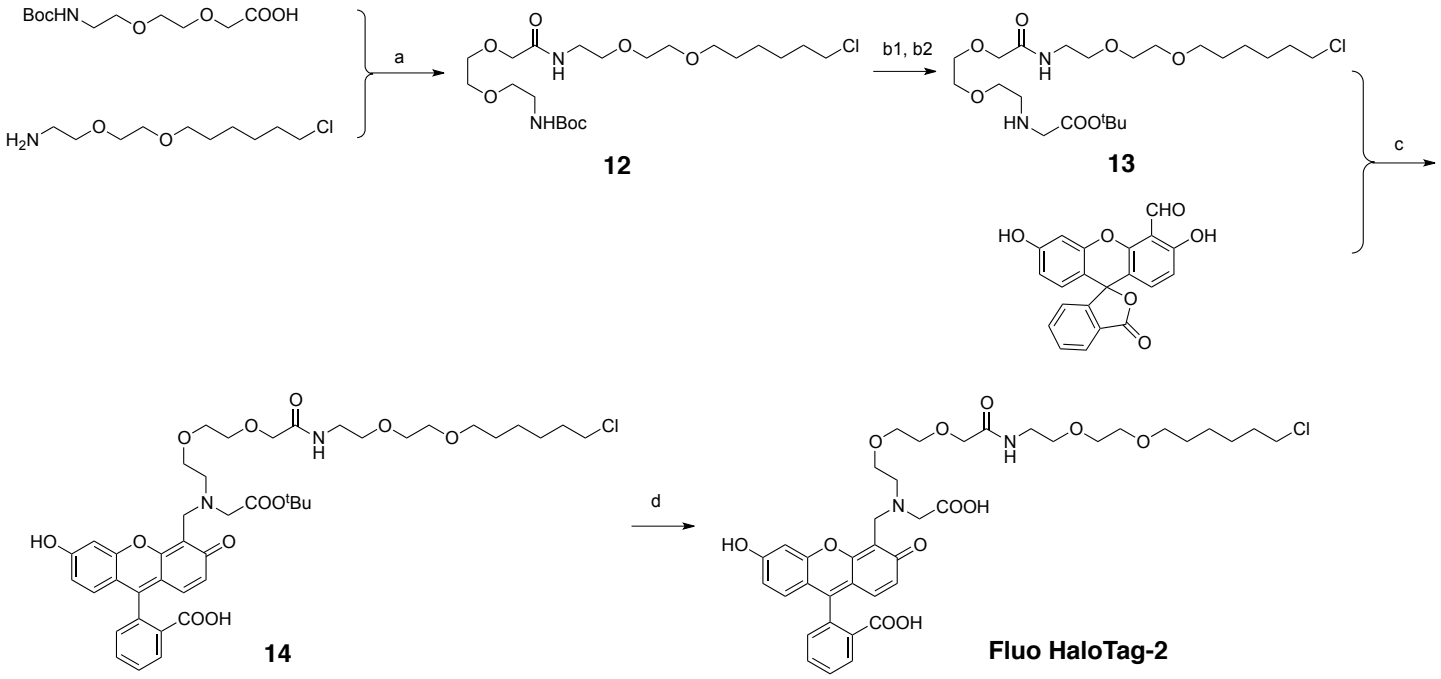
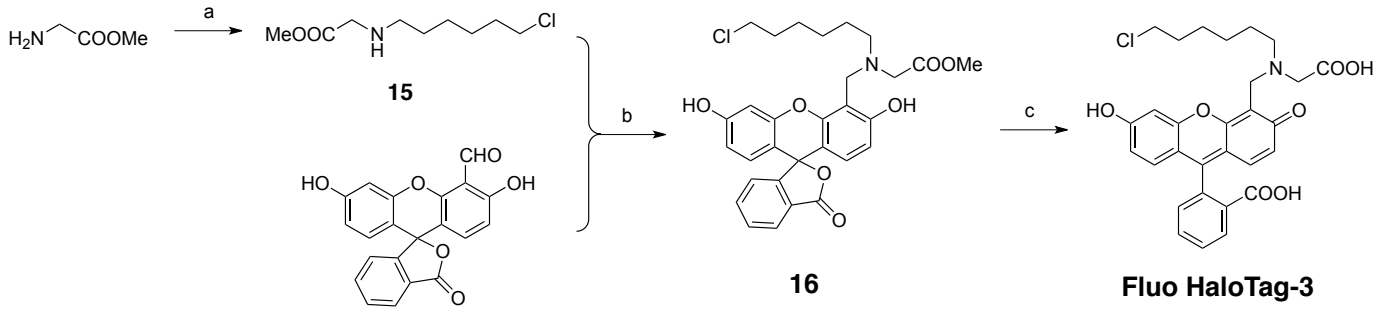
Supplementary information



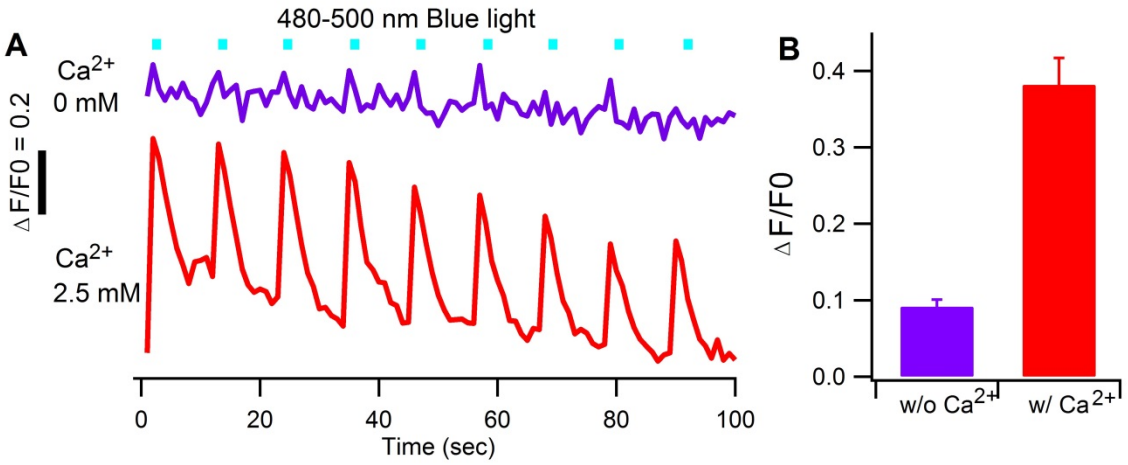
Supplementary Figure 1. Synthesis of Fluo HaloTag-1. (a) $ClCH_2CH_2Cl$, $BrCH_2COOEt$, Cs_2CO_3, r.t., overnight, 32%; (b) $NaBH(OAc)_3$, HOAc, $ClCH_2CH_2Cl$, r.t., overnight, 72%; (c) LiOH (4 M), THF, r.t., 4 h, 84%.



Supplementary Figure 2. Synthesis of Fluo HaloTag-2. (a) EDC, NHS, DIEA, $ClCH_2CH_2Cl$, 13%; (b1) TFA/CH_2Cl_2, 8h; (b2) CH_3CN, K_2CO_3, $BrCH_2COO^tBu$, r.t., 42%; (c) $NaBH(OAc)_3$, HOAc, $ClCH_2CH_2Cl$, r.t., 40%; (d) TFA/DCM, 100%



Supplementary Figure 3. Synthesis of Fluo HaloTag-3. (a) Et_3N, 1-chloro-6-iodohexane, $ClCH_2CH_2Cl$, 0 °C, 8 h, 14%; (b) $NaBH(OAc)_3$, HOAc, $ClCH_2CH_2Cl$, r.t., overnight, 50%; (c) LiOH (4 M), THF, r.t., 4 h, 80%.



Supplementary Figure 4. Photo-activation of ChR2(T159C) in MIN6 cells caused intracellular Ca^{2+} elevation through Ca^{2+} entry. **(A)** Representative fluorescence enhancements of R-GECO1.2 to photo-activation (490/20 nm, 1 sec duration, indicated by the solid bars on top) in MIN6 cells expressing ChR2(T159C)-R-GECO1.2 either in Ca^{2+} free SAB buffer containing 2 mM EGTA (top blue trace), or in normal SAB buffer containing 2.5 mM Ca^{2+} (bottom red trace). (**B**) The average peak signal of R-GECO1.2 after photo-activating ChR2(T159C) in Ca^{2+} free SAB or in normal SAB with 2.5 mM Ca^{2+} (N= 8 cells for each condition). The minute signal increase of R-GECO1.2 detected in the Ca^{2+} free medium was likely due to the photo-activation of R-GECO1.2 per se[1] under the setting used here for activating ChR2(T159C) (490/20 nm for 1 sec).

Supplementary Movie 1

Two color imaging of R-GECO1.2 (red) and HaloTag (green) in MIN6 beta cells during repetitive photo-activation of ChR2(T159C). The movie corresponded to 0 sec – 100 sec of Figure 6B.

Supplementary Movie 2

Two color imaging of R-GECO1.2 (red) and HaloTag (green) in MIN6 beta cells without photo-activation. The movie corresponded to 150 sec – 250 sec of Figure 6B.

CHEMICAL SYNTHESIS

All reagents were purchased from Aldrich or VWR. Anhydrous solvents were stored over activated molecular sieves (3 Å or 4 Å). TLC was performed on precoated silica gel 60F-254 glass plates (EM Science). Reaction products were purified by low-pressure flash chromatography (FC) using silica gel 60 (63–200 μm; EM Science). 1H-NMR spectra were acquired on a Varian 400-MHz or 500-MHz spectrometer. Chemical shifts (δ, ppm) were reported against tetramethylsilane (0 ppm). MALDITOF MS was performed on a Voyager-DE PRO biospectrometry workstation (Applied Biosystems) using 2,5-dihydroxy benzoic acid as the matrix.

Synthesis of Fluo HaloTag-1 (Supplementary Figure 1)

Compound **10**: Ethyl bromoacetate (116 μL, 2 mmol) was added dropwise to a mixture of chloro-amine (2.68 mmol) [2] and Cs_2CO_3 (874 mg, 2.68 mmol) in 1,2-dichloroethane at room temperature (RT). After stirring at RT overnight, the reaction mixture was purified by FC with DCM/MeOH (50:1 to 10:1) to yield the product as an oily residue (88.6 mg; 32%) . ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.15 (q, *J* = 7.2 Hz, 2H), 3.58-3.45 (m, 8H), 3.42 (t, *J* = 6.8 Hz, 2H), 3.39 (s, 2H), 2.77 (t, *J* = 5.2 Hz, 2H), 1.76-1.71 (m, 2H), 1.58-1.52 (m, 2H), 1.43-1.37 (m, 2H), 1.37-1.30 (m, 2H), 1.24 (t, *J* = 7.2 Hz, 3H).

Compound **11**: Compound **10** (19 mg, 61.1 μmol) in 1,2-dichloroethane (1 mL) was added dropwise to a mixture of fluorescein monoaldehyde [3] (20 mg, 55.5 μmol) and HOAc (12.7 μL) in 1,2-dichloroethane (2 mL). After stirring at RT for 2 h, $NaBH(OAc)_3$ (8.8 mg, 140 μmol) was added and the reaction was continued at RT overnight. The solvent was then removed under vacuum and the mixture was purified with FC (DCM/MeOH, 50:1 to 20:1) to give the product as a yellow foam (26 mg, 71.6%). ^{1}H NMR (CD_3OD , 400 MHz): δ 8.00 (d, *J* = 7.2 Hz, 1H), 7.73-7.65 (m, 2H), 7.20 (d, *J* = 7.6 Hz, 1H), 6.69 (d, *J* = 2.4 Hz, 1H), 6.66-6.60 (m, 2H), 6.55-6.50 (m, 2H), 4.29 (dd, *J* = 4, 18.8 Hz, 2H), 4.15 (q, *J* = 7.2 Hz, 2H), 3.68 (t, *J* = 5.2 Hz, 2H), 3.59-3.40 (m, 20H),

2.96 (t, *J* = 5.2 Hz, 2H), 1.76-1.71 (m, 2H), 1.58-1.52 (m, 2H), 1.44-1.38 (m, 2H), 1.36-1.31 (m, 5H). MS analysis: 653.24 calculated for $C_{35}H_{40}ClNO_9$; observed: 654.49 $(M + H)^+$, 676.42 $(M + Na)^+$.

Fluo HaloTag-1: An aqueous LiOH solution (4 M, 1 mL) was added to compound 11 (10 mg, 16 μmol) in tetrahydrofuran (THF, 2mL). The solution was concentrated 4h later under vacuum and the resulting residue was purified by a reverse phase C18 column ($MeOH/H_2O$, 0:100 to 80:20) to yield the product as an orange solid (8 mg, 84%). ^1H NMR (CD_3OD , 400 MHz): δ 8.03-7.98 (m, 1H), 7.56-7.52 (m, 2H), 7.20-7.17 (m, 1H), 7.08-7.02 (m, 3H), 6.56-6.50 (m, 2H), 3.88 (q, *J* = 12 Hz, 2H), 3.64-3.45 (m, 12H), 2.77 (t, *J* = 6.0 Hz, 2H), 1.76-1.71 (m, 2H), 1.58-1.52 (m, 2H), 1.44-1.38 (m, 2H), 1.36-1.31 (m, 2H). MS analysis: 625.21 calculated for $C_{33}H_{36}ClNO_9$; observed: 626.29 $(M + H)^+$, 345.18 $(M- C_{12}H_{22}ClNO_4)^+$.

Synthesis of Fluo HaloTag-2 (Supplementary Figure 2)

Compound 12: Boc-protected amino acid (0.647 g, 2.46 mmol; prepared according to a known procedure [4]) was mixed with 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC, 513 mg, 2.68 mmol), N-hydroxysuccinimide (NHS, 309 mg, 2.68 mmol) and N,N-Diisopropylethylamine (DIEA, 553 μL, 3.35 mmol) in anhydrous 1,2-dichloroethane (5 mL). Chloro-amine (0.5 g, 2.23 mmol) [2] was added to the mixture which was stirred at RT overnight. The reaction mixture was then washed with saturated saline and the aqueous phase was extracted with $CHCl_3$ (3 X 10 mL). The combined organic phases was dried with anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by FC to give the product as an oil (134.6 mg, 12.9%). ^1H NMR ($CDCl_3$, 400 MHz): δ 3.98 (s, 2H), 3.66-3.38 (m, *18*H), 3.32-3.28 (m, 2H), 1.76-1.71 (m, 2H), 1.58-1.52 (m, 2H), 1.44-1.38 (m, 2H), 1.41 (s, 9H), 1.36-1.31 (m, 2H).

Compound 13: Compound **12** (0.3 mmol) was treated with trifluoroacetic acid (TFA/DCM 1:1, 2 mL) at RT for 8 h to remove Boc protecting group. After removing solvents under high vacuum, the oily product was dissolved in CH_3CN (1 mL). To this solution K_2CO_3 (207.3 mg, 1.5 mmol) and tert-butyl bromoacetate (16.5 μL,

0.1 mmol) was added. The reaction was continued at RT overnight. Solvents were then removed under vacuum and the resulting residue was purified with FC (DCM/MeOH, 100:1 to 20:1) to give the product as an oil (20 mg, 41% based on tert-butyl bromoacetate). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.98 (s, 2H), 3.66-3.32 (m, 22H), 1.76-1.71 (m, 2H), 1.58-1.52 (m, 2H), 1.44-1.38 (m, 2H), 1.42 (s, 9H), 1.36-1.31 (m, 2H). MS analysis: 482.28 calculated for $C_{22}H_{43}ClN_2O_7$; observed: 483.46 $(M + H)^+$, 505.45 $(M + Na)^+$.

Compound 14: This was synthesized from compound **13** and fluorescein monoaldehyde in 40% yield following the same procedure as preparing compound **11**. ^{1}H NMR (CD_3OD , 400 MHz): δ 8.01 (d, *J* = 6.4 Hz, 1H), 7.78 (t, *J* = 6.0 Hz, 1H), 7.71 (t, *J* = 6.0 Hz, 1H), 7.23 (d, *J* = 6.4 Hz, 1H), 6.74 (s, 1H), 6.59-6.53 (m, 4H), 4.32 (dd, *J* = 11.2, 25.2 Hz, 2H), 3.98 (s, 2H), 3.74-3.49 (m, 20H), 3.04 (t, *J* = 4.0 Hz, 2H), 1.74-1.68 (m, 2H), 1.57-1.53 (m, 2H), 1.50 (s, 9H), 1.44-1.40 (m, 2H), 1.36-1.31 (m, 2H). MS analysis: 826.34 calculated for $C_{43}H_{55}ClN_2O_{12}$; observed: 827.49 $(M + H)^+$.

Fluo HaloTag-2: This was prepared in quantitative yield after treating compound **14** (5 mg) with TFA/dichloromethane (1:1, 1 mL) at RT overnight. ^{1}H NMR (CD_3OD , 400 MHz): δ 8.03 (s, 1H), 7.81-7.67 (m, 2H), 7.25 (d, *J* = 6.0 Hz, 1H), 7.01 (s, 1H), 6.77-6.57 (m, 4H), 3.95 (s, 2H), 3.89 (s, 2H), 3.75-3.42 (m, 22H), 1.74-1.68 (m, 2H), 1.57-1.53 (m, 2H), 1.44-1.40 (m, 2H), 1.36-1.31 (m, 2H). MS analysis: 770.28 calculated for $C_{39}H_{47}ClN_2O_{12}$; observed: 771.55 $(M + H)^+$, 793.55 $(M + Na)^+$.

Synthesis of Fluo HaloTag-3 (Supplementary Figure 3)

Compound 15: At 0 °C, 1-chloro-6-iodohexane (304 μL, 2 mmol) was added dropwise to a solution of glycine methyl ester hydrochloride (1.005 g, 8 mmol) and Et_3N (1.12 mL, 8 mmol) in 1,2-dichloroethane. The reaction as continued at 0 °C for 8 h. The reaction mixture was purified with FC (DCM/MeOH, 50:1 to 20:1) to give the product as an oil (59 mg, 14%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.71 (s, 3H), 3.50 (t, *J* = 6.8 Hz, 2H),

3.41 (s, 2H), 2.60 (t, *J* = 7.2 Hz, 2H), 1.77-1.72 (m, 2H), 1.52-1.48 (m, 2H), 1.46-1.40 (m, 2H), 1.39-1.32 (m, 2H).

Compound 16: This was synthesized from compound **15** and fluorescein monoaldehyde in 50% yield following the same procedure as preparing compound **11**. ^{1}H NMR (CD_3OD , 400 MHz): δ 8.01 (d, *J* = 6.4 Hz, 1H), 7.75 (p, *J* = 4.8 Hz, 1H), 7.69 (t, *J* = 6.0 Hz, 1H), 7.22 (d, *J* = 6.4 Hz, 1H), 6.69 (d, *J* = 2.0 Hz, 1H), 6.66-6.60 (dd, *J* = 7.2, 10.8 Hz, 2H), 6.57-6.54 (m, 2H), 4.21 (s, 2H), 3.75 (s, 3H), 3.49 (t, *J* = 5.2 Hz, 2H), 2.70 (t, *J* = 6.4 Hz, 2H), 1.74-1.68 (m, 2H), 1.62-1.58 (m, 2H), 1.41-1.36 (m, 2H), 1.33-1.29 (m, 5H).

Fluo HaloTag-3: This was prepared in 80% yield from compound **16** using the same procedure as preparing Fluo HaloTag-1. ^{1}H NMR (CD_3OD , 400 MHz): δ 7.94 (dd, *J* = 1.6, 8.0 Hz, 1H), 7.63-7.58 (m, 2H), 7.18 (dd, *J* = 0.8, 6.4 Hz, 1H), 7.10 (t, *J* = 9.2 Hz, 2H), 6.63-6.56 (m, 3H), 3.95 (dd, *J* = 12.4, 42.4 Hz, 2H), 3.44-3.37 (m, 2H), 3.34 (s, 2H), 2.56-2.50 (m, 2H), 1.64-1.55 (m, 4H), 1.33-1.22 (m, 4H). MS analysis: 537.14 calculated for $C_{29}H_{28}ClNO_7$; observed: 538.36 $(M + H)^+$.

Synthesis of ZIMIR HaloTag (Figure 3)

Compound 1: Potassium tert-butoxide (2.25 g, 20 mmol) was added to a solution of tert-butyl (2-(2-hydroxyethoxy)ethyl)carbamate (4.065 g, 20 mmol) in anhydrous THF (50 mL) at 0 ^{o}C. One hour later, ethyl bromoacetate (2.7 ml, 24 mmol) was added. The reaction was warmed up to RT and continued overnight. The reaction mixture was then concentrated under vacuum, re-dissolved in 100 mL of $CHCl_3$, and washed with saturated saline. The organic phase was dried with anhydrous Na_2SO_4 and concentrated. The resulting mixture was purified by FC with hexane(Hex)/Ethyl Acetate(EA) (10:1 to 1:1) to give the product as a colorless oil (3.461 g, 60%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.20 (q, *J* = 7.2 Hz, 2H), 4.12 (s, 2H), 3.71-3.67 (m, 2H), 3.66-3.62 (m, 2H), 3.55-3.51 (m, 2H), 3.32-3.28 (m, 2H), 1.42 (s, 9H), 1.27 (t, *J* = 7.2 Hz, 3H).

Compound 3: Compound **1** (1.2 g, 4 mmol) was deprotected in TFA/DCM (1:1, 5 mL) at RT overnight. After removing the solvent under vacuum, the crude compound 2 was redissolved in $CHCl_3$ (4 mL) and used directly for the next step without further purification. K_2CO_3 (2.764 g, 20 mmol) and tert-butyl bromoacetate (0.222 mL, 1.56 mmol) were then added to the solution. The reaction mixture was stirred at RT overnight, diluted with 50 mL of $CHCl_3$, washed with saturated saline, dried with anhydrous Na_2SO_4 and concentrated under vacuum. The resulting oily residue was purified by FC with DCM/MeOH (50:1 to 20:1) to give the product as a colorless oil (238.2 mg, 50%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.22 (q, *J* = 7.2 Hz, 2H), 4,13 (s, 2H), 3.70 (m, 2H), 3.65 (m, 2H), 3.58 (t, *J* = 5.2 Hz, 2H), 3.30 (s, 2H), 2.80 (t, *J* = 5.2 Hz, 2H), 1.44 (s, 9H), 1.26 (t, *J* = 7.2 Hz, 3H). MS analysis: 305.18 calculated for $C_{14}H_{27}NO_6$; observed: 306.25 $(M + H)^+$, 328.22 $(M + Na)^+$.

Compound 4: Under argon, 2-methylresorcinol (2.01 g, 16.18 mmol) and 4-nitrophthalic anhydride (2.92 g, 15.12 mmol) were mixed in dry nitrobenzene (50 mL). After cooling the mixture to 0°C, we added $AlCl_3$ (4.7 g, 35.3 mmol) in one portion. The reaction was warmed up to RT and stirred for 16 h before it was poured into a mixture of hexane (60 mL) and an aqueous solution of HCl (1 M, 200 mL) under vigorous stirring. The precipitate was filtered and recrystallized twice from methanol/water to yield a powder containing a mixture of two nitro regioisomers (4.385 g, 91%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.83 (d, *J* = 2.0 Hz, 0.7H), 8.48 (d, *J* = 8.8 Hz, 0.7H), 8.40 (d, *J* = 8.8 Hz, 0.3H), 8.23 (d, *J* = 8.8 Hz, 0.3H), 8.18 (d, *J* = 2.0 Hz, 0.3H), 7.60(d, *J* = 8.4 Hz, 0.7H), 6.78 (d, *J* = 8.8 Hz, 0.3H), 6.74 (d, *J* = 8.8 Hz, 0.7H), 6.27-6.22 (m, 1H), 2.06 (s, 3H). MS analysis: 317.05 calculated for $C_{15}H_{11}NO_7$; observed: 318.18 $(M + H)^+$.

Compound 5: Compound **4** (3.172 g, 10 mmol) and resorcinol (1.156 g, 10.5 mmol) was dissolved in methanesulfonic acid (20 mL) and was heated at 90 ^{o}C in a sealed tube for 24 h. After cooling to RT, the mixture was poured into ice-cold water (200 mL). The resulting precipitate was filtered, washed with water (3 X 50 mL) and dried to yield a dark red powder (3.7 g, 94%). In addition to generating the desired compound **5** as a major product (~ 50%; MS analysis: 391.07 calculated for $C_{21}H_{13}NO_7$; observed: 392.12 $(M + H)^+$), this

reaction also yielded two other side products: 5(6)-nitro-carboxyfluorescein (~25%; MS analysis: 377.05 calculated for $C_{20}H_{11}NO_7$; observed: 378.10 $(M + H)^+$) and 4',5'-dimethyl-5(6)-nitro-carboxyfluorescein (~ 25%; MS analysis: 405.08 calculated for $C_{22}H_{15}NO_7$; observed: 406.14 $(M + H)^+$). The mixture was used directly for the following three steps. The unwanted isomers were removed by column separation during the preparation of intermediate compound **8.**

Compound 6a: (*Step f1, Figure 3*) The above mixed isomers containing compound **5** (3.92 g, 10 mmol) was added to a suspension of Cs_2CO_3 (7.16 g, 22 mmol) in dimethylformamide (DMF, 40 mL). Pivalic anhydride (4.5 mL) was added to this solution. Two hours later, the reaction mixture was filtered and washed with MeOH (20 mL). The filtrate was concentrated under vacuum. The resulting residue was extracted with $CHCl_3$ (3 × 50 mL) which was combined and washed with saturated brine. The organic layer was dried over Na_2SO_4, concentrated, and purified by FC (hexane/EtOAc, 20:1 – 4:1) to provide 5-nitro isomers (0.805 g, 14.4%), 6-nitro isomers (0.594 g, 10.6%), and the mixture of both isomers (1.276 g, 22.8%) as white solids. 6-nitro-4'-methyl fluorescein dipivaloyl ester (Compound **6a**): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.48 (dd, *J* = 1.2 7.2 Hz, 1H), 8.20 (d, *J* = 7.2 Hz, 1H), 8.00 (d, *J* = 1.2 Hz, 1H), 7.21 (q, *J* = 0.8, 1H), 6.84-6.65 (m, 4H), 2.31 (s, 3H), 1.39 (s, 18H); MS analysis: 559.18 calculated for $C_{31}H_{29}NO_9$; observed: 560.40 $(M + H)^+$. Each of the nitro regioisomer also contained two side products: 5-(or 6-)nitro-4',5'-dimethyl fluorescein dipivaloyl ester (described in Ref. [5]) and 5-(or 6-)nitro fluorescein dipivaloyl ester: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.50 (dd, *J* = 1.2 7.2 Hz, 1H), 8.22 (d, *J* = 7.2 Hz, 1H), 8.00 (d, *J* = 1.2 Hz, 1H), 7.12 (d, *J* = 1.6 Hz, 2H), 6.84-6.78 (m, 4H), 1.37 (s, 18H); MS analysis: 545.17 calculated for $C_{30}H_{27}NO_9$; observed: 546.39 $(M + H)^+$.

Compound 6: (*Step f2, Figure 3*) Compound **6a** (212 mg, 0.378 mmol; containing two other side products as minor components) was mixed with N-bromosuccinimide (102 mg, 0.569 mmol) and benzoyl peroxide (10 mg) in CCl_4 (10 mL). The mixture was refluxed for 4 h, cooled down and filtered. The solid residue was washed with Et_2O. The filtrate was concentrated and purified by FC (hexane/EtOAc, 10:1 – 8:1) to afford the product (203 mg, 84.3%; containing a bis-brominated side product derived from 6-nitro-4',5'-dimethyl

fluorescein dipivaloyl ester). A small sample of pure compound **6** was used for analysis. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.48 (dd, *J* = 1.2 7.2 Hz, 1H), 8.20 (d, *J* = 7.2 Hz, 1H), 8.00 (d, *J* = 1.2 Hz, 1H), 7.21 (q, *J* = 0.8, 1H), 6.84-6.65 (m, 4H), 4.67 (s, 2H), 1.39 (s, 18H).

Compound 7: Compound **6** (160 mg, 0.251 mmol; containing a side product as a minor component) was mixed with NaI (42 mg, 0.276 mmol), compound **3** (84.2 mg, 0.276 mmol) and proton sponge (60 mg, 0.276 mmol) in anhydrous acetonitrile (10 mL). The mixture was refluxed overnight and concentrated under vacuum. The resulting residue was purified by FC (hexane/EtOAc, 10:1 – 3:2) to yield the product as a yellow oil (142 mg, 70%; containing a side product as the minor component). A small sample of pure compound **7** was used for analysis. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.48 (dd, *J* = 1.6 6.8 Hz, 1H), 8.22 (d, *J* = 6.8 Hz, 1H), 8.04 (d, *J* = 1.6 Hz, 1H), 7.19 (d, *J* = 1.6, 1H), 6.84-6.79 (m, 2H), 6.76-6.70(m, 2H), 4.18 (q, *J* = 6.0 Hz, 2H), 4.14-4.04 (m, 4H), 3.68-3.65 (m, 2H), 3.60-3.52 (m, 4 H), 5.52 (s, 2H), 3.05-3.00 (m, 2H), 1.46 (s, 9H), 1.40 (s, 9H), 1.38 (s, 9H), 1.25 (t, *J* = 6.0 Hz, 3H). MS analysis: 862.35 calculated for $C_{45}H_{54}N_2O_{15}$; observed: 863.70 $(M + H)^+$, 885.70 $(M + Na)^+$.

Compound 8: Sodium hydrosulfide hydrate (400 mg) was added to a solution of the above compound **7** (60 mg, 70 μmol) in MeOH/THF/H_2O (4:1:1, 3 mL). The mixture was refluxed for 1.5 h. The solvent was concentrated under vacuum, and the residue was purified by reversed phase column chromatography (LiChroprep RP-18, EMD Chemicals) to yield the product as a red solid (40 mg, 90%; containing ~ 10% of bis-substituted side product). A small sample of pure compound **8** was used for analysis. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.84 (d, *J* = 8.4 Hz, 1H), 7.14-7.09 (m, 2H), 6.80 (dd, *J* = 2.4, 8.4 Hz, 1H), 6.56-6.49 (m, 3H), 6.40 (d, *J* = 2.4 Hz, 1H), 3.96-3.84 (m, 4H), 3.75-3.58 (m, 6H), 3.27 (s, 2H), 2.87-2.76 (m, 2H), 1.26 (s, 9H). MS analysis: 636.23 calculated for $C_{33}H_{36}N_2O_{11}$; observed: 637.57 $(M + H)^+$, 659.56 $(M + Na)^+$.

Compound 9: Compounds **8** (30 mg, 47 μmol) and **8a** (47 mg, 141 μmol; prepared as in Ref. [5]) were mixed in anhydrous MeOH (1.4 mL) containing an excess amount of dried Na_2SO_4 (100 eq). The mixture was stirred at

RT for two hours under argon. $NaCNBH_3$ (15 mg, 235 μmol) was then added. The reaction was continued at RT overnight, and the mixture was filtered. The filtrate was concentrated, and the resulting residue was purified by reversed phase column chromatography (LiChroprep RP-18). Compound **9** was obtained as a red film in 34% yield and ~ 10 mg of starting material was also recovered. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.34 (d, *J* = 3.6 Hz, 1H), 8.27 (d, *J* = 3.6 Hz, 1H), 7.67-7.58 (m, 3H), 7.25-7.14 (m, 4H), 6.79-6.75 (m, 2H), 6.68-6.64 (m, 2H), 6.61-6.54 (m, 2H), 6.11 (d, *J* = 1.6 Hz, 1H), 4.39 (d, *J* = 2.8 Hz, 2H), 3.99 (s, 2H), 3.92 (s, 2H), 3.80-3.74 (m, 2H), 3.68-3.63 (m, 6H), 3.19-3.13 (m, 4H), 3.02 (t, *J* = 5.6 Hz, 2H), 2.91 (t, *J* = 5.2 Hz, 2H), 2.85 (t, *J* = 4.8 Hz, 2H), 1.47 (s, 9H). MS analysis: 875.37 calculated for $C_{48}H_{53}N_5O_{11}$; observed: 876.27 $(M + H)^+$, 898.27 $(M + Na)^+$.

Compound 9a: (*Step j1*) PyBOP (12.2 mg, 23.4 μmol) was added to a DMF solution (0.5 mL) containing compound **9** (10 mg, 11.4 μmol) and a chloro-amino linker (5.1 mg, 22.8 μmol, prepared as in Ref.[2]). The mixture was stirred at RT overnight. DMF was then removed under vacuum and the resulting residue was purified by reversed phase column chromatography (LiChroprep RP-18). The product was obtained as a red film in 50% yield. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.31 (d, *J* = 4.8 Hz, 1H), 8.28 (d, *J* = 4.8 Hz, 1H), 7.73 (d, *J* = 8.8 Hz, 1H), 7.60 (td, *J* = 2.0 7.8 Hz, 1H), 7.60 (td, *J* = 1.6 8.0 Hz, 1H), 7.20-7.09 (m, 4H), 6.85-6.81 (m, 2H), 6.79 (dd, *J* = 2.4 8.8 Hz, 1H), 6.62 (d, *J* = 2.4 Hz, 1H), 6.55-6.48 (m, 2H), 6.10 (d, J = 2.0 Hz, 1H), 4.15 (q, J = 13.2 Hz, 2H), 3.98 (s, 2H), 3.75 (s, 2H), 3.71-3.37 (m, 22H), 3.06 (t, J = 6.4 Hz, 2H), 2.97-2.89 (m, 2H), 2.87 (s, 2H), 2.70 (t, J = 6.0 Hz, 2H), 1.79-1.66 (m, 2H), 1.60-1.50 (m, 2H), 1.47-1.36 (m, 2H), 1.41 (s, 9H), 1.34-1.26 (m, 2H). MS was performed: 1080.50 calculated for $C_{58}H_{73}ClN_6O_{12}$; observed: 1103.3 $(M+Na)^+$.

ZIMIR HaloTag: (*Step j2*) Compound 9a (5 mg) was dissolved in DCM/TFA (1:1, 1 mL). The mixture was stirred at RT for 3 h. After removing the solvents under vacuum, the product was obtained in quantitative yield. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.56 (d, *J* = 6.0 Hz, 1H), 8.45 (d, *J* = 5.2 Hz, 1H), 8.23 (td, *J* = 1.6 8.0 Hz, 1H), 8.02 (td, *J* = 1.6 8.0 Hz, 1H), 7.72-7.65 (m, 3H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.55-7.51 (m, 1H), 6.90-6.79 (m, 3H), 6.72-6.56 (m, 3H), 6.23 (d, J = 2.0 Hz, 1H), 4.15 (dd, J = 13.2 22.0 Hz, 2H), 4.45 (s, 2H), 4.23 (s, 2H), 4.01-

3.91(m, 6H), 3.81-3.65 (m, 8H), 3.60-3.31 (m, 14H), 3.23 (t, J = 6.0 Hz, 2H), 1.75-1.67 (m, 2H), 1.59-1.49 (m, 2H), 1.46-1.39 (m, 2H), 1.39-1.32 (m, 2H). MS analysis: 1024.43 calculated for $C_{54}H_{65}ClN_6O_{12}$; observed: 1025.03 $(M+H)^+$.

Reference:

1. Wu, J.; Liu, L.; Matsuda, T.; Zhao, Y.; Rebane, A.; Drobizhev, M.; Chang, Y. F.; Araki, S.; Arai, Y.; March, K.; Hughes, T. E.; Sagou, K.; Miyata, T.; Nagai, T.; Li, W. H.; Campbell, R. E., Improved Orange and Red Ca Indicators and Photophysical Considerations for Optogenetic Applications. *ACS chemical neuroscience* **2013,** *4* (6), 963-972.

2. So, M. K.; Yao, H.; Rao, J., HaloTag protein-mediated specific labeling of living cells with quantum dots. *Biochem Biophys Res Commun* **2008,** *374* (3), 419-23.

3. Wang, W.; Rusin, O.; Xu, X.; Kim, K. K.; Escobedo, J. O.; Fakayode, S. O.; Fletcher, K. A.; Lowry, M.; Schowalter, C. M.; Lawrence, C. M.; Fronczek, F. R.; Warner, I. M.; Strongin, R. M., Detection of homocysteine and cysteine. *J Am Chem Soc* **2005,** *127* (45), 15949-58.

4. Schmidt, F.; Rosnizeck, I. C.; Spoerner, M.; Kalbitzer, H. R.; Konig, B., Zinc(II)cyclen-peptide conjugates interacting with the weak effector binding state of Ras. *Inorg. Chim. Acta* **2011,** *365* (1), 38-48.

5. Li, D.; Chen, S.; Bellomo, E. A.; Tarasov, A. I.; Kaut, C.; Rutter, G. A.; Li, W. H., Imaging dynamic insulin release using a fluorescent zinc indicator for monitoring induced exocytotic release (ZIMIR). *Proc Natl Acad Sci U S A* **2011,** *108* (52), 21063-68.