Cytoplasmic delivery and selective, multi-component labeling with oligoarginine-linked protein tags.

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Experimental Methods.

1.0 Materials and analyses.

Unless otherwise noted, chemicals were obtained from Sigma-Aldrich, Inc., peptides were obtained from AnaSpec, Inc., and cell culture reagents were purchased from Invitrogen, Inc. The NHS ester of Lumi4 was prepared as previously described.[1] BG-PEG-NH2 (SNAP-Tag substrate) and BC-PEG-NH2 (CLIP-Tag substrate) were purchased from New England Biolabs. H2B-eDHFR plasmid DNA was provided by Prof. V.W. Cornish, and pcDNA3-TagRFP-T was obtained from Prof. R.Y. Tsien. MDCKII, HeLa and NIH3T3 cells were obtained from the American Type Culture Collection.

Electrospray ionization (ESI) high-resolution mass spectra (HRMS) were obtained by the Mass Spectrometry Facility, College of Chemistry, University of California, Berkeley, CA. MALDI-TOF mass spectra were obtained by the Mass Spectrometry Facility, Dept. of Chemistry, University of Illinois at Urbana. Flash chromatography was performed using EM Science Silica Gel 60 (230- 400 mesh). NMR spectra were obtained using a DRX-500 spectrometer operating at 500 (125) MHz for 1H (or 13C) respectively. 1H (or 13C) chemical shifts are reported in parts per million (ppm) relative to the solvent resonances, taken as δ 7.26 (δ 77.0) and δ 2.49 (δ 39.5) for $CDCl_3$ and $(CD_3)_2SO$, respectively. For the Lumi4 intermediates (**6**, **7**, **12**, **13**), the observed NMR spectra were very complicated due to the presence of differing conformers/isomers in solution, and are not reported.[1] Analytical HPLC was performed on an Agilent 1200 instrument (Agilent, Santa Clara, CA) equipped with a diode array detector (λ = 254, 280, 315 and 340 nm, 600 nm reference), a thermostat set at 25 °C, and a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 μm, Agilent, Santa Clara, CA). The mobile phase of a binary gradient (2-40% B/20 min; solvent A, 0.1% TFA; solvent B, ACN) at a flow rate of 1 mL/min was used for analytical HPLC. UV absorption measurements were recorded on a Varian Cary 300 double beam spectrometer using quartz cells of 1 cm path length.

2.0 Synthesis.

2.1 Preparation of N-Lumi4-cysteine amide of SNAP-Tag, $CysArg_9$ derivative 8.

N-FMOC-cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **3** (Scheme S1). In a 10 mL round bottom flask, N-hydroxysuccinimide (2.7 mg, 24 μmol) and N-FMOC-cysteine S-monomethoxytrityl ether **1** (9.9 mg, 16 μmol, Anaspec, Inc.) were dried together in vacuo. In a separate flask, BG-PEG-NH2 **2** (6.0 mg, 12.3 μmol) was dissolved in DMF (600 μL) and diisopropylethylamine (5 μL). Dimethylformamide (100 μL) and diisopropylcarbodiimide (3 μL, 29.2 μmol) were added to the flask containing compound **1**, and the resulting solution was allowed to stir under nitrogen for 9.5 hr. The solution containing compound **2** was transferred via syringe to this solution and stirred. After 16 hr, solvent was removed under reduced pressure, and the residue purified by silica gel chromatography using 7.5% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide N-FMOC-cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **3** (8 mg, 60%). 1H NMR (500 MHz, $CDCl_3$): δ = 11.85 (br s, 4H, NH), 7.68 (m, 4H, ArH), 7.51 (m, 4H, ArH), 7.34 – 7.16 (m, 17H, ArH), 6.83 (d, 2H, ArH), 6.74 (m, 3H, NH), 4.22 – 3.95 (m, 8H, CH, ArCH, $ArCH_2$), 3.79 (s, 3H, OCH_3), 3.70 – 3.20 (m, 20H, CH_2O, CH_2S), 3.26 (m, 4H, CH_2N). 13C NMR (500 MHz, $CDCl_3$): δ = 158.7, 147.1, 139.2, 129.2, 127.8, 127.1, 117.6, 113.2, 81.7, 55.2, 29.7. LC-MS pEI: calculated for $C_{60}H_{64}N_9O_9S$ $[MH]^+$, 1086.45, found, 1086.3.

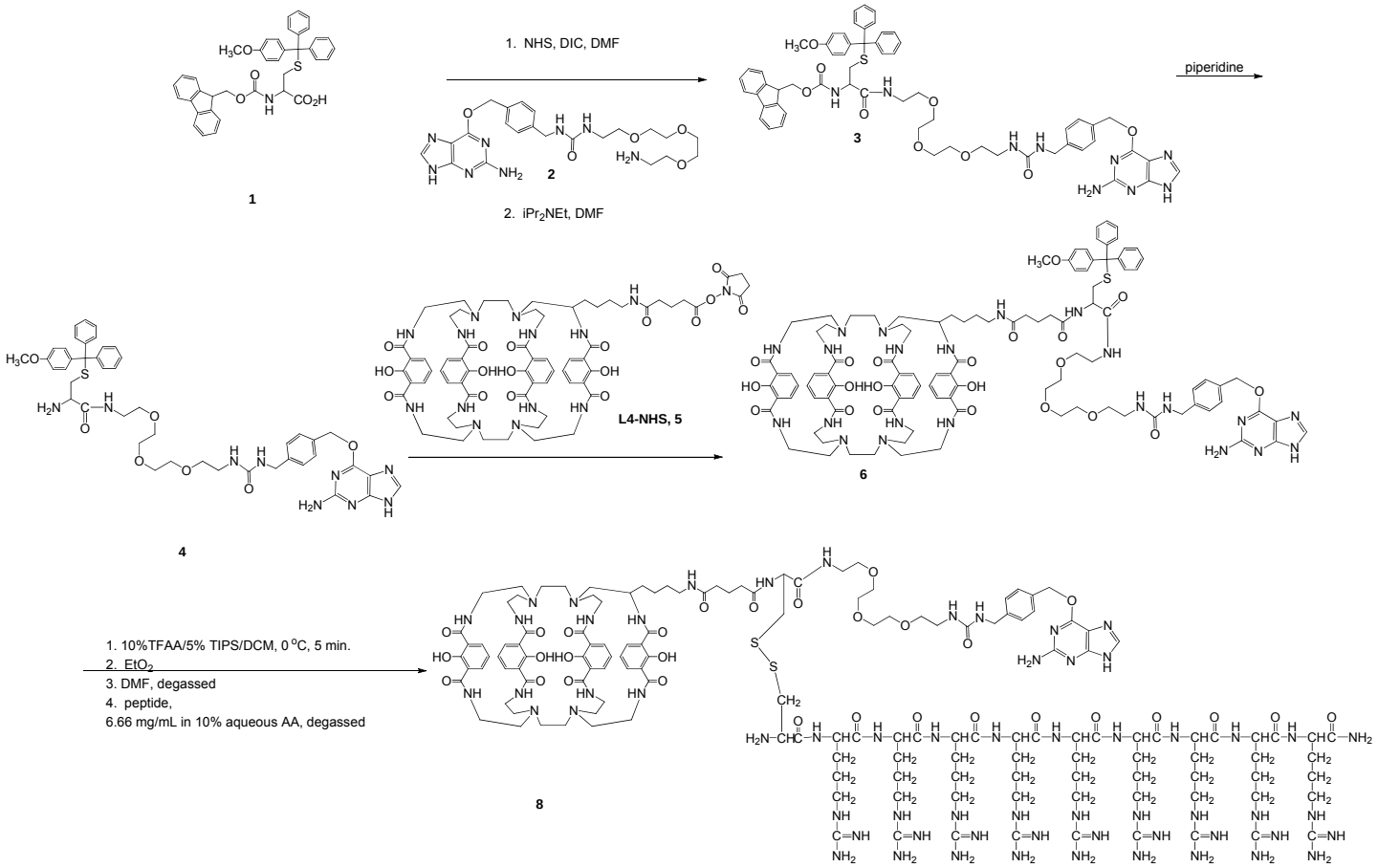
Cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **4** (7 mg, 66%) also eluted from the column in the last step upon changing solvent to 10% methanol in dichloromethane. LC-MS pEI: calculated for $C_{45}H_{54}N_9O_7S$ $[MH]^+$, 864.39, found, 864.3.

N-Lumi4-cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **6** (Scheme S1). Cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **4** (7 mg, 8 μmol) was dissolved in dimethylformamide (500 μL) and diisopropylethylamine (14 μL, 146 μmol), and then added to the NHS ester of Lumi4 (**5**, 5.7 mg, 4.3 μmol)

in an O-ring microcentrifuge tube. The resulting solution was shaken at ambient temperature for 48 hrs, whereupon the solution was transferred to 2 O-ring capped microcentrifuge tubes using 100 μL methanol, and ether was added (1.5 mL per tube). After standing for 4 hr at 4 °C, the tubes were centrifuged for 3 min at 12,000 rpm, and the supernatant was decanted and the pellets washed with ether. The pellets were allowed to air dry, and dissolved in methanol (300 uL) and dimethylformamide (100 μL/tube). Ether was added (1.5 mL/tube) to form a precipitate, and the tubes were allowed to stand overnight at 4 °C. The tubes were centrifuged for 3 min at 12,000 rpm, and the supernatant was decanted. The pellets were washed with ether, allowed to air dry, and then dried overnight in vacuo to provide compound **6** (6.2 mg, 70%). FTMS pEI: calculated for $C_{106}H_{132}N_{22}O_{21}S$ $[M+2H]^{2+}$, 1040.4824, found, 1040.4838.

N-Lumi4-cysteine, SNAP-Tag amide **7** (Scheme S1). A solution of 5% triisopropylsilane and 10% trifluoroacetic acid in dichloromethane (200 μL) was cooled to 0 °C, and added to N-Lumi4-cysteine-S-monomethoxytrityl ether, SNAP-Tag amide **6** (550 nmol). The resulting solution was blanketed with nitrogen, and shaken at 600 rpm for 5 min, whereupon ethyl ether (1.5 mL) was added. The suspension resulting upon mixing was stored briefly at 4 ^{o}C, and centrifuged at 12,000 rpm for 2 min. The supernatant was removed and the solids were washed with ethyl ether (1.2 mL). The suspension was centrifuged and supernatant was removed. The resulting solids were air dried briefly then dried in vacuo to provide the trifluoroacetate salt of compound **7**. Compound **7** was stored under nitrogen and used in the next step without further purification. LC-MS pEI: calculated for $C_{86}H_{115}N_{22}O_{20}S$ $[M+H]^{+}$, 1807.8, found, 1808.3.

N-Lumi4-cysteine amide of SNAP-Tag, $CysArg_9$ derivative **8** (BG-Lumi4-R_9, Scheme S1). The peptide with sequence H_2N-L-Cys(NPys)-$[Arg]_9$-$CONH_2$, (1 mg, 595 nmol,) was dissolved in a solution of 10% acetic acid in sterile water (150 μL) and degassed with nitrogen. A solution of N-Lumi4-cysteine, SNAP-Tag amide **7** (550 nmol) in dimethylformamide (40 uL), degassed with nitrogen, was added and the resulting solution was mixed at 800 rpm. The progress of the reaction was monitored using reversed phase HPLC. After 18 hr, the crude peptide was purified by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the compound (162 nmol, 29%). FTMS pEI: calc. for $C_{143}H_{232}N_{60}O_{30}S_2$ $[M+4H]^{4+}$, 833.4473, found, 833.4476.



Scheme S1

2.2 Preparation of N-Lumi4-cysteine amide of CLIP-TAG, $CysArg_9$ derivative 14. (Scheme S2).

N-FMOC-cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **10** (Scheme S2). In a 10 mL round bottom flask, N-hydroxysuccinimide (2.3 mg, 20 µmol) and N-FMOC-cysteine S-monomethoxytrityl ether **1** (10.7 mg, 17.3 µmol, Anaspec, Inc.) were dried together in vacuo. In a separate flask, BC-PEG-NH2 **9** (6.0 mg, 13 µmol) was dissolved in DMF (400 µL) and diisopropylethylamine (3.5 µL). Dimethylformamide (500 µL) and diisopropylcarbodiimide (3 µL, 29.2 µmol) were added to the flask containing compound **1**, and the resulting solution was allowed to stir under nitrogen for 9.5 hr. The solution containing compound **9** was transferred via syringe to this solution and stirred. After 18 hr, solvent was removed under reduced pressure, and the residue purified by silica gel chromatography using 5% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide N-FMOC-cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **10** (11 mg, 78%). 1H NMR (300 MHz, $CDCl_3$): δ = 11.84 (s, 1H, NH), 9.19 (s, 1H, NH), 7.99 (s, 1H, CH), 7.70 (m, 2H, ArH), 7.53 (m, 2H, ArH), 7.35 – 7.08 (m, 20H, ArH), 6.81 (d, 1H, ArH), 6.75 (d, 2H, ArH), 6.50 (s, 1H, NH), 5.59 (s, 1H, NH), 5.41 (s, 2H, $PhCH_2O$), 5.08 (s, 2H, $PhCH_2N$), 4.24 (m, 3H, NH, $ArCH_2$), 4.14 (m, 1H, ArCH), 4.00 (m, 1H, CH), 3.71 (s, 3H, OCH_3), 3.51 – 3.30 (m, 16H, CH_2O, CH_2NCO), 2.60 (m, 2H, CH_2S). 13C NMR (75 MHz, $CDCl_3$): δ = 178.4, 177.5, 175.0, 172.1, 170.7, 158.4, 157.3, 156.2, 145.0, 143.9, 141.5, 136.7, 132.4, 131.0, 129.7, 129.2, 128.3, 128.1, 127.4, 127.1, 125.4, 120.3, 118.1, 113.5, 70.9, 70.5, 70.2, 69.8, 67.3, 66.9, 63.5, 63.0, 55.5, 53.2, 49.3, 47.3, 39.7, 36.8, 34.2, 32.2, 31.7, 30.0, 25.9, 25.2. FTMS pEI: calculated for $C_{59}H_{64}N_7O_9S$ $[MH]^+$, 1046.4481, found, 1046.4460.

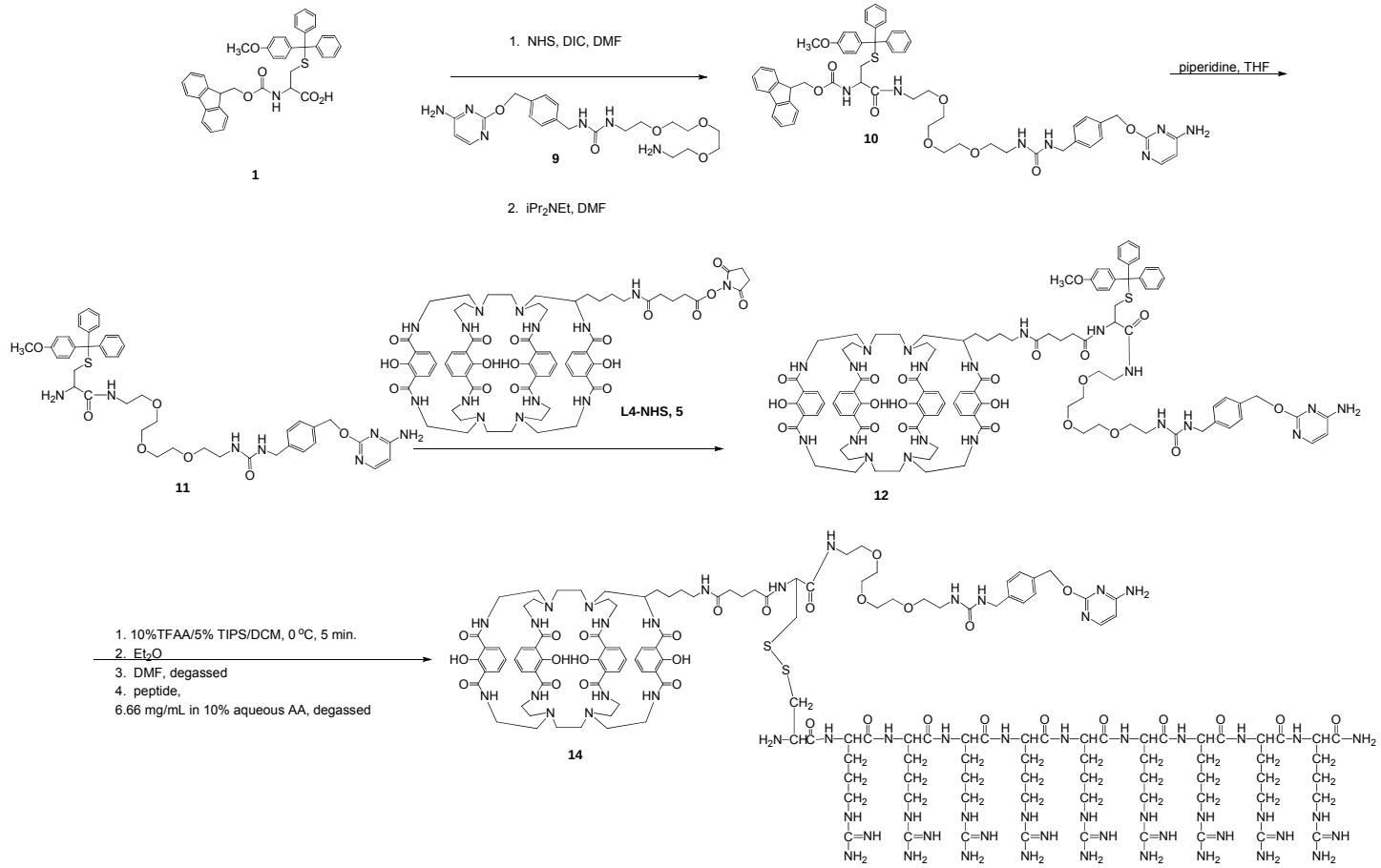
Cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **11** (Scheme S2). N-FMOC-cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **10** (11 mg, 11 umol) was dissolved in tetrahydrofuran (1 mL). Piperidine (10.4 µL) was added, and the resulting solution was stirred at ambient temperature for 21 hr.

Solvents were removed under reduced pressure, and the residue dried in vacuo. The residue was purified by silica gel chromatography using 5 – 10% methanol in dichloromethane as eluent. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide compound **11** (6 mg, 69%). FTMS pEI: calculated for $C_{44}H_{54}N_7O_7S$ $[MH]^+$, 824.3800, found, 824.3790.

N-Lumi4-cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **12** (Scheme S2). Cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **11** (5 mg, 6 μmol) was dissolved in dimethylformamide (500 μL) and diisopropylethylamine (10 μL), and then added to the NHS ester of Lumi4 (**5**, 6.2 mg, 4.7 μmol) in an O-ring microcentrifuge tube. The resulting solution was shaken at ambient temperature for 24 hrs, whereupon the solution was divided between 2 O-ring capped microcentrifuge tubes, and ether was added (1.2 mL per tube). After standing for 2 hr at 4 °C, the tubes were centrifuged for 3 min at 12,000 rpm, and the supernatant was decanted and the pellets washed with ether. The pellets were allowed to air dry, and dissolved in methanol (200 uL) and dimethylformamide (50 μL/tube). Ether was added (1.2 mL/tube) to form a precipitate, and the tubes were allowed to stand overnight at 4 °C. The tubes were centrifuged for 3 min at 12,000 rpm, and the supernatant was decanted. The pellets were washed with ether, allowed to air dry. The pellets were dissolved in methanol (200 uL) and dimethylformamide (50 μL/tube) and sampled for quantification by UV-vis absorbance. Ether was added (1.2 mL/tube) to form a precipitate, and the tubes were allowed to stand overnight at 4 °C. The tubes were centrifuged for 3 min at 12,000 rpm, and the supernatant was decanted. The pellets were washed with ether, allowed to air dry, and then dried overnight in vacuo. Compound **12** was quantified by UV-vis absorbance assuming λ_{max} in MeOH at 315 nm = 26,000 M^{-1} cm^{-1} (2.90 umol, 62%). FTMS pEI: calculated for $C_{105}H_{132}N_{20}O_{21}S$ $[M+2H]^{2+}$, 1020.4793, found, 1020.4794.

N-Lumi4-cysteine, CLIP-Tag amide **13** (Scheme S2). A solution of 5% triisopropylsilane and 10% trifluoroacetic acid in dichloromethane (200 μL) was cooled to 0 °C, and added to N-Lumi4-cysteine-S-monomethoxytrityl ether, CLIP-Tag amide **12** (730 nmol). The resulting solution was blanketed with nitrogen, and stored on ice for 5 min, whereupon ethyl ether (1.5 mL) was added. The suspension resulting upon mixing was stored briefly at 4 ^oC, and centrifuged at 12,000 rpm for 3 min. The supernatant was removed and the solids were washed with ethyl ether (1.2 mL). The suspension was centrifuged and supernatant was removed. The resulting solids were air dried briefly then dried in vacuo to provide the trifluoroacetate salt of compound **13**. Compound **13** was stored under nitrogen and used in the next step without further purification. FTMS pEI: calculated for $C_{85}H_{116}N_{20}O_{20}S$ $[M+2H]^{2+}$, 884.4192, found, 884.4190.

N-Lumi4-cysteine amide of CLIP-Tag, $CysArg_9$ derivative **14** (BC-Lumi4-R_9, Scheme S2). The peptide with sequence H_2N-L-Cys(NPys)-$[Arg]_9$-$CONH_2$, (1 mg, 595 nmol,) was dissolved in a solution of 10% acetic acid in sterile water (150 μL) and degassed with nitrogen. A solution of N-Lumi4-cysteine, CLIP-Tag amide **13** (730 nmol) in dimethylformamide (40 uL), degassed with nitrogen, was added and the resulting solution was mixed at 800 rpm. The progress of the reaction was monitored using reversed phase HPLC. After 18 hr, the crude peptide was stored at 4 °C and purified by HPLC using a reversed phase column and 0.1% trifluoroacetic acid and acetonitrile gradient. Fractions containing product were combined, lyophilized, and the purified peptide conjugate was dissolved in sterile water. Lumi4 absorbance at 340 nm (ε_{340} = 26,000, pH 7.4) was used to quantify the compound (151 nmol, 25%). FTMS pEI: calc. for $C_{142}H_{232}N_{58}O_{30}S_2$ $[M+4H]^{4+}$, 823.4458, found, 823.4456.



Scheme S2

2.5 Metallation with terbium and conjugate luminescence characterization.

Lyophilized conjugates were dissolved in sterile water (conc. = 1 mM) in 1.5 mL microfuge tubes. $TbCl_3$ (~1.2 equiv.) in water was added, and the samples were vortexed ~5 min. and allowed to rest at RT for ~30 min. All samples exhibited bright green luminescence following dissolution in water and addition of terbium when exposed to light from a hand-held UV lamp.

3.0 Plasmids.

H2B-mCherry-eDHFR. The gene encoding mCherry was subcloned from H2B-mCherry[13] to pRSETB-EGFP-eDHFR to generate pRSETb-mCherry-eDHFR. A 700 bp BamHI to BsrGI fragment encoding mCherry was amplified from H2B-mCherry using the primers 5'-G CAT ACG TCG GAT CCC ATG GTG AGC AAG GGC GA-3' (BamHI, coding strand) and 5'-G CAT ACG TCT GTA CAC CTT GTA CAG CTC GTC CAT-3' (BsrGI, non-coding strand). This fragment was inserted between the BamHI site and the BsrGI site in pRSETb-EGFP-eDHFR to get pRSETb-mCherry-eDHFR.

The gene encoding mCherry-eDHFR was subcloned from pRSETb-mCherry-eDHFR to H2B-eDHFR to generate pH2B-mCherry-eDHFR. A 1203 bp BamHI to NotI fragment encoding mCherry-eDHFR with an N-terminal linker (Ala-Ser-Ala-Ser-Ser-Ser) was amplified by PCR from pRSETb-mCherry-eDHFR using the primers 5'-T CGT GAT CTG GAT CCC GCT TCT GCT TCG TCT TCG ATG GTG AGC AAG GGC-3' (BamHI, coding strand) and 5'-GCT TTG TTA GCG GCC GCA CCA TGC TTA GAA TCC TTA CCG -3' (NotI, non-coding strand). This fragment was inserted between the BamHI site and the NotI site in H2B-eDHFR to get pH2B-mCherry-eDHFR. Plasmid integrity was confirmed by direct sequencing.

pH2B-GFP-SNAP and pH2B-GFP-CLIP. The gene encoding EGFP was subcloned from plasmid pH2B-GFP-eDHFR to pCLIPf-H2B and pSNAPf-H2B to construct pH2B-GFP-CLIP and pH2B-GFP-SNAP, respectively. A 753bp NheI to AgeI fragment encoding EGFP with and an N-terminal GCT TCT GCT TCG TCT TCG linker and a C-terminal GGA TCT GGA GGA TCT GGA linker was prepared by PCR from pH2B-GFP-eDHFR using the primers 5'-GCT TTG TTT GCT AGC GCT TCT GCT TCG TCT TCG-3' (NheI, coding strand) and 5'-GCT TTG TTT ACC GGT TCC AGA TCC TCC AGA TCC-3' (AgeI, non-coding strand). This fragment was inserted between the NheI site and the AgeI site in pCLIPf-H2B or pSNAPf-H2B to yield pH2B-GFP-CLIP or pH2B-GFP-SNAP. Plasmid integrity was confirmed by DNA sequencing.

4.0 Cell culture and transfection and labeling.

MDCKII, NIH3T3, and HeLa cells were cultured in DMEM (+) (Dulbecco's Modified Eagle Media supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/mL penicillin and 100 μg/mL of streptomycin) at 37 °C and 5% CO_2. For all labeling studies, cells were passaged fewer than ten times following thawing from frozen stocks. For transient transfection, cells were seeded at 10^5 cells per well into a 6-well plate. After ~18 h incubation at 37 °C and 5% CO_2, adherent cells (~80% confluent) were transfected with 2 μg of desired plasmid DNA using Lipofectamine2000™ (Invitrogen) according to manufacturer's instructions. Approximately 6 h after transfection, cells were trypsinized and reseeded at 15,000 cells/well into 8-well chambered coverglasses (Nunc™, 12-565-470) and incubated at 37 °C and 5% CO_2 overnight.

Stable expression of H_2B-EGFP-eDHFR and H2B-mCherry-eDHFR in MDCKII cells. MDCKII cells were grown to ca. 70-80% confluency in a 10 cm dish. The cells were transfected with 24 μg of H2B-EGFP-eDHFR or H2B-mCherry-eDHFR plasmid using 60 μL of Lipofectamine2000™. The stable transfectants were selected with culture media containing 2.5 mg/mL G418 and individual clones were isolated after approximately 2 weeks. The stable clones were maintained in media containing 0.4 mg/mL G418.

Incubation of cells with peptides. For labeling with peptides, transiently or stably transfected cells growing in DMEM (+) in 8-well chambered coverglasses were washed 1X with PBS and re-immersed in DMEM (-) (same as DMEM (+) without FBS) at the desired temperature (37 °C or 22 °C) containing peptides at the indicated concentration. Cells were then incubated at 37 °C and 5% CO_2 (in ambient atmosphere for 22 °C studies), washed 2X in PBS and reimmersed in DMEM (+) supplemented with 1 mM Patent Blue™ dye to quench extracellular luminescence. Following incubation and washing, cells were maintained at 37 °C and 5% CO_2 until imaging.

5.0 Microscopy, image processing and analysis.

Imaging of adherent live cells was performed using a previously described, modified epifluorescence microscope (Zeiss Axiovert 200).[2] All images were acquired using an EC Plan Neofluar, 63X, 1.25 N.A. objective (Carl Zeiss, Inc.) Filter cubes containing the appropriate excitation and emission filters and dichroics allowed for wavelength selection. Continuous wave fluorescence images were acquired using an Axiocam MRM CCD camera (Carl Zeiss, Inc.). For time-gated luminescence, pulsed excitation light from a UV LED (λ_{em}, 365 nm; illumination intensity, 0.5 W/cm^2 at the sample plane) was synchronized with the intensifier component of an ICCD (Mega-10EX, Stanford Photonics, Inc.) such that a 10 μs delay was inserted between the end of the LED pulse and the intensifier start-time. For each acquisition, the signal from multiple excitation/emission events was accumulated on the ICCD sensor and read out at the end of the camera frame. The source/camera timing parameters were the same for all of the time-gated images and data presented here: excitation pulse width = 1500 μs; pulse period = 3000 μs; delay time = 10 μs; intensifier on-time = 1480 μs. The sensitivity of time-gated imaging is dependent on the number of excitation/detection events integrated on the CCD during a single camera frame and on the intensifier gain voltage. Frame length was varied in increments of

66.7 ms up to a maximum of 2000 ms, and the number of excitation/detection events per frame clock (66.7 ms time increment) was fixed at 22. Frame summing was used to increase the signal-to-noise ratio and to remove ion-feedback noise from the intensifier. Each frame summed effectively increases the bit depth of the resulting image in increments of 1024 (i.e., 1 frame yields bit depth equal to 1024, 2 frames, 2048, etc.). All time-gated images reported here comprised four summed frames and have a bit-depth of 4096 (12-bit).

ICCD images (tagged image file format, .TIF) were captured with Piper control software (v2.4.05, Stanford Photonics, Inc.) and Axiocam images (.ZVI) were captured with Zeiss AxioVision software (v4.6). Images were processed using NIH ImageJ (v1.42). For the data presented in Figure 3C of the main text, it was necessary to standardize pixel intensity values from time-gated images obtained at varying exposure times and intensifier gain voltages. A calibration specimen consisting of a concentrated fluorescein solution (0.15 g/mL in 0.1 N NaOH) sealed between a coverslip and a microscope slide was used to provide a consistent field of illumination. Images of the fluorescein specimen were obtained by pulsing the LED excitation on for 60 ms during a single, 66.7 ms camera frame, and exposure time was varied by changing the intensifier on-time during the LED pulse. A series of 4-frame images at different exposures were captured such that mean pixel gray values spanned the 12-bit dynamic range. Calibration curves were generated for each intensifier gain level by plotting mean pixel gray value vs. exposure time. Pixel values were normalized by dividing raw pixel gray level by the slope of the corresponding calibration curve and by the frame length.

The mean background value was subtracted from all time-gated images using the Image Math feature of NIH ImageJ. Correction of Tb^{3+} donor bleedthrough was accomplished by measuring signals from cells containing Tb probe in both the Tb and mCherry emission channels (**Supporting Figure S2**). The mCherry channel signal was plotted as a function of the Tb channel signal, a line was fit to the data, and the slope of that line was taken to be the correction constant. In samples where Tb-to-mCherry FRET was measured, the background-subracted Tb channel image was multiplied by the bleedthrough correction constant, and the resulting image was subtracted from the background-subtracted mCherry channel image to yield the true mCherry FRET image.

6.0 References.

1 Xu, J.; Corneillie, T. M.; Moore, E. G.; Law, G. L.; Butlin, N. G.; Raymond, K. N. *J Am Chem Soc* **2011**, *133*, 19900-19910.

2. Gahlaut, N.; Miller, L.W. *Cytometry A* **2010**, *77*, 1113-1125.

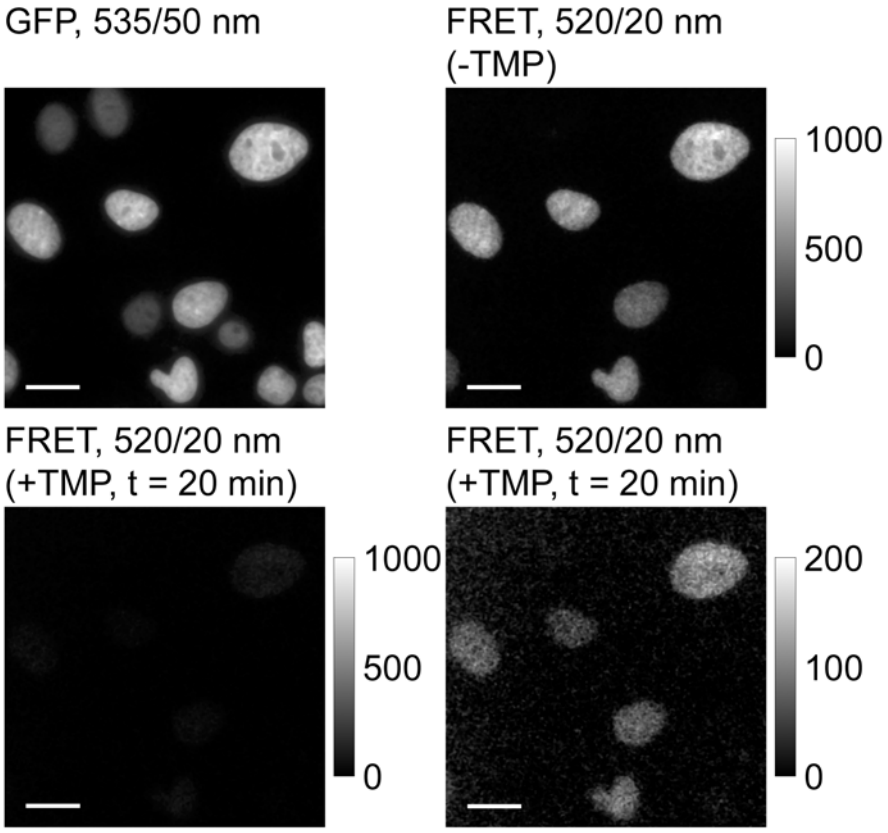


Figure S1. Addition of TMP to culture substantially reduces binding of TMP-Lumi4 to eDHFR and associated FRET signal, as evidenced by continuous wave (upper left) and time-gated, Tb^{3+}-to-GFP FRET (upper right and bottom) images of MDCKII cells stably expressing H2B-GFP-eDHFR that were incubated with TMP-Lumi4-R_9 (in DMEM w/o serum, 10 μM, 30 min), washed and imaged. The FRET signal before addition of TMP to the growth medium is shown in the upper right image. Twenty min after TMP addition (final conc., 100 μM), the FRET signal is reduced ca. 90% (bottom images). Micrographs: upper left, continuous wave fluorescence (λ_{ex} = 535/15 nm); upper right and bottom, time-gated Tb^{3+}-to-GFP FRET (delay = 10 μs, λ_{ex} = 365 nm). Emission wavelengths as indicated. Scale bars, 20 μm. Bottom images represent same raw data rendered at different contrast levels, as indicated by accompanying intensity displays.

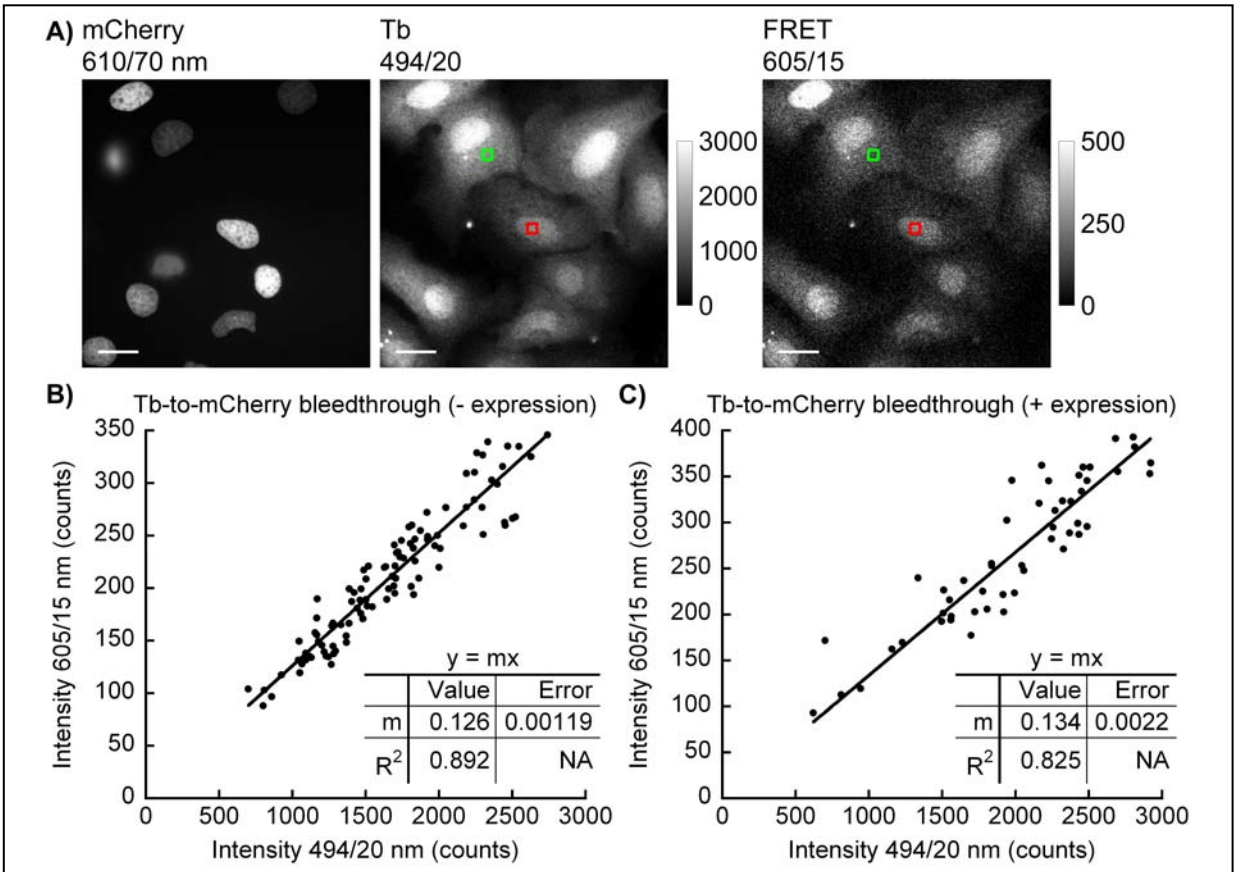


Figure S2. Bleedthrough of Tb^{3+} signal into the mCherry channel and measurement of diffusion enhanced energy transfer between Lumi4-Tb^{3+} and mCherry. **A)** Representative images showing MDCKII cells stably expressing H2B-mCherry-eDHFR that were incubated with BG-Lumi4-R_9 (in DMEM w/o serum, 10 μM, 30 min), washed and imaged. Mean pixel gray values were measured in the Tb and FRET channels in corresponding regions of interest (ROIs) that coincided with locations of fusion protein expression (red squares) and non-expression (green squares). Micrographs: left, continuous wave fluorescence (λ_{ex} = 535/15 nm); middle, time-gated Tb^{3+} luminescence (delay = 10 μs, λ_{ex} = 365 nm); right, time-gated Tb^{3+}-to-mCherry FRET (delay = 10 μs, λ_{ex} = 365 nm). Emission wavelengths as indicated. Scale bars, 20 μm. **B)** and **C)** Plots of FRET channel intensities vs. Tb channel intensities measured in non-expressing ROIs **(B)** or expressing ROIs **(C)**. Lines are linear fits to data with y-intercepts forced to 0. The slope of the plot in **(B)** is the bleedthrough correction factor applied to the mCherry FRET image in Figure 4 of the paper.

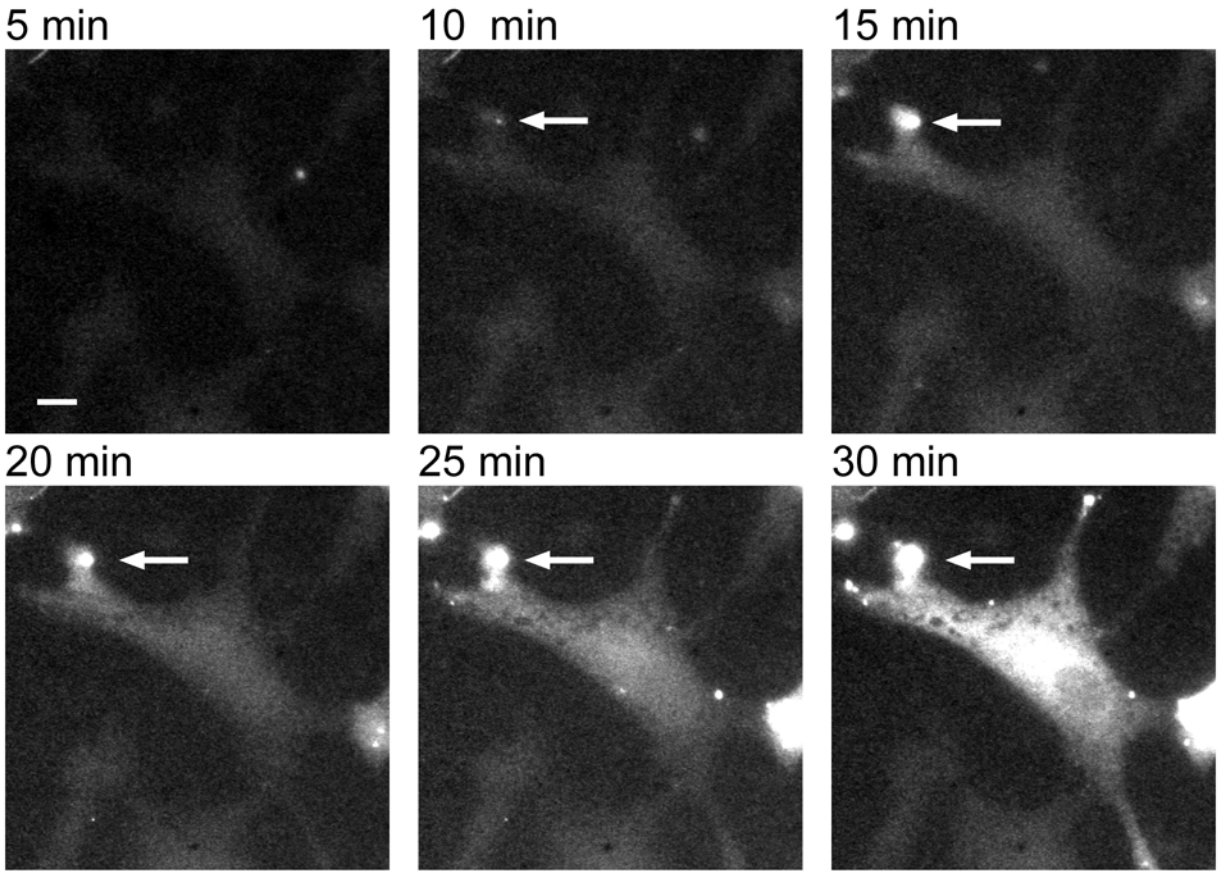


Figure S3. Transduction of TMP-Lumi4-R_9 into the cytoplasm. NIH3T3 cells were exposed to TMP-Lumi4-R_9 at a concentration of 10 μM in serum-free DMEM and time-gated images of Tb^{3+} luminescence were obtained every 5 min. Six frames recorded at 5, 10, 15, 20, 25 and 30 min are shown. Arrows indicate the spatially confined area of the plasma membrane from which the peptides spread throughout the cytoplasm.