Photolabile Fluorescently Quenched Peptide Cassettes

Hsien-Ming Lee, Melanie A. Priestman, and David S. Lawrence*

Department of Chemistry, Division of Medicinal Chemistry and Natural Products, Department of Pharmacology University of North Carolina, Chapel Hill, NC 27599

Supporting Information

EXPERIMENTAL PROCEDURES

All amino acids, NovaSyn TGR resin, 5-chloro-1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HCTU), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) were obtained from NovaBiochem or Peptide International. 4-{4-[1-(9 Fluorenylmethyloxycarbonylamino)ethyl]-2-methoxy-5-nitrophenoxy}butanoic acid (Fmoc-photolabile linker **5**) and 3-Nα-Fmoc-Amino-3-(2-nitrophenyl) propionic acid (Fmoc-photolabile linker **6**) were from Advanced ChemTech. N,N-dimethylformamide (DMF), methylene chloride (CH_2Cl_2), 10 X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4), and other general solvents were obtained from Fisher Scientific. N,N-diisopropylethylamine (DIEPA), 4-dimethylaminophenylazophenyl-4'-maleimide (Dabcyl maleimide, **10**) , and dithiothreitol (DTT) were purchased from Sigma. Fluorescein-5-isothiocynate (5-FITC, **3**), QSY7-maleimide **7**, QSY9-maleimide **8** and QSY-35 maleimide **9,** were purchased from Invitrogen. 5-Carboxyltetramethylrhodamine (5-TAMRA, **4**) was purchased from ChemPep. Bovine mitochondria was purchased from MitoSciences.

High pressure liquid chromatography (HPLC) analysis was performed using a Waters 600 solvent delivery system and Waters Delta 600 controller with a 2996 Photodiode Array detector. Analyses and purification were carried out either on analytical (Apollo C18, 4.6 X 250 mm) or semipreparative (Apollo C18, 19 X 250 mm) column. The HSQC/HMBC 2D NMR of **4-5-8** (DMSO-d_6) was recorded using an INOVA 500. ESIMS were recorded using Agilent 6100 Series Single Quad LC/MS system. Fluorescence was measured using the Photon Technology International QuantaMaster 40 spectrofluorometer. Photolysis experiments were performed using an Oriel Hg arc lamp (NewPort, power supply model 69907, with a 200 Watts Hg lamp) or a photocrosslinker light box (Stratagene, model UV Stratalinker 1800) for the FRET cassette library photolysis. A 360 nm color glass filter (300 - 400 nm band pass, NewPort, model UG-1) was used for all photolysis experiments. Photolysis power was measured using a Coherent FieldMaxII-TO power meter equipped with a PS19 thermal pile power sensor. Mitochondria fluorescence was measured using a BD LSRII flow cytometer.

Peptide Library Synthesis, Purification and Characterization.

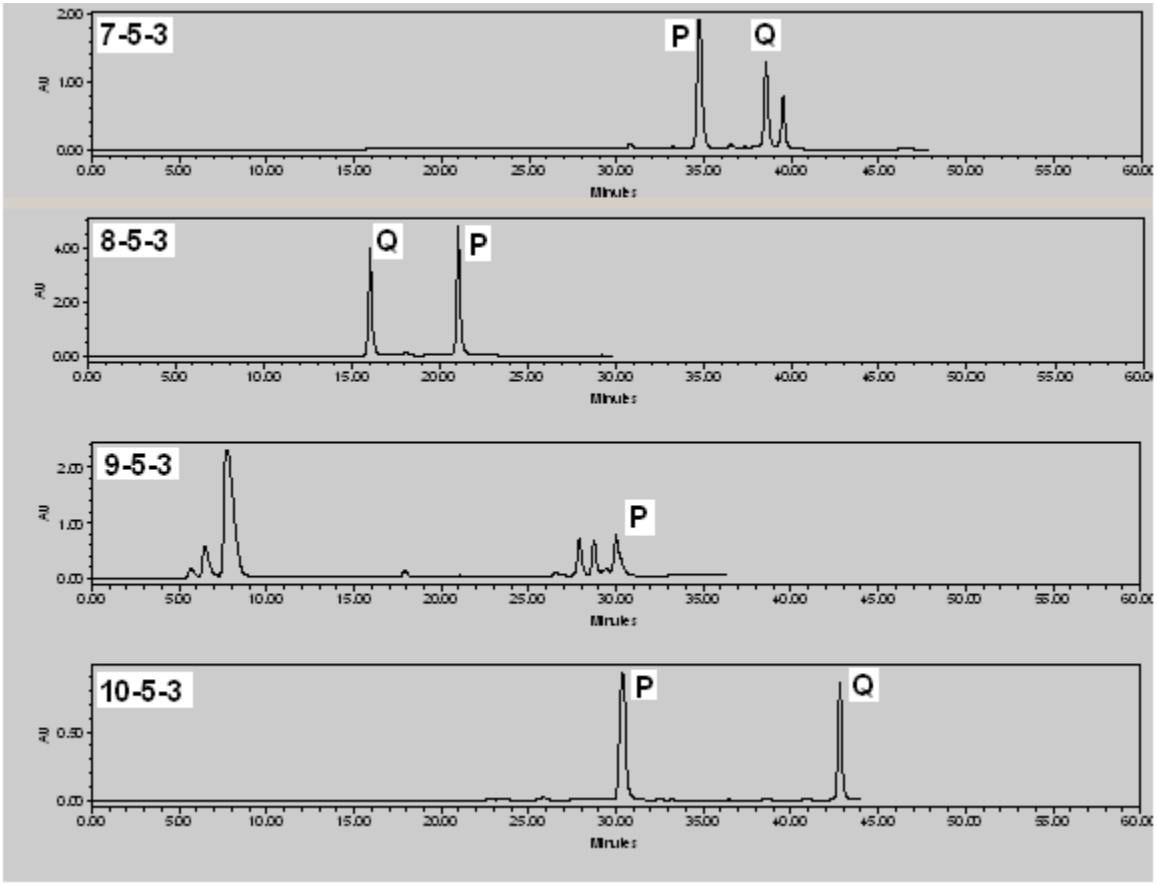
Fmoc-Cys(Trt)-OH, Fmoc-Lys(ivDde)-OH, and Fmoc-photolabile linkers were used to make the four different peptide backbone configurations (Table S1, column 1) on the TGR resin (0.2 mmol/g, 500 mg resin, 0.1 mmol scale). A standard peptide coupling protocol was used for all amino acid couplings including the Fmoc-photolabile linkers (Fmoc-**5** and Fmoc-**6**). Amino acid (3 equiv/equiv resin), HCTU/6-Cl-HOBt (3 equiv/equiv resin) and DIEPA (8 equiv/equiv resin) were mixed with resin in DMF and reacted for 2 h at RT. Fmoc deprotection was achieved using 20% piperidine in DMF for 20 min at RT. The N-terminus was capped using acetic anhydride with 20% DIEPA for 20 min.

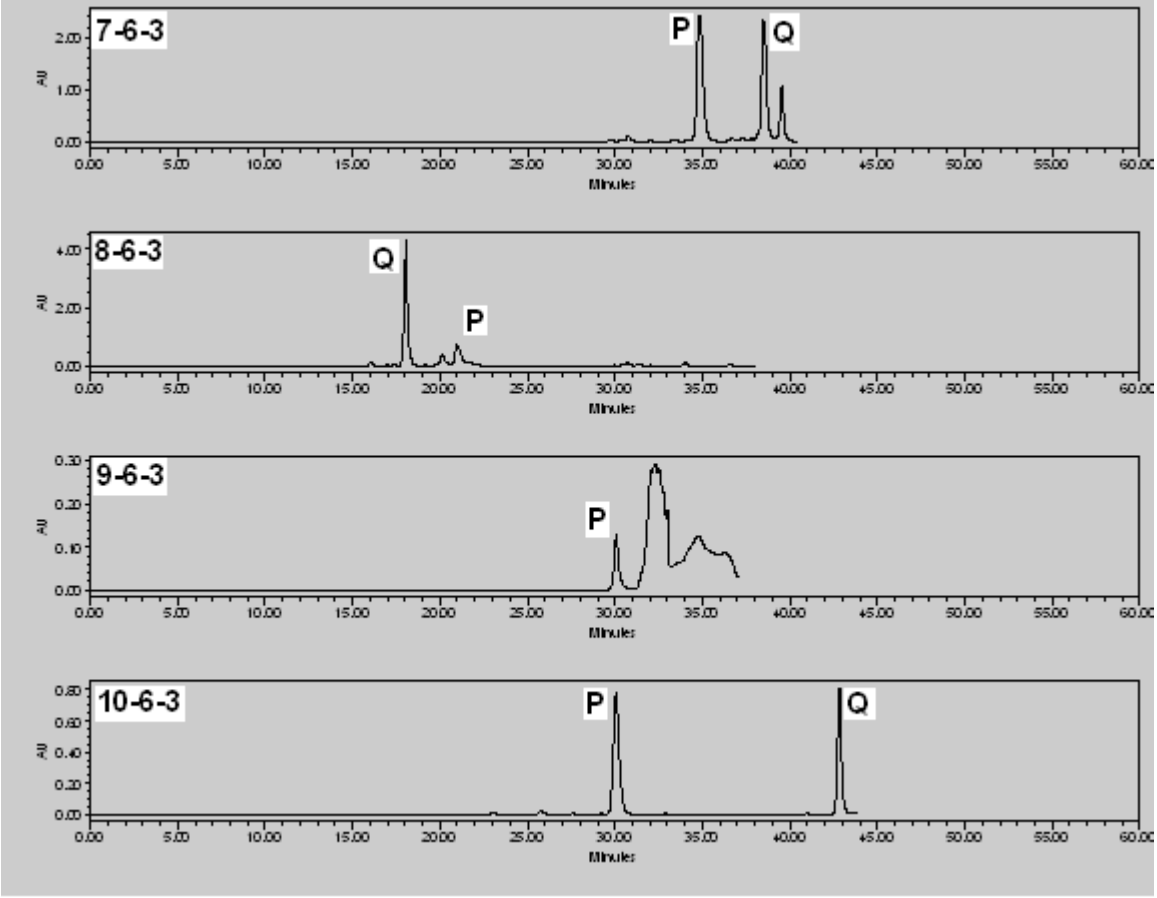
Following N-terminus acetylation, the Lys side-chain (ivDde) protecting group was deprotected using 2% hydrazine monohydrate in DMF (25 mL/g resin of hydrazine solution, 3 X 3 min). Each of the four backbone peptidyl resins were split in half (50 μmol scale) for the fluorophore labeling reactions (Table S1, column 2). For 5-FITC (**3**) labeling, the Lys side chain deprotected peptidyl resins were reacted with 5-FITC (3 equiv, 150 μmol) with DIEPA (8 equiv) in DMF for 2 h at RT. For the 5-TAMRA (**4**) labeling, 5-TAMRA (3 equiv, 150 μmol) was preactivated using HCTU (3 equiv, 150 μmol) with DIEPA (8 equiv) in DMF for 1 min and reacted with the Lys deprotected peptidyl resins for 2 h.

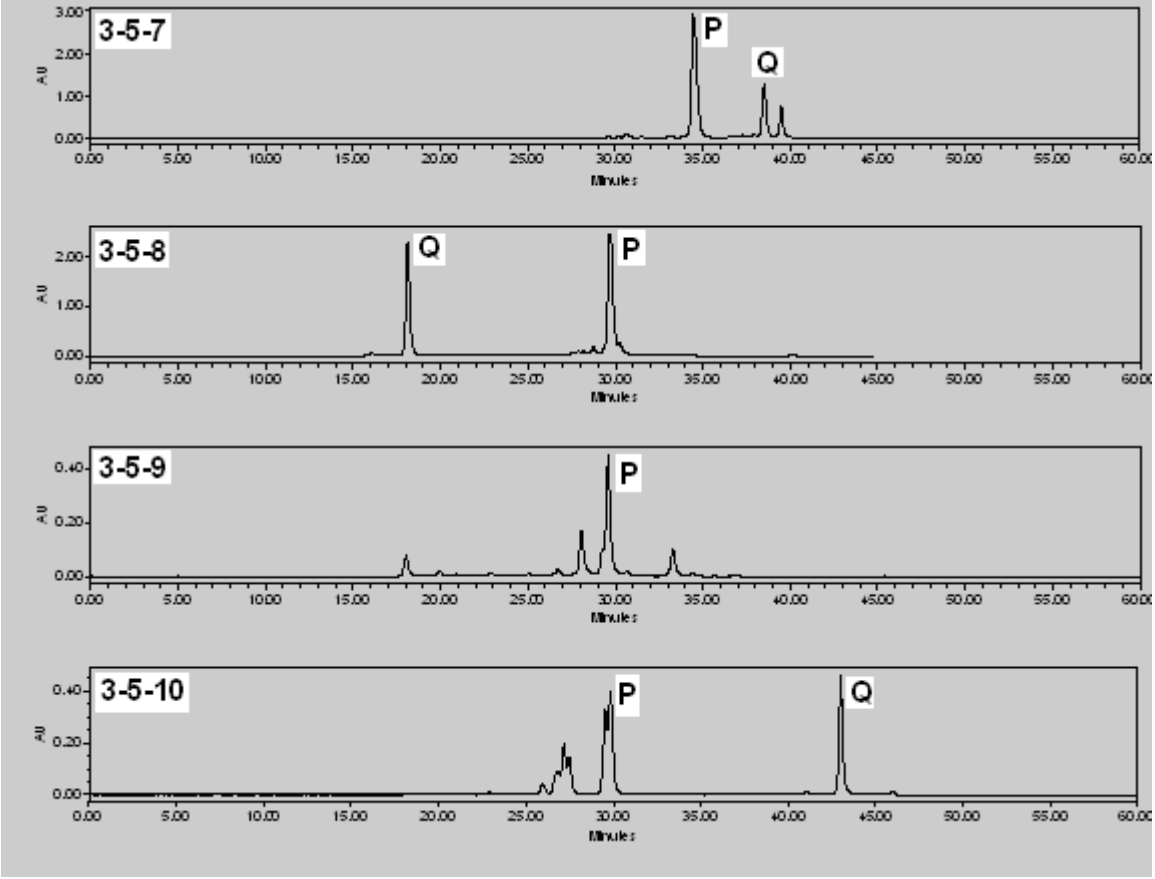
After the fluorophore labeling, the eight peptidyl resins were dried and subsequently cleaved in a solution of 95% TFA, 2.5% H_2O and 2.5% triisopropylsilane. The fluorophore-labeled peptides were HPLC (semiprepative C-18 column) purified using a linear gradient binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 80:20 (0 min) to 30:70 (30 min) (Fig. S-1). The major fraction for each peptide was collected and freeze-dried. The eight individual peptides were dissolved in DMF (10 mM stock solution) and further aliquoted into 4 vials (400 μL, 4 μmol each vial, total 32 vials) for the quencher labeling reaction (Table S1, column 3). The thiol-reactive quenchers **7**, **8**, **9**, or **10** (2 equiv, 8 μmol) was added to the vials and the peptide/quencher mixtures further diluted to 1 mL with 50% DMF/ 50% PBS at pH 7.5, and reacted for 2 h to generate the thirty-two peptide member library. All members of the library were purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 80:20 (0 min) to 30:70 (30 min). The major fraction from each reaction was collected and characterized by ESIMS (Table S-2). The subsequently prepared DMF stock solutions employed the HPLC purified peptides. Peptide concentrations were obtained by using (1) the extinction coefficient of the λ_{max} of quenchers or (2) the extinction coefficient summation of quenchers + fluorophores at the λ_{max} of quenchers if there is an overlap between the fluorophore and quencher absorbance spectra:

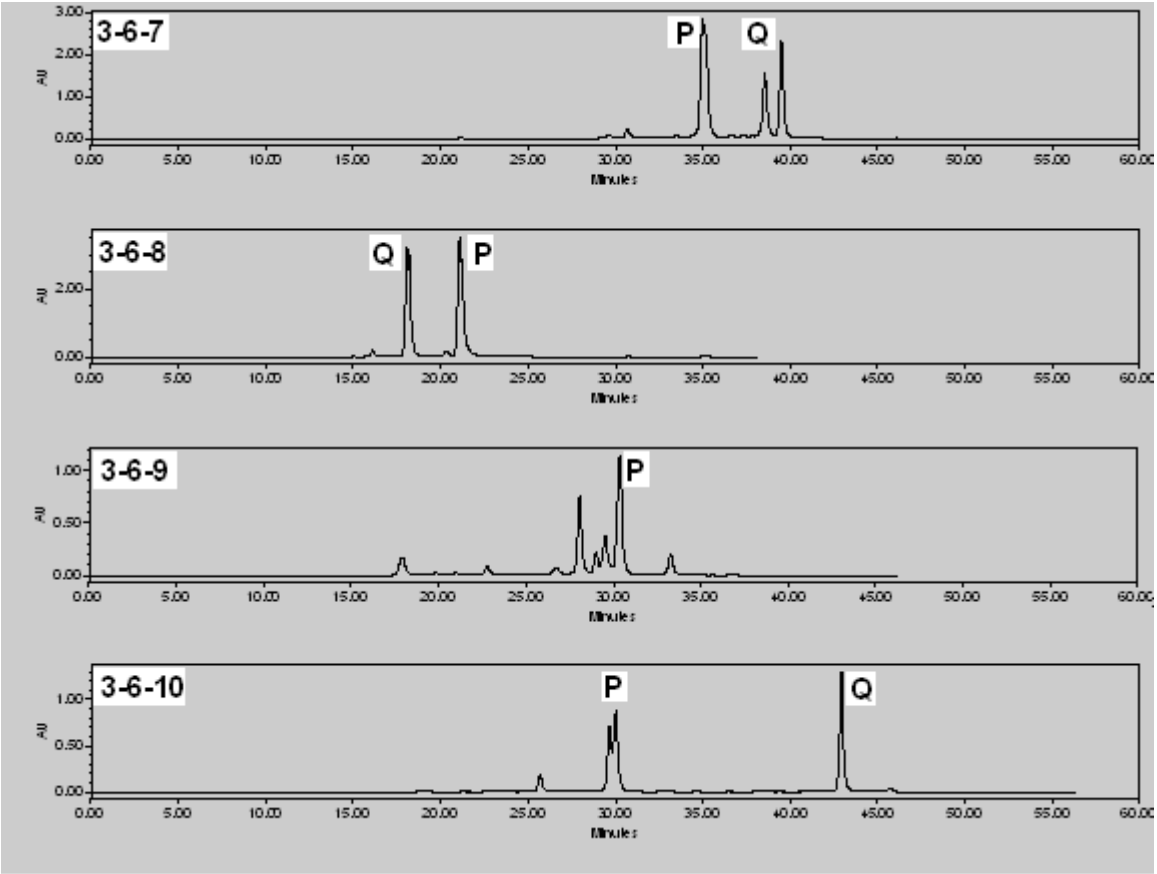
For the fluorescein cassettes, the concentrations of QSY 7/9 containing cassettes (**7-5-3, 8-5-3, 7-6-3, 8-6-3, 3-5-7, 3-6-8, 3-6-7, 3-6-8**) were determined using $\varepsilon_{QSY7/9}$ = 90000 $cm^{-1}M^{-1}$ @ 560 nm. The concentrations of QSY 35 cassettes (**9-5-3**, **9-6-3**, **3-5-9**, **3-6-9**) were determined using $\varepsilon_{QSY35+fluorescein}$ = 73083 $cm^{-1}M^{-1}$ @ 476 nm. The concentrations of dabcyl containing cassettes (**10-5-3**, **10-6-3**, **3-5-10**, **3-6-10**) were determined using $\varepsilon_{Dabcyl+fluorescein}$ = 51017 $cm^{-1}M^{-1}$ @ 452 nm. For TAMRA cassettes, the concentrations of QSY7/9 containing cassettes (**7-5-4, 8-5-4, 7-6-4, 8-6-4, 4-5-7, 4-5-8, 4-6-7, 4-6-8**), concentrations were determined using $\varepsilon_{QSY7/9+TAMRA}$ = 166000 $cm^{-1}M^{-1}$. The concentration QSY 35 containing cassettes (**9-5-4**, **9-6-4**, **4-5-9**, **4-6-9**) were determined using only ε_{QSY35} (24000 $cm^{-1}M^{-1}$ @ 476 nm), and the concentration of dabcyl containing cassettes (**10-5-4**, **10-6-4**, **4-5-10**, **4-6-10**) were deterermined using only ε_{Dabcyl} (32000 $cm^{-1}M^{-1}$ @ 452 nm).

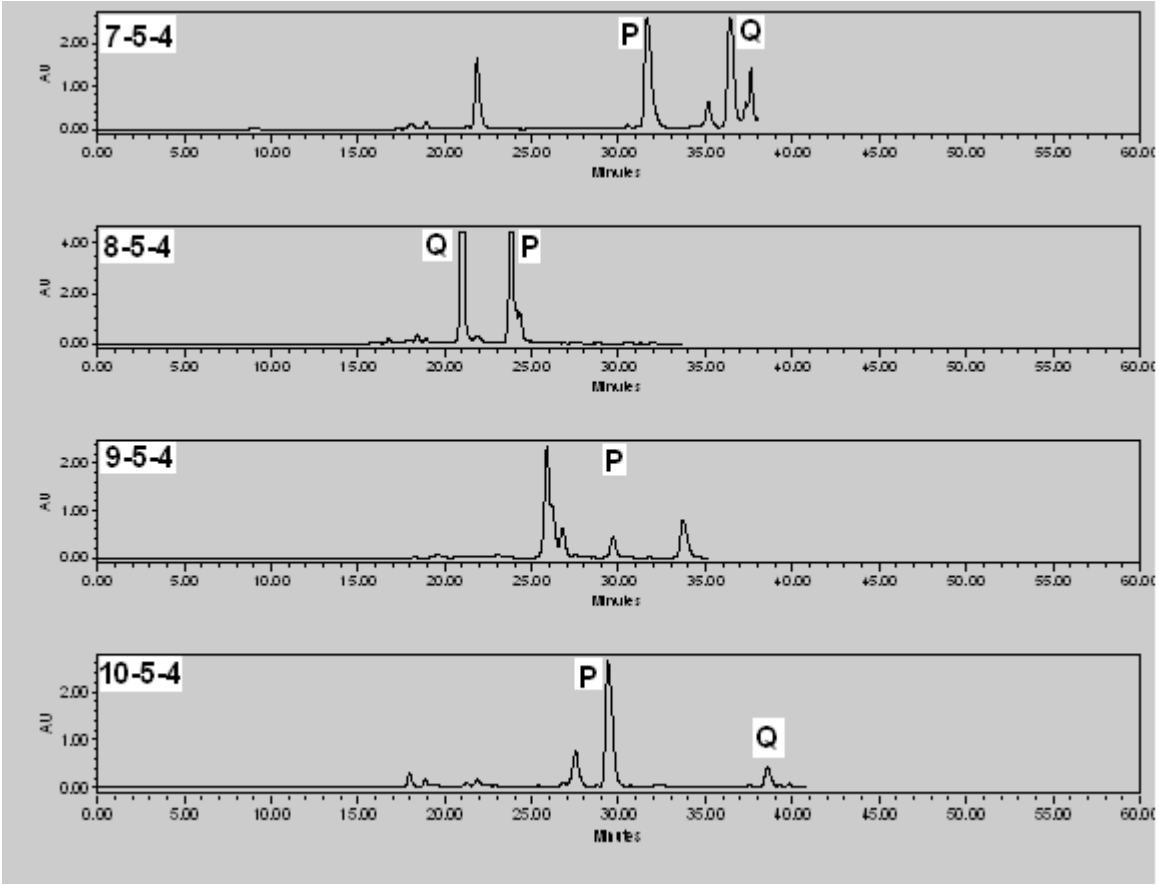
Figure S-1. HPLC of crude peptide cassette libraries. All cassettes were purified using the following gradient: 80% solvent A/20% solvent B (0 min) to 30% solvent A/70% solvent B (30 min) to 10% solvent A/90% solvent B (40 min), except **7-6-4**, **10-6-4, 4-6-7** where the gradient setting was 20% solvent B (0 min) to 30% solvent B (5 min) to 70% solvent B (35 min) to 90% solvent B (45 min). The peptide cassette (P) and excess quencher (Q) are labeled in the chromatograms (characterized and confirmed by mass spectrometry).

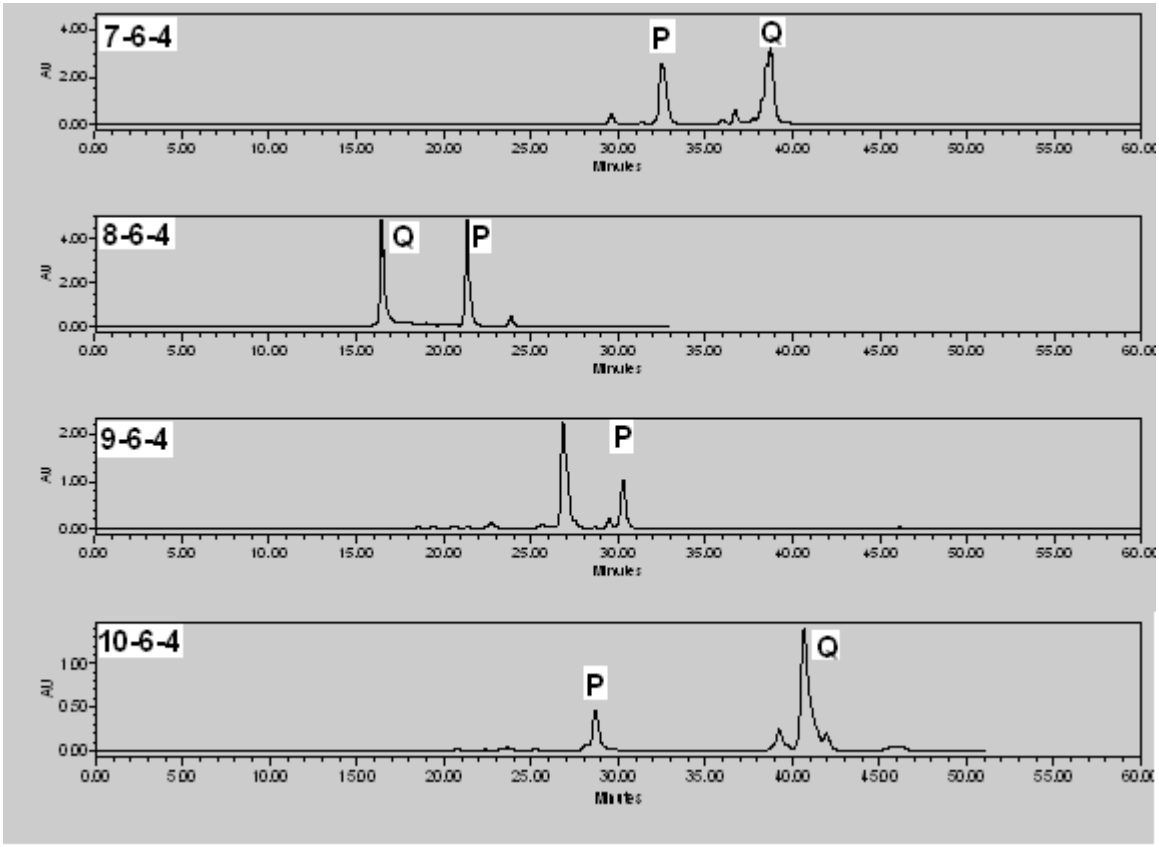


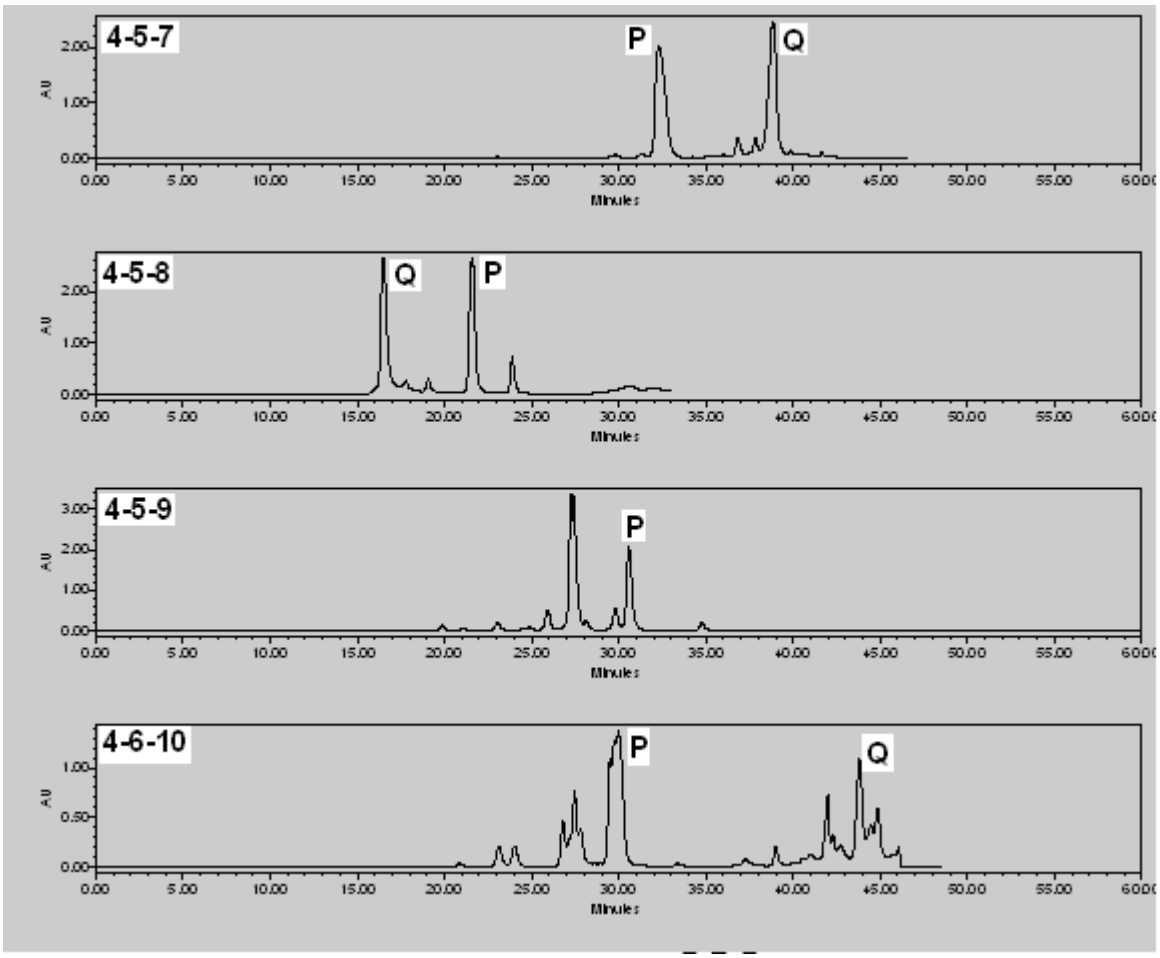

7-6-3
P
Q
8-6-3
Q
P
9-6-3
P
10-6-3
P
Q
AU
Minutes


3-5-7
P
Q
3-5-8
Q
P
3-5-9
P
3-5-10
P
Q
AU
Minutes


3-6-7
P
Q
3-6-8
Q
P
3-6-9
P
3-6-10
P
Q
AU
Minutes


7-5-4
P
Q
8-5-4
Q
P
9-5-4
P
10-5-4
P
Q
AU
Minutes


7-6-4
P
Q
8-6-4
Q
P
9-6-4
P
10-6-4
P
Q
AU
Minutes


4-5-7
P
Q
4-5-8
Q
P
4-5-9
P
4-6-10
P
Q
AU
Minutes

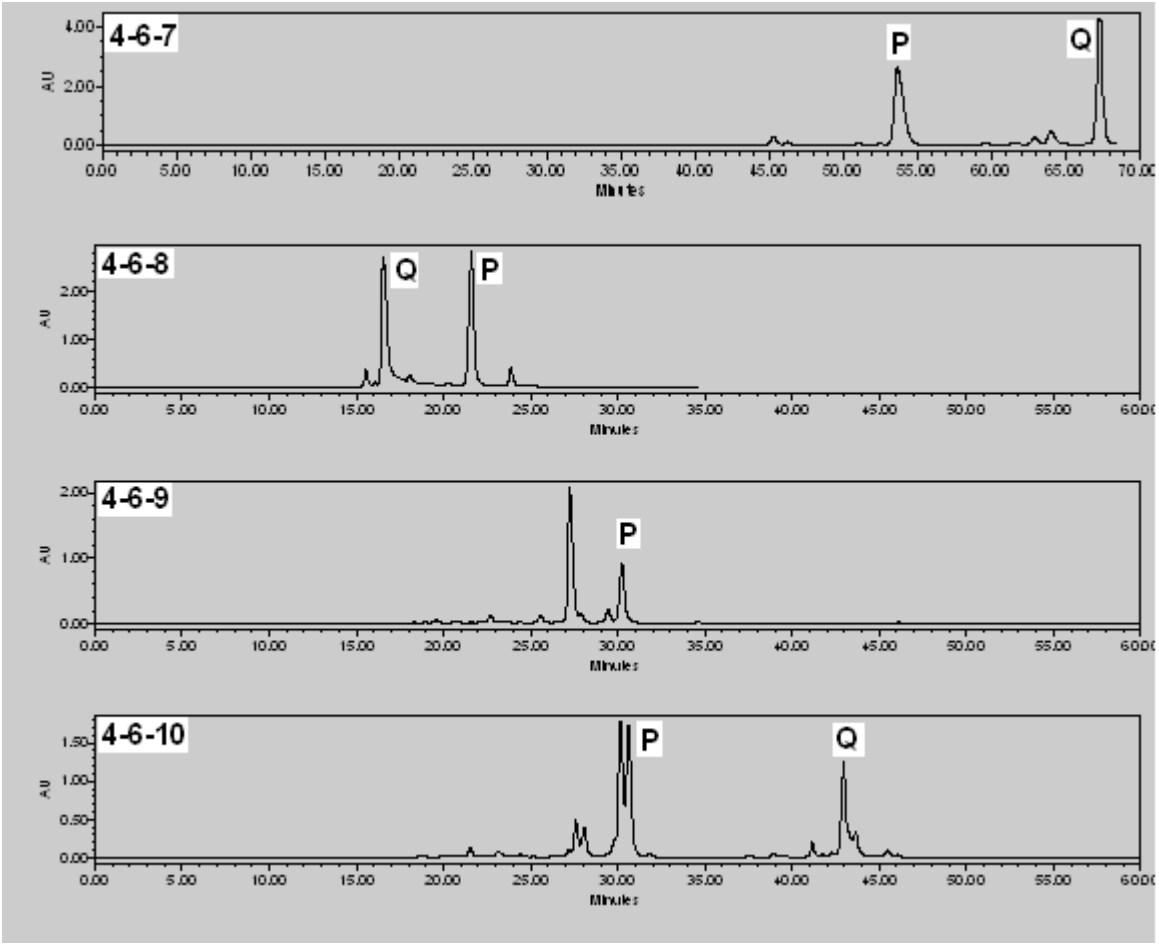

4-6-7
P
Q
AU
Minutes
4-6-8
Q
P
AU
Minutes
4-6-9
P
AU
Minutes
4-6-10
P
Q
AU
Minutes

Table S-1. Synthetic splitting pattern for the fluorescently quenched peptide cassette library. Note: Red signifies fluorescein and green signifies TAMRA library members.

Tripeptide Backbone	Fluorophore Labeled	Quencher Labeled
Ac-Cys-**5**-Lys-Resin	Ac-Cys-5-3-Resin	7-5-3
		8-5-3
		9-5-3
		10-5-3
	Ac-Cys-5-4-Resin	7-5-4
		8-5-4
		9-5-4
		10-5-4
Ac-Cys-**6**-Lys-Resin	Ac-Cys-6-3-Resin	7-6-3
		8-6-3
		9-6-3
		10-6-3
	Ac-Cys-6-4-Resin	7-6-4
		8-6-4
		9-6-4
		10-6-4
Ac-Lys-**5**-Cys-Resin	Ac-3-5-Cys-Resin	3-5-7
		3-5-8
		3-5-9
		3-5-10
	Ac-4-5-Cys-Resin	4-5-7
		4-5-8
		4-5-9
		4-5-10
Ac-Lys-**6**-Cys-Resin	Ac-3-6-Cys-Resin	3-6-7
		3-6-8
		3-6-9
		3-6-10
	Ac-4-6-Cys-Resin	4-6-7
		4-6-8
		4-6-9
		4-6-10

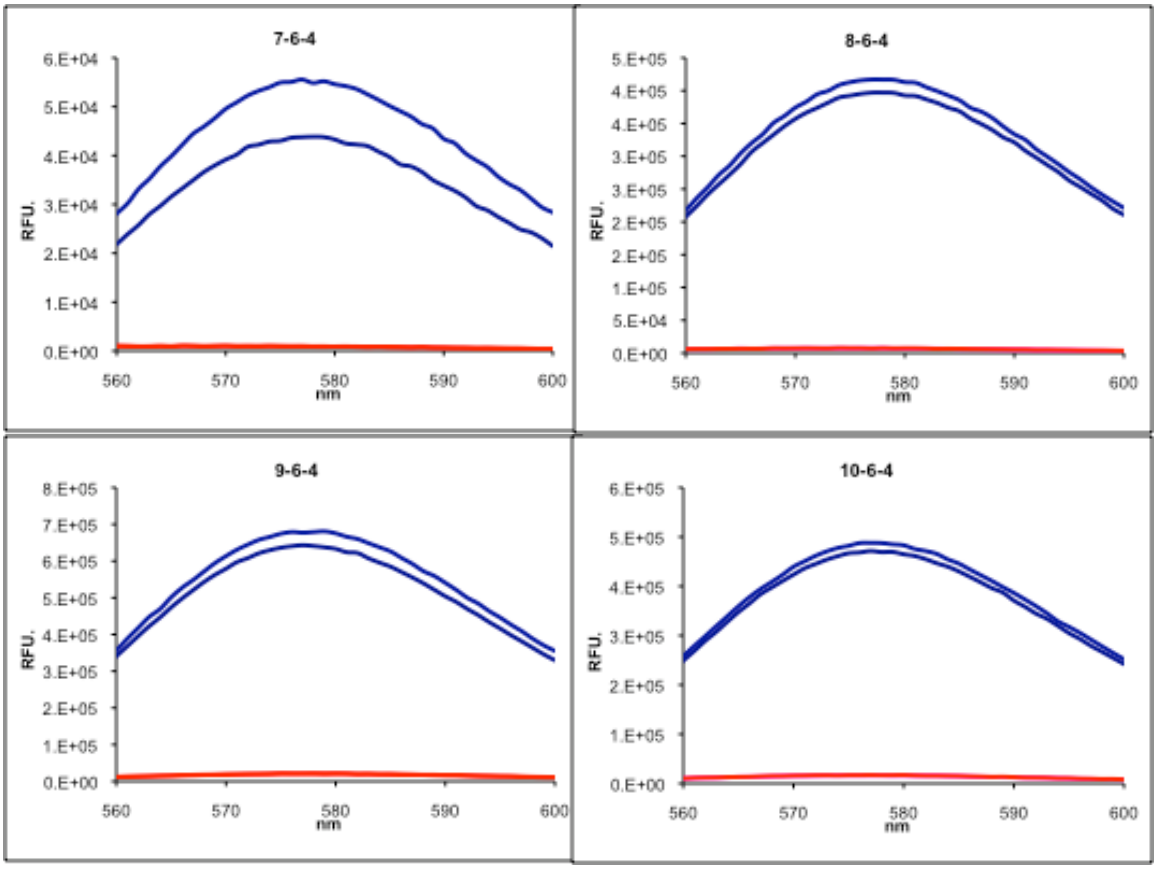
Table S-2. The ESI MS results of the fluorescently quenched peptide cassette library. Note: Red signifies fluorescein and green signifies TAMRA library members.

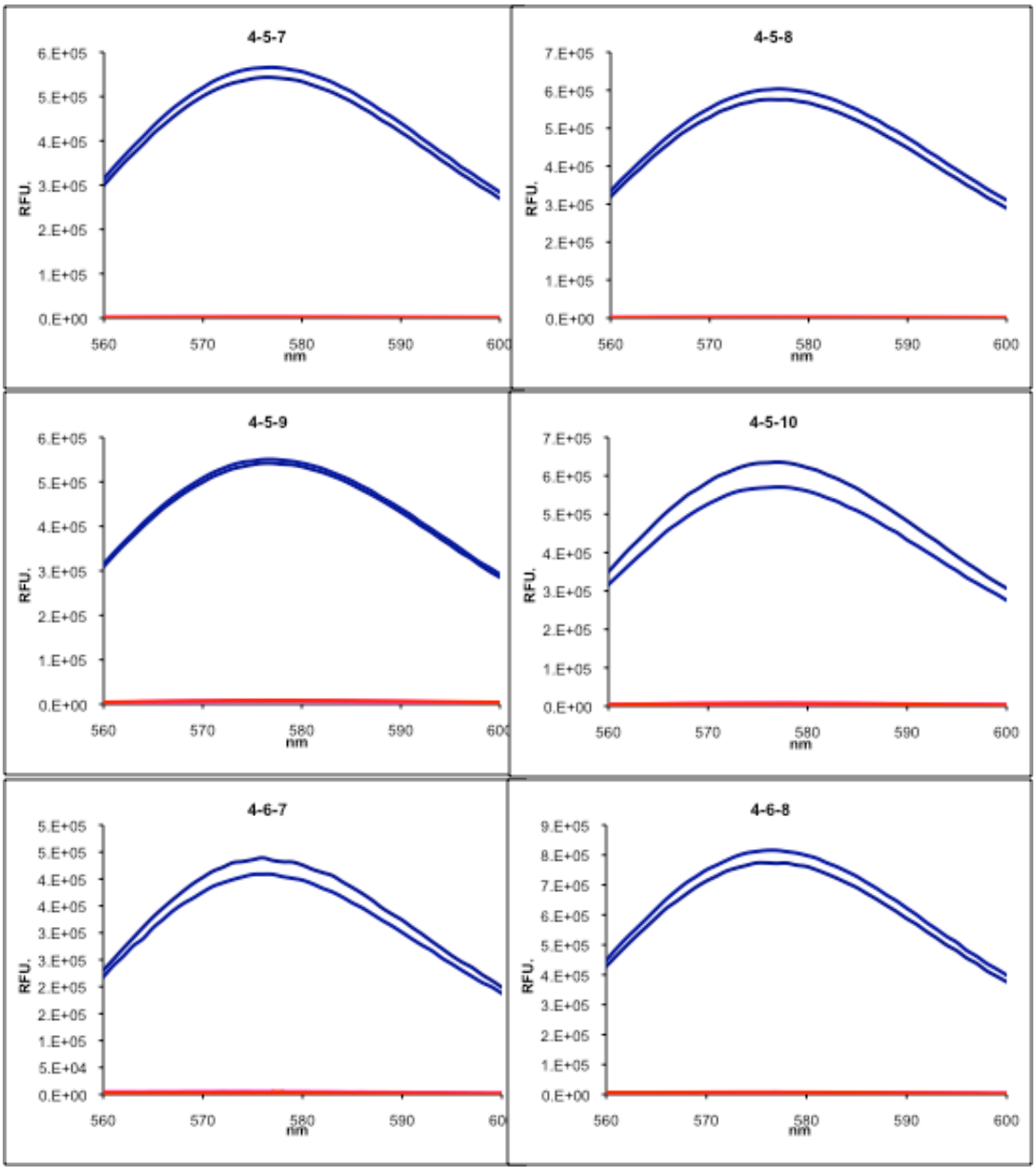
7-5-3 $C_{93}H_{97}N_{12}O_{19}S_3^+$ calculated 1781.61 Found 1781	**8-5-3** $C_{93}H_{96}N_{12}O_{25}S_5^+$ Calculated 1940.52 Found 1941	**9-5-3** $C_{60}H_{60}N_{12}O_{17}S_2$ calculated 1284.36 Found 1284.5	**10-5-3** $C_{63}H_{65}N_{11}O_{15}S_2$ calculated 1279.41 Found 1280
7-6-3 $C_{89}H_{89}N_{12}O_{17}S_3^+$ Calculated 1693.56 Found 1694	**8-6-3** $C_{89}H_{89}N_{12}O_{23}S_5^+$ calculated 1853.48 Found 1853.4	**9-6-3** $C_{56}H_{52}N_{12}O_{15}S_2$ calculated 1196.31 Found 1196.4	**10-6-3** $C_{59}H_{57}N_{11}O_{13}S_2$ calculated 1191.36 Found 1191.4
3-5-7 $C_{93}H_{97}N_{12}O_{19}S_3^+$ calculated 1781.61 Found 1781.6	**3-5-8** $C_{93}H_{96}N_{12}O_{25}S_5^+$ Calculated 1940.52 Found 1940.4	**3-5-9** $C_{60}H_{60}N_{12}O_{17}S_2$ calculated 1284.36 Found 1284.4	**3-5-10** $C_{63}H_{65}N_{11}O_{15}S_2$ calculated 1279.41 Found 1279.5
3-6-7 $C_{89}H_{89}N_{12}O_{17}S_3^+$ calculated 1693.56 Found 1693.6	**3-6-8** $C_{89}H_{89}N_{12}O_{23}S_5^+$ calculated 1853.48 Found 1853.6	**3-6-9** $C_{56}H_{52}N_{12}O_{15}S_2$ calculated 1196.31 Found 1196.4	**3-6-10** $C_{59}H_{57}N_{11}O_{13}S_2$ calculated 1191.36 Found 1191.4
7-5-4 $C_{97}H_{107}N_{13}O_{18}S_{22}^+$ calculated 1805.73 Found 1805.6	**8-5-4** $C_{97}H_{105}N_{13}O_{24}S_4$ calculated 1963.63 Found 1963.4	**9-5-4** $C_{64}H_{70}N_{13}O_{16}S^+$ calculated 1308.48 Found 1308.4	**10-5-4** $C_{67}H_{75}N_{12}O_{14}S^+$ calculated 1303.52 Found 1303.4
7-6-4 $C_{93}H_{99}N_{13}O_{16}S_{22}^+$ calculated 1717.68 Found 1717.6	**8-6-4** $C_{93}H_{97}N_{13}O_{22}S_4$ calculated 1875.58 Found 1875.4	**9-6-4** $C_{60}H_{62}N_{13}O_{14}S^+$ calculated 1220.43 Found 1220.3	**10-6-4** $C_{63}H_{67}N_{12}O_{12}S^+$ calculated 1215.47 Found 1215.5
4-5-7 $C_{97}H_{107}N_{13}O_{18}S_{22}^+$ calculated 1805.73 Found 1805.9	**4-5-8** $C_{97}H_{105}N_{13}O_{24}S_4$ calculated 1963.63 Found 1963.6	**4-5-9** $C_{64}H_{70}N_{13}O_{16}S^+$ calculated 1308.4 Found 1308.4	**4-5-10** $C_{67}H_{75}N_{12}O_{14}S^+$ calculated 1303.52 Found 1303.4
4-6-7 $C_{93}H_{99}N_{13}O_{16}S_{22}^+$ calculated 1717.68 Found 1717.6	**4-6-8** $C_{93}H_{97}N_{13}O_{22}S_4$ calculated 1875.58 Found1875.4	**4-6-9** $C_{60}H_{62}N_{13}O_{14}S^+$ calculated 1220.43 Found 1220.4	**4-6-10** $C_{63}H_{67}N_{12}O_{12}S^+$ Calculated 1215.47 Found 1215.4

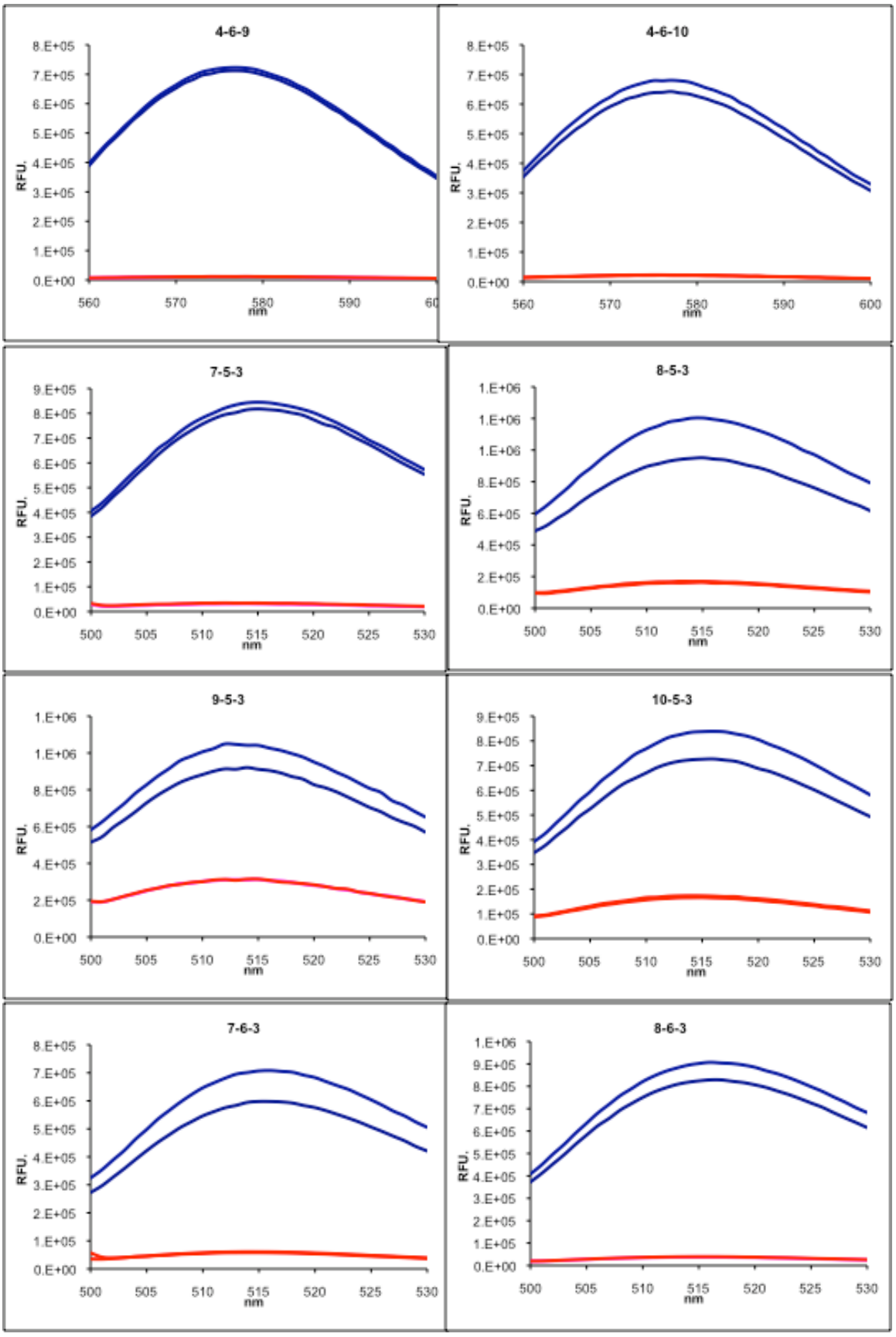
Photolysis and Fluorescence of the Fluorescently Quenched Peptide Cassette Library.

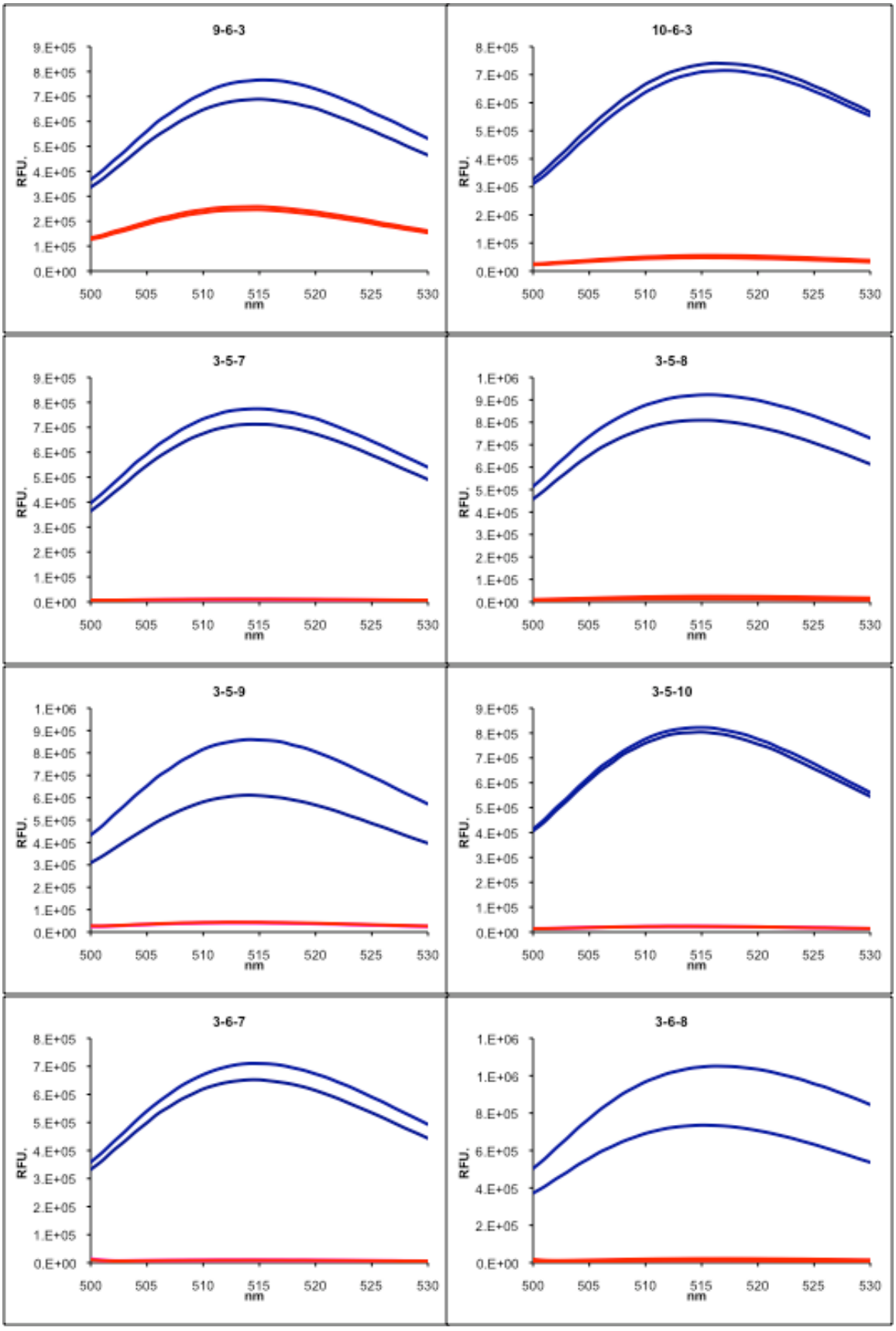
The DMF stock solutions of the HPLC purified peptide library were aliquoted into a pH 7.5 100 mM Tris buffer, 20 mM DTT, a peptide concentration of 1 μM in a 96 well plate (200 μL final volume per well). The plates were irradiated from the top in a photocrosslinker light box (5 mW/cm^2). A 360 nm color glass filter covered the top of the plate, and the plate was placed in an ice bath. Irradiation proceeded for 150 min at 5 mW/cm^2. Fluorescence of caged and photo-uncaged samples was measured at 25 °C. For the FITC-labeled peptides, λ_{ex} = 490 nm and λ_{em} = 500 – 530 nm. For the TAMRA labeled peptides, λ_{ex} = 540 nm and λ_{em} = 550 nm - 600 nm.

Figure S-2. Pre-photolysis (red) and post-photolysis fluorescence spectra (in duplicate) for library cassettes.




4-5-7
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
560
570
580
590
600
nm
4-5-8
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
560
570
580
590
600
nm
4-5-9
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
560
570
580
590
nm
4-5-10
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
560
570
580
590
600
nm
4-6-7
5.E+05
5.E+05
4.E+05
4.E+05
3.E+05
3.E+05
2.E+05
2.E+05
1.E+05
5.E+04
0.E+00
RFU.
560
570
580
590
nm
4-6-8
9.E+05
8.E+05
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
560
570
580
590
600
nm


4-6-9
4-6-10
7-5-3
8-5-3
9-5-3
10-5-3
7-6-3
8-6-3
RFU.
nm


9-6-3
10-6-3
3-5-7
3-5-8
3-5-9
3-5-10
3-6-7
3-6-8
RFU.
nm
500
505
510
515
520
525
530

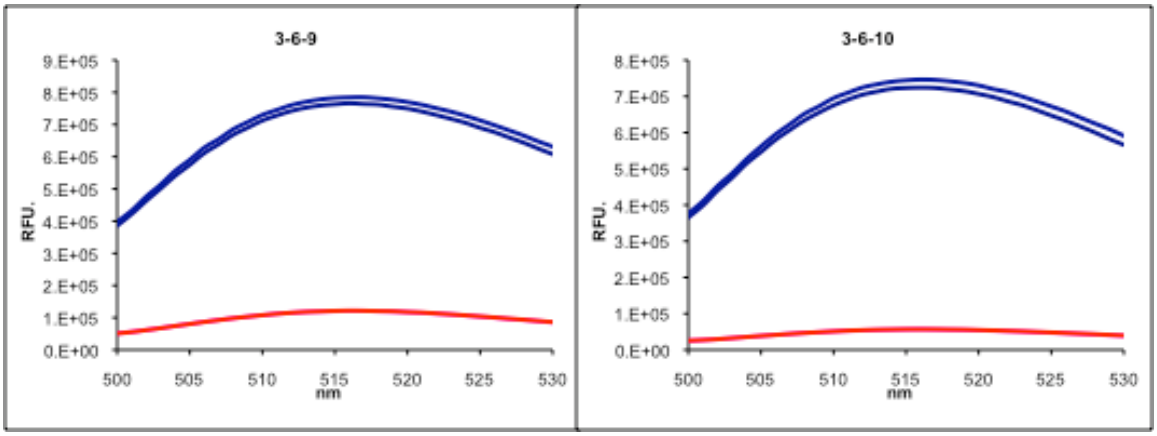
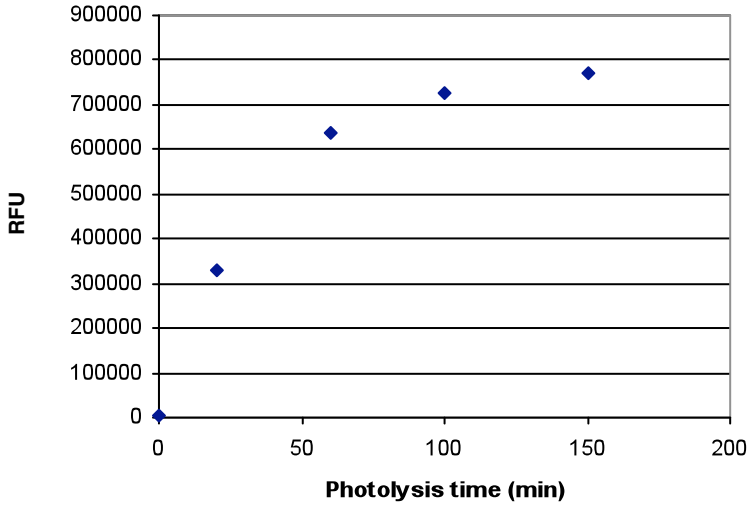

3-6-9
9.E+05
8.E+05
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
500
505
510
515
520
525
530
nm
3-6-10
8.E+05
7.E+05
6.E+05
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
RFU.
500
505
510
515
520
525
530
nm

Figure S-3. Fluorescence increase vs. photolysis time of Cassette **4-5-7.** (A) Y axis in RFU, (B) Y axis in Fluorescence Fold Increase.

A



B

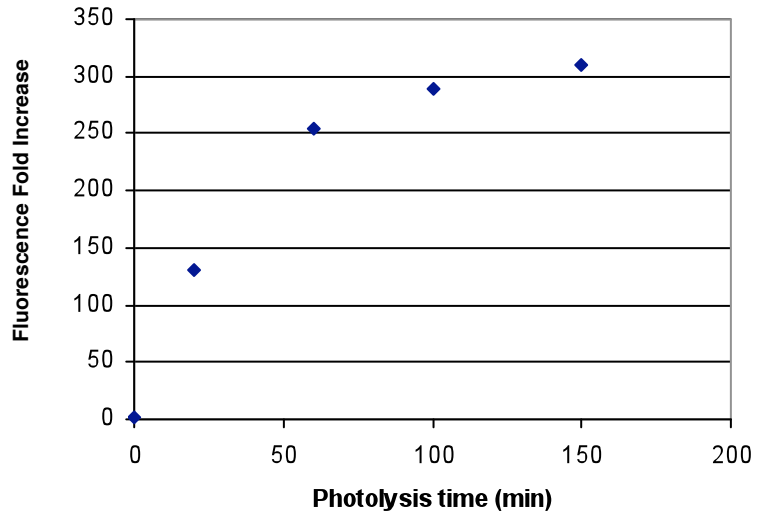


Figure S-4. Chromatograms of cassette **4-5-7** photolyzed for 0, 20, 60, 100 and 150 min (top to bottom). Elution conditions: 10% Acetonitrile to 95% Acetonitrile for 35 min. ESI-MS indicate peak @ 23 min is **4-5-7** (found 1806.6)**,** peak @16.2 min is Ac-Lys(TAMRA)-$CONH_2$ (found 600.3), both peaks in 23.3 and 23.5 min have masses that correspond to photolyzed nitosobenzyl-(Cys QSY7) ketone product (minus 17 amu; found 1189).

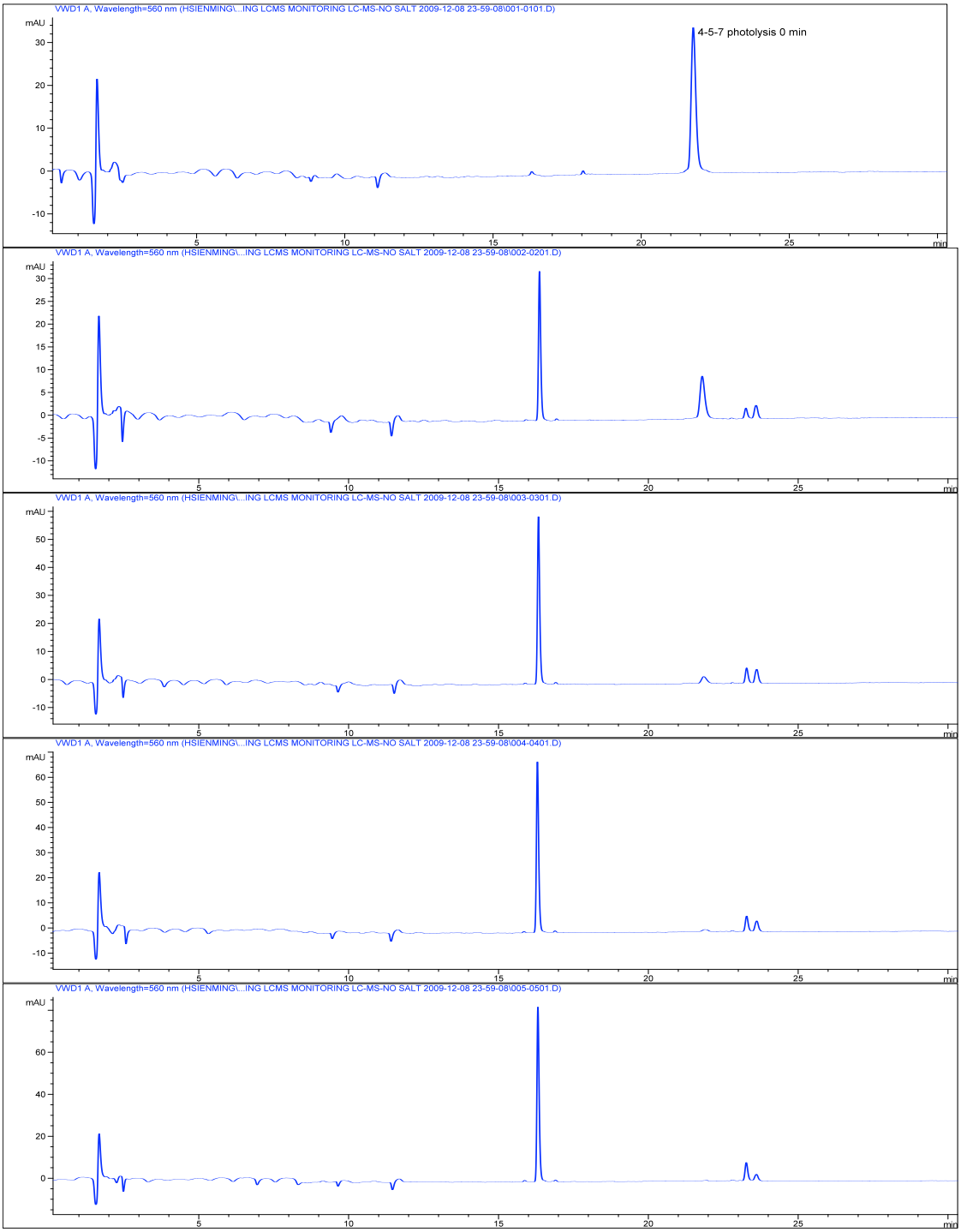


Figure S-5. Bar graph of fluorescein cassette library fluorescence dequenching by photolysis (conversion from Figure S-1). Top graph is in the absolute fluorescence scale and the bottom graph is in the log fluorescence scale.

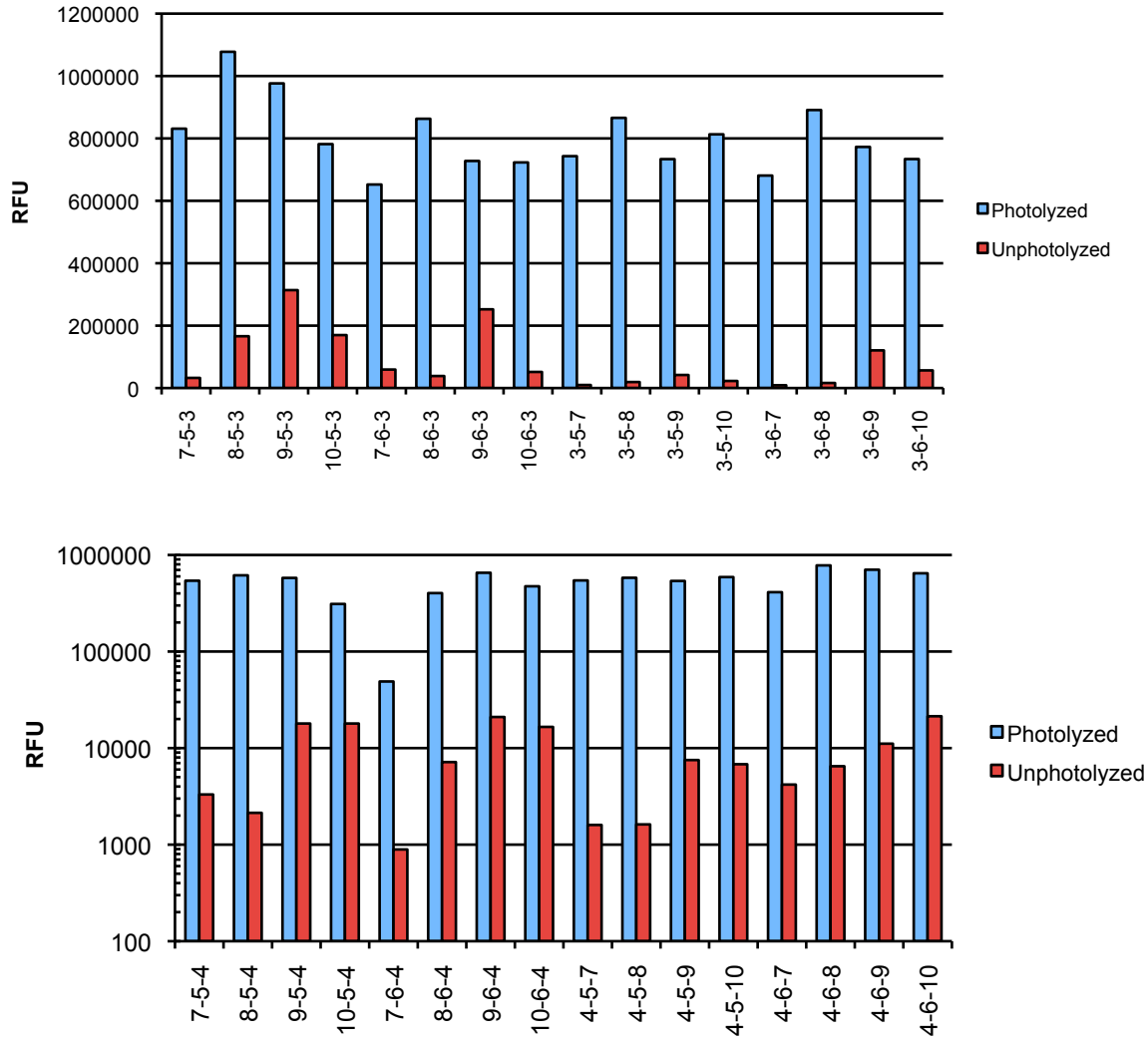
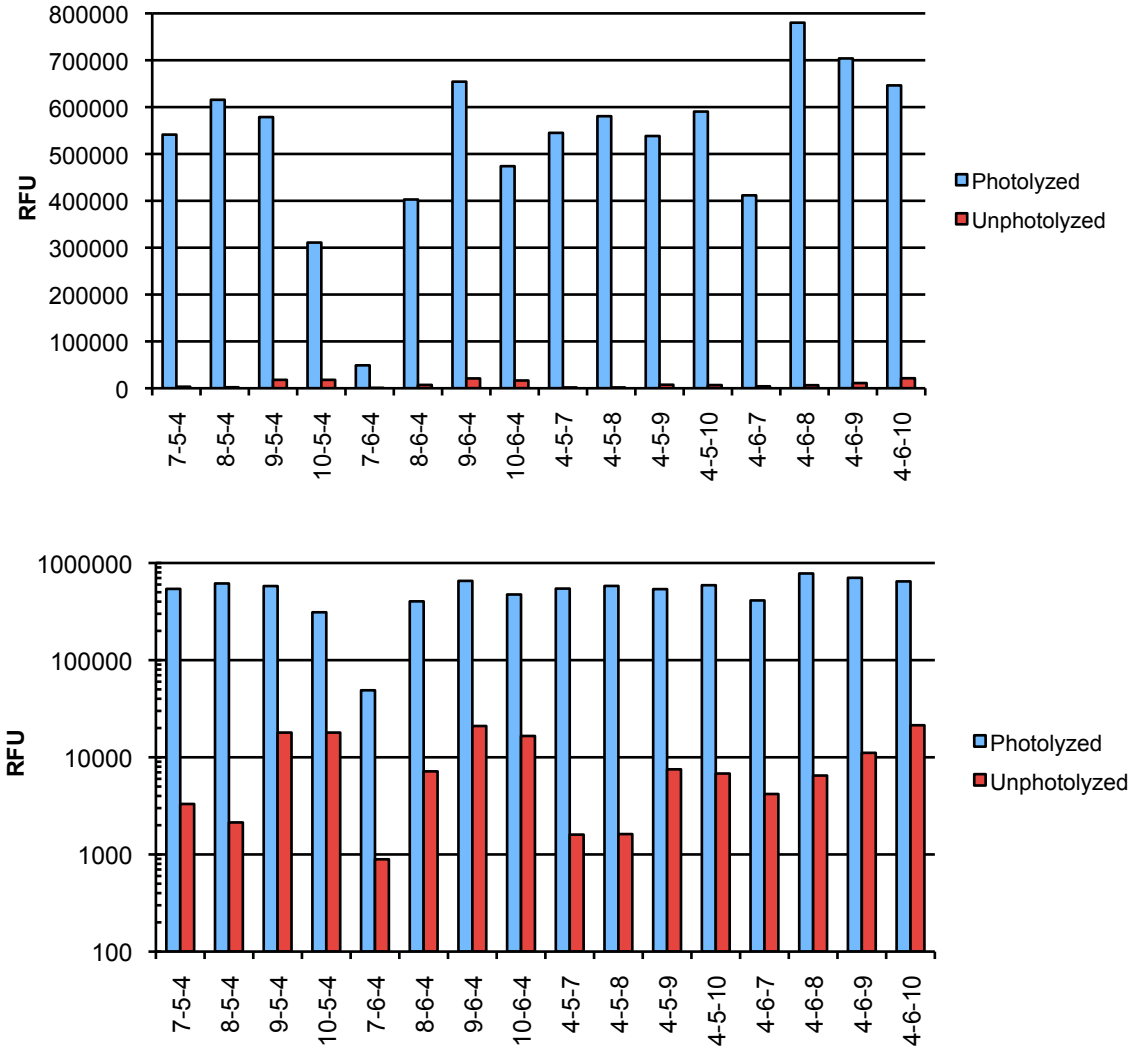


Figure S-6. Bar graph of TAMRA cassette library fluorescence dequenching by photolysis (conversion from Figure S-1). Top graph is in the absolute fluorescence scale and the bottom graph is in the log fluorescence scale.



Synthesis and Characterization of MLS-PEG-4-5-8 and MLS-PEG-8-5-4.

Fmoc amino acids were used to synthesize the mitochondrial localization sequence (MLS), PEG as a flexible linker, Fmoc-Lys(ivDde)-OH as the residue for fluorophore **4** attachment, Fmoc-photolabile linker **5** as the photolabile linker, and Fmoc-Cys(Trt)-OH as the residue for quencher **8** attachment. MLS = MLALLGWWWFSRKK.

A 40 μmol scale of NovaSyn TGR resin was used to synthesize MLS-PEG-**4**-**5**-**8** and MLS-PEG-**8**-**5**-**4**. The N-terminus was acetylated prior to Lys(ivDde) deprotection. Lys(ivDde) was selectively deprotected using 2% hydrazine monohydrate in DMF (25 mL/g resin, 3 x 3 min). 5-TAMRA **4** (3 eq, 120 μmol, 52 mg) was coupled to the peptidyl resin using HCTU (3 equiv, 120 μmol, 50 mg) and DIEPA (12 equiv, 480 μmol, 61 mg) for 2 h. The peptides were subsequently dried and then cleaved with the 95% TFA, 2.5% TIS, 2.5% H_2O for 2 h. The peptides were purified by HPLC with a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 70:30 (0 min) to 30:70 (60 min) using a semipreparative C-18 column.

MLS-PEG-**4-5-Cys** and MLS-PEG-**Cys-5-4** were collected and freeze-dried. MLS-PEG-**4-5-Cys** and MLS-PEG-**Cys-5-4** (1.7 μmol, 5 mg) were dissolved in 200 μL of DMF, mixed with **9**-maleimide DMF solution (3 equiv, 5.1 μmol, 5 mg, 200 μL), and further diluted to 1 mL with 50% DMF/ 50% PBS at pH 7.5. The reaction mixture was reacted for 2 h. The quencher-labeled peptides were purified by HPLC with a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 70:30 (0 min) to 30:70 (60 min) using a semipreparative C-18 column. ESIMS m/z peptide MLS-PEG-**4**-**5**-**8** and MLS-PEG-**8-5-4** both calculated at 4234.95, and both displayed 4234.8.

Synthesis and Characterization of CPMLS-4-5-7

Fmoc amino acids were used to synthesize the cell permeable mitochondrial localization sequence (CPMLS), where CPMLS = Ac-Fx-r-Fx-K-Fx-r-Fx-K. A 40 μmol scale of NovaSyn TGR resin and 120 μmol of Lys [Fmoc-K(Boc); except at position 4, where FmocK(ivDde) was employed], D-Arg [r(Pbf)], Cys [C(Trt)], and cyclohexylalanine (Fx) were used to synthesize Ac-Fx-r-Fx-K-Fx-r-Fx-K-**4-5-7** (CPMLS-**4-5-7**). The N-terminus of the sidechain protected H_2N-Fx-r(Pbf)-Fx-K(Boc)-Fx-r(Pbf)-Fx-K(Boc)-K(ivDde)-**5**-C(Trt)-resin was acetylated prior to Lys(ivDde) deprotection. Lys(ivDde) was selectively deprotected using 2% hydrazine monohydrate in DMF (25 mL/g resin, 3 x 3 min). TAMRA (3 eq, 120 μmol, 52 mg) was coupled to the peptidyl resin using HCTU (3 equiv, 120 μmol, 50 mg) and DIEPA (12 equiv, 480 μmol, 61 mg) for 2 h. The peptide was subsequently dried and cleaved with the 95% TFA, 2.5% TIS, 2.5% H_2O for 2 h. The peptides were purified by HPLC using a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 70:30 (0 min) to 10:90 (60 min) using a semipreparative C-18 column.

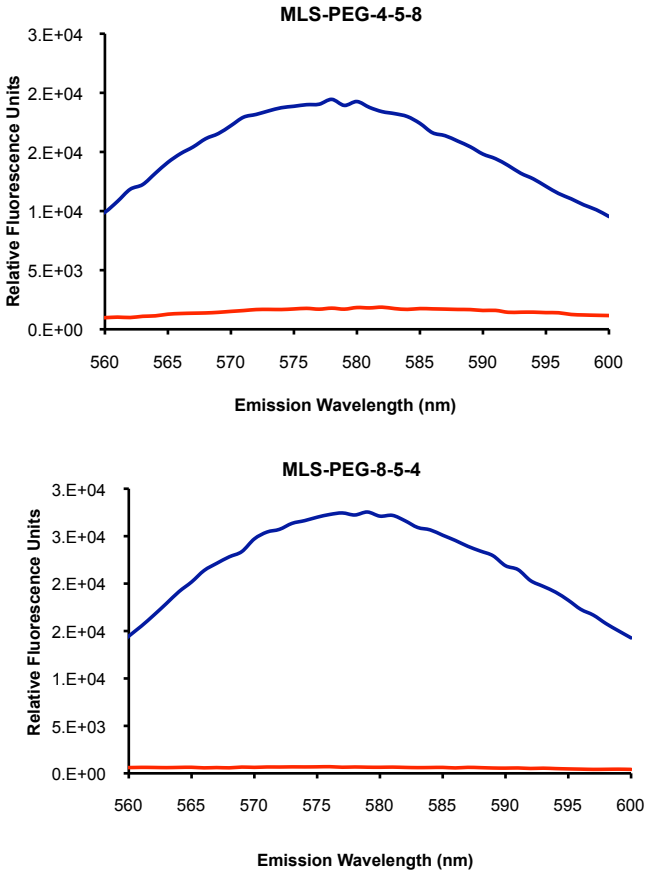
After Ac-Fx-r-Fx-K-Fx-r-Fx-K-**4-5-Cys** was collected and freeze-dried, 20 mg (9.3 μmol) were dissolved in 200 μL of DMF, mixed with **QSY7**-maleimide DMF solution (1.1 equiv, 10.2 μmol, 10 mg, 200 μL), and further diluted to 1 mL using 50% DMF/50% PBS at pH 7.5. The reaction mixture was shaken for 2 h. CPMLS-**4-5-7** was purified by HPLC using a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.l% TFA/CH_3CN) with a ratio of A:B that varied from 70:30 (0 min) to 10:90 (60 min) using a semipreparative C-18 column. ESI-MS of CPMLS-**4-5-7**: m/z calculated 2988.7, found 2987.9.

Additional peptides prepared include MLS-PEG-4-5-Cys (calculated 3253.84, found 3253.6) and CPMLS-4-5-Cys (calculated 2164.70, found 2165.5), which lack covalently appended quencher.

Photolysis of MLS-PEG-4-5-8 and MLS-PEG-8-5-4.

MLS-PEG-**4-5-8** and MLS-PEG-**8-5-4** stock solutions (0.2 mM) were diluted using 200 μL PBS (with 20 mM DTT) to a final peptide concentration of 2 μM. The solutions were irradiated at 0 °C for 15 min with a Hg arc lamp. A 360 nm glass filter was positioned between the lamp and the peptide solutions. The power delivered to the reaction vessel was 300 mW/cm^2. The fluorescence of the unphotolyzed and photolyzed peptide solutions was measured using a PTI spectrofluorometer (Fig. S-7).

Figure S-7. (Top) MLS-PEG-**4-5-8** fluorescence emission scan curve before (red) and after (blue) photolysis in buffer. An 11-fold increase was observed. (Bottom) MLS-PEG-**8-5-4** fluorescence emission scan curve before (red) and after (blue) photolysis in buffer. A 42-fold increase in fluorescence was observed.



Photolysis of CPMLS-**4-5-7**

CPMLS-**4**-**5**-**7** stock solutions (4.4 mM) were diluted using 200 μL PBS (with 20 mM DTT) to a final peptide concentration of 2 μM. The solutions were irradiated at 0 °C for 15 min with a Hg arc lamp. A 360 nm glass filter was positioned between the lamp and the peptide solutions. The fluorescence of the unphotolyzed and photolyzed peptide solutions was measured using a PTI spectrofluorometer (Fig. S-8).

Figure S-8. CPMLS-**4-5-7** (1 μM) fluorescence emission scan curve before (red) and after (blue) photolysis in buffer. A 51-fold increase in fluorescence was observed.

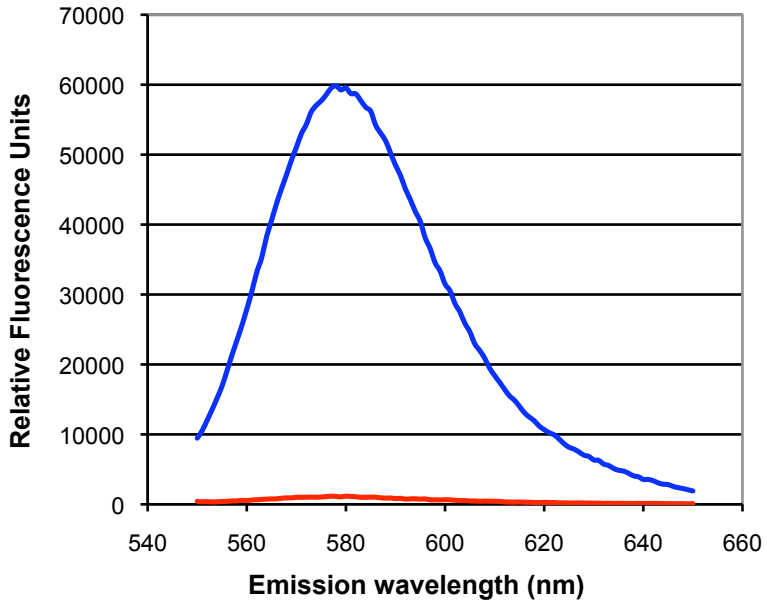


Figure S-9. Fluorescence of (A) MLS-PEG-**4**-**5**-Cys (red) relative to TAMRA (black) and (B) CPMLS-**4**-**5**-Cys (blue) relative to TAMRA (black). Peptide and TAMRA concentrations are 2 μM. TAMRA conjugated to MLS-PEG peptide is only 0.2% as bright as free TAMRA, which we ascribe to the presence of the Trp triplet in the MLS sequence. By contrast, TAMRA conjugated to the CPMLS peptide is 30% as bright as free TAMRA.

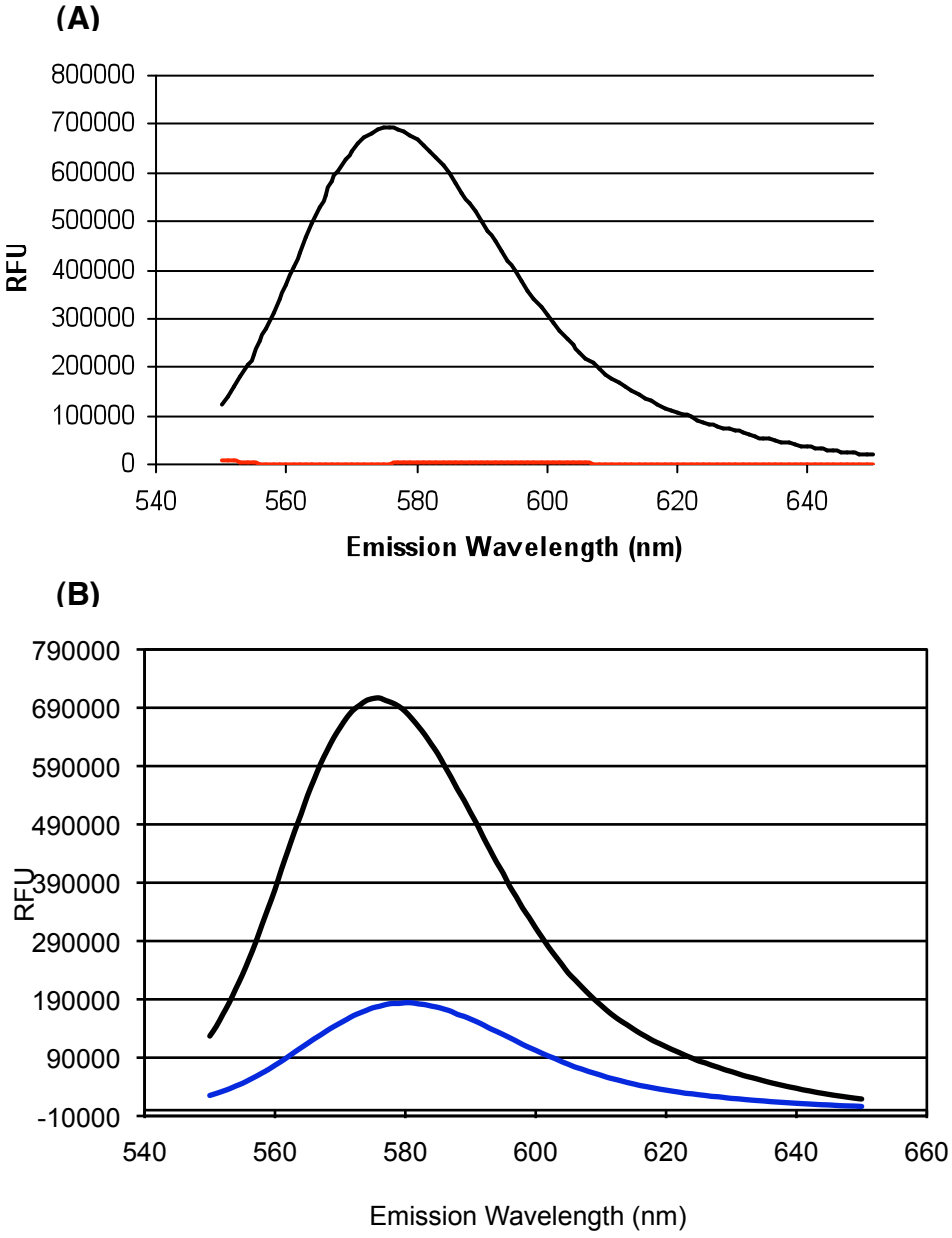
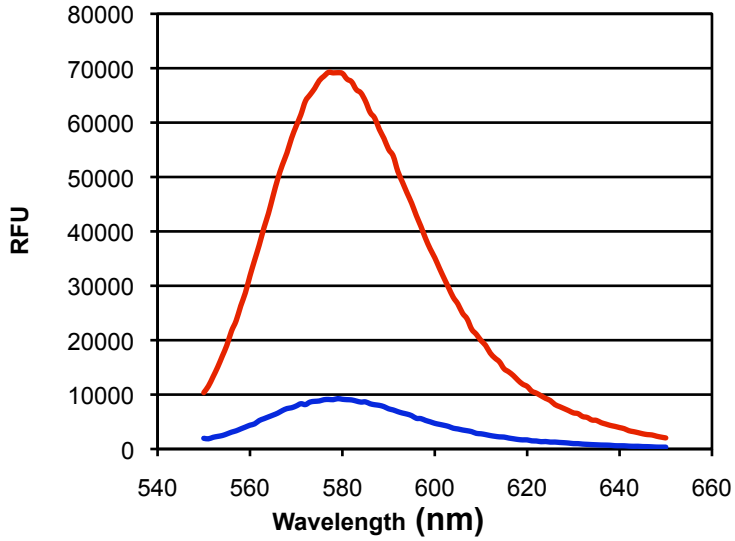
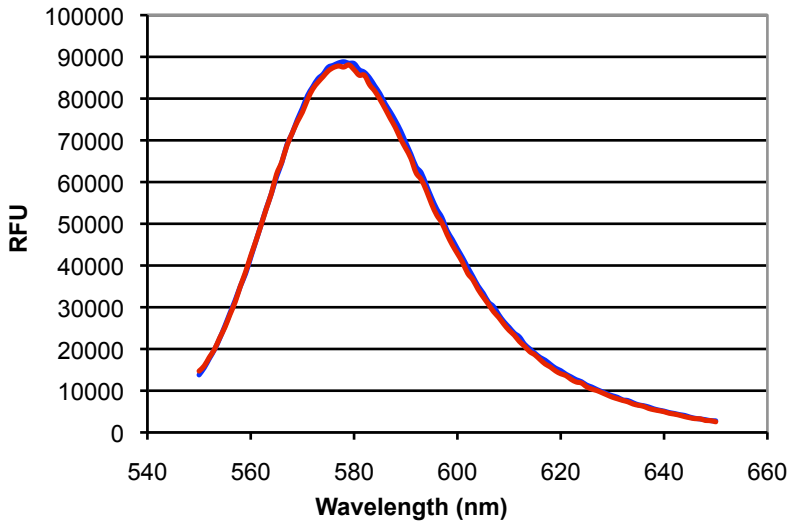


Fig. S-10. (A) Association of CPMLS-**4-5**-Cys with mitochondria**.** Fluorescence of CPMLS-**4-5**-Cys (2 μM) in 200 μL PBS buffer containing 20 mM DTT (red) and fluorescence of supernatant (blue) of CPMLS-**4-5**-Cys with mitochondria under identical conditions. 86% of CPMLS-**4-5**-Cys fluorescence is cleared from the supernatant in the presence of mitochondria. The estimated CPMLS-**4-5**-Cys associated with the mitochondria is (0.4 nmol x 0.86)/0.11mg mitochondria = 3.12 nmol/mg. (B) Experiment identical to that performed in (A) except with the **4-5**-Cys cassette lacking the MLS sequence: **4-5**-Cys alone (blue) and in the presence of mitochondria (red), indicating that in the absence of the MLS sequence the peptide does not associate with mitochondria.

(A)



(B)



Photolysis of MLS-PEG-4-5-8-Embedded Mitochondria.

MLS-PEG-**4-5-8** was diluted in 200 μL PBS (20 mM DTT) to a final concentration of 2 μM. 30 μL of a mitochondria suspension (MitoSciences, 5.5 mg/mL) was mixed with the peptide solution and incubated for 5 min on ice. The peptide-embedded mitochondria were washed four times using 200 μL PBS (20 mM DTT) with repeated centrifugation (4000 X g for 4 min), buffer replacement, and mitochondrial resuspension. The fluorescence of the supernatant of the peptide-embedded mitochondria was measured prior to photolysis-induced fluorophore release. The mitochondria were then irradiated at 0 °C for 30 min with a Hg arc lamp in the presence of a 360 nm glass filter placed between the lamp and the reaction vessel. The power delivered to the reaction vessel was 300 mW/cm^2. After photolysis, the mitochondria were centrifuged at 4000 X g for 4 min and supernatant fluorescence measured (Fig. S-11). Absorbance and visual changes are provided in Fig. S-12.

Figure S-11. Fluorescence scan of MLS-PEG-**4-5-8** embedded-mitochondria supernatant before (red) and after (blue) photolysis.

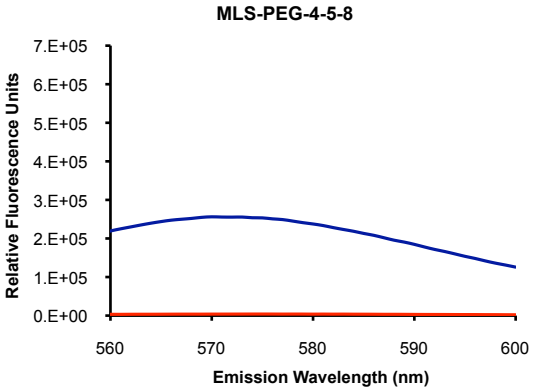
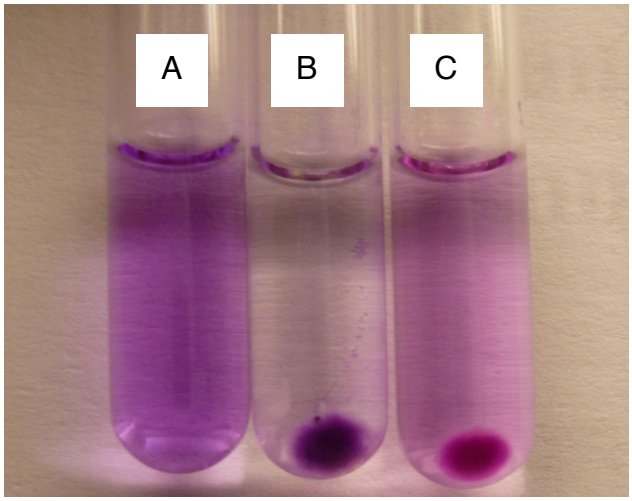
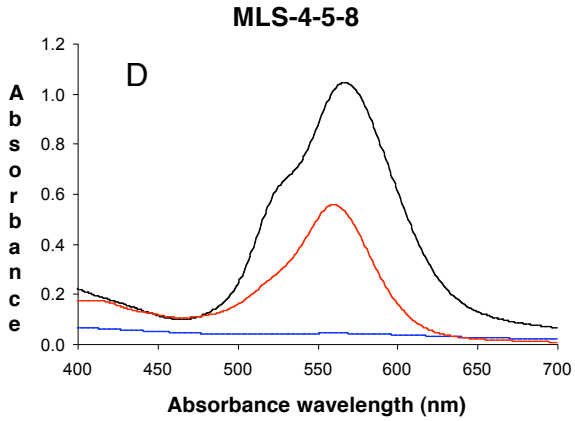


Figure S-12. Unphotolyzed and photolyzed MLS-PEG-**4-5-8**-embedded mitochondria in PBS buffer. (A): MLS-PEG-**4-5-8** (10 μM) (B) MLS-PEG-**4-5-8** and mitochondria (110 μg), (C) photolyzed MLS-PEG-**4-5-8** and mitochondria, and (D) absorbance spectra of the supernatant in A (black), B (blue), and C (red). Since the ε of **8** and **4** are nearly identical and photolysis furnishes a ~50% absorbance release into the supernatant, the photolytic yield is approximately 100%.

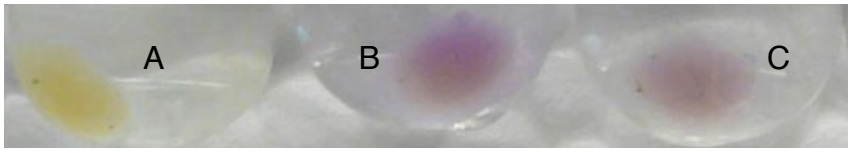


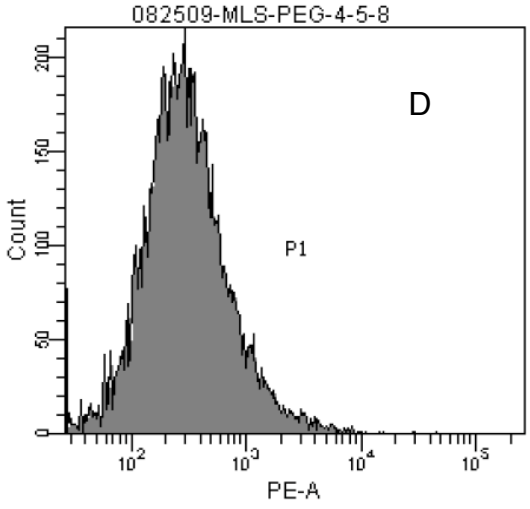


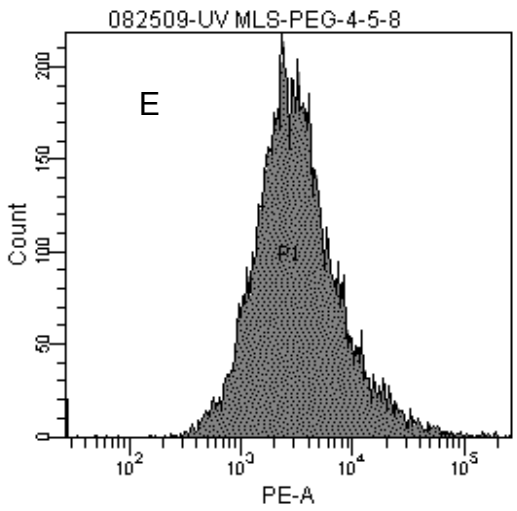
Mitochondrial Fluorescence Prior To and Following Photolysis as Assessed By Flow Cytometry.

A BD LSRII flow cytometer was used to record the fluorescent intensity of mitochondria before and after the photolysis. A 532 nm excitation laser and the 570 nm PMT channel (phycoerythrin “PE” channel) were used to measure mitochondrial fluorescence distribution. Pre-photolysis fluorescence was measured using mitochondria incubated with the MLS peptide and washed three times with 200 μL PBS (20 mM DTT) followed by centrifugation (4000 X g, 4 min) (Fig. S-13). The mitochondrial pellet was then resuspended in 5 mL of PBS buffer and loaded into the flow cytometer. Post-photolysis fluorescence was measured following irradiation and subsequent washing with PBS as described above (Fig. S-13). The irradiated mitochondrial pellet was resuspended in 5 mL PBS buffer and loaded into the flow cytometer.

Figure S-13. Mitochondria treated with MLS-PEG-**4-5-8** and subsequently photolyzed. These experiments were performed identically to those shown in Fig. S-12 except that the (A) untreated mitochondria were washed with PBS buffer (B) following exposure to MLS-PEG-**4-5-8** (i.e. peptide removed from supernatant) and (C) following photolysis. The mitochondria in C display a 13.4-fold enhanced fluorescence relative to those in B as measured by flow cytometry: (D) before and (E) after photolysis.







Cell Culture

HeLa cells were passaged by treatment with 0.5% trypsin with 0.53 mM EDTA before reaching confluence and maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum supplemented with 1X nonessential amino acids, gentamycin, and kanamycin at 37 ºC in 5% CO_2 incubator. On the day prior to the microscopy experiments, the cells were plated into either MatTek 6-well glass bottom dishes for experiments with cell permeable reagents or into MatTek 50 mm grided glass bottom dishes for experiments requiring microinjection of cell impermeable reagents. On the day of the microscopy experiments, the media was replaced with Leibovitz's L-15 medium without phenol red.

Fluorescent Microscopy

All fluorescent microscopy imaging experiments were performed using an inverted Olympus IX81 microscope equipped with a Hamamatsu C8484 camera, a 60X oil immersion Plan S-Apo objective, and Cy3 and Cy5.5 filter cubes (Semrock). Microinjection was performed using an Eppendorf FemtoJet and Injectman NI 2 system. Photolysis studies were carried out using a diode pumped, Q-switching, solid state neodymium doped yttrium lithium fluoride (Nd:YLF) laser at 349 nm from Spectra-Physics that is integrated with the microscope. The laser was controlled using L-win software with burst control. All imaging analyses and overlays were performed using Metamorph and Image J software.

TAMRA Photobleaching Curve. HeLa cells were microinjected with free TAMRA (100 μM in needle; injection pressure of 100 hPa for 0.5 s) to obtain a photobleaching curve (Fig. S-12). After injection, cells were centered in the laser path and images were taken at 100 ms exposures using the Cy3 filter cube. Photolysis was carried out with a Nd:YLF laser at 3.02 A resulting in 90 - 100 μJ of energy per pulse at 10 pulses/burst. A repetition rate of 100 Hz with a delay of 5 s between bursts was employed. Images were acquired over a 0 – 200 burst interval. The photobleaching curve was generated using the same photolysis and imaging conditions as for the cassette and the CPMLS-**4-5-7**. The fluorescence increase upon photolysis in all subsequent studies was obtained by subtracting both the initial cell fluorescence and the photobleaching curve (Fig. S-14) from the acquired fluorescence for each photolyzed cell. All cassette and photobleaching data are averaged from 3 - 4 independent experiments. Fig. S-15 shows the subcellular distribution of TAMRA. The fluorescence intensity is greatest in the nuclear region, which may simply be a consequence of the longer path length associated with this region of the cell.

Figure S-14. TAMRA photobleaching curve.

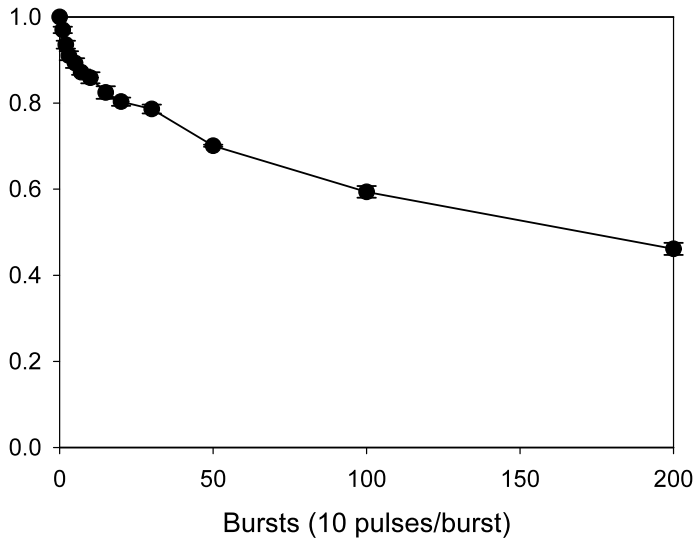
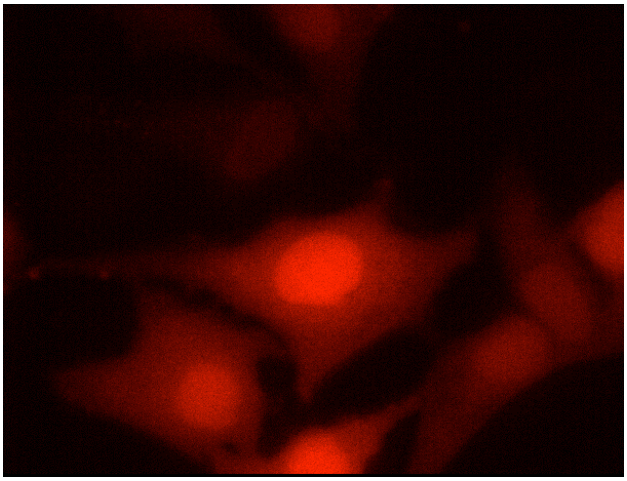
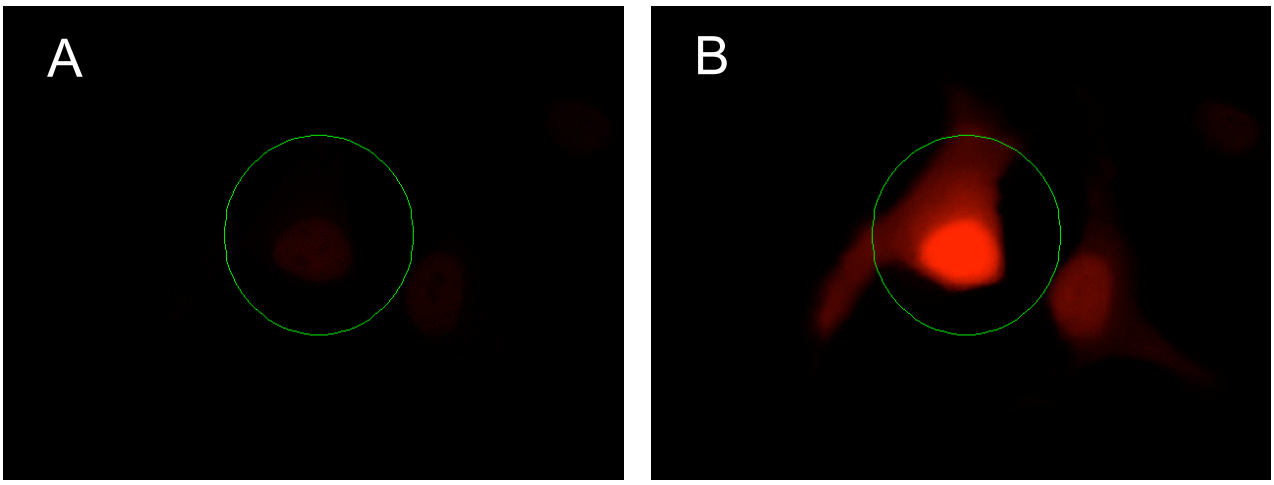


Figure S-15. TAMRA distribution in HeLa cells.



Photolyzed 4-5-8 Subcellular Localization and Fluorescence Change. The quenched cassette (**4-5-8**), which lacks a mitochondrial localization sequence, was used as a control to assess whether the photolyzed product displays any subcellular localization properties. The cell impermeable cassette (100 μM in needle) was microinjected into HeLa cells, photolyzed, and imaged as described above for TAMRA. A fluorescence enhancement of 12.6 ± 1.2 fold was observed (4 cells total). The localization pattern for the photolyzed cassette (Fig. S-16) is diffuse and indistinguishable from that of TAMRA.

Fig. S-16. Fluorescence in HeLa cells of the quenched cassette **4-5-8** before (A) and after (B) photolysis with 50 laser bursts. Green circle represents the focus of the laser.



Photolyzed CPMLS-4-5-7 Subcellular Localization and Fluorescence Change. The intracellular localization and light-induced fluorescent increase of CPMLS-**4**-**5**-**7** was established by loading HeLa cells with 50 nM Mitotracker FarRed (Invitrogen) at 37º C for 20 min in L-15 medium. Cells were subsequently incubated with 2 μM CPMLS-**4**-**5**-**7** at 37º C for 20 min in L-15 medium, washed three times with PBS to remove excess fluorophore, and centered in the laser path. Images were conducted as described above for the TAMRA photobleaching curve experiments. The Cy3 images corresponding to CPMLS-**4**-**5**-**7** were overlayed with the Cy5.5 images of Mitotracker FarRed and intensity correlation analysis was run using Image J to generate Pearson coefficients for each photolysis experiment. A fluorescence enhancement of 11.8 ± 0.7 fold was observed (4 cells total). The subcellular localization pattern for the photolyzed CPMLS-**4**-**5**-**7** (Fig. 2 and S-17) overlaps with that of Mitotracker FarRed (Pearson coefficient: 0.878 ± 0.027).

Fig. S-17. Enlarged version of Fig. 2 (Row B, Column C) in which the Cy 3 (photolyzed CPMLS-**4**-**5**-**7**) and Cy 5.5 (mitotracker FarRed) windows are merged. Red represents regions in which mitotracker FarRed predominates, green regions in which photolyzed CPMLS-**4**-**5**-**7** predominates, and orange in which both photolyzed CPMLS-**4**-**5**-**7** are present. The laser focus for photolysis is represented as a green circle. Note that the region highlighted by the arrow, which is outside of the photolysis region, is predominantly red, indicating that this portion of the cell contains comparatively little photolyzed CPMLS-**4**-**5**-**7**.