Supporting Information for:

Releasable SNAP-tag Probes for Studying Endocytosis and Recycling

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Supplemental Information:

Materials: O^6-[4-(aminomethyl)benzyl]guanine (BG-NH_2) was from Toronto Research Chemicals. The heterobifunctional crosslinker LC-SPDP was from Thermo Scientific (Pierce Protein Research Products). Cysteamine (2-mercaptoethylamine), N, N dimethylformamide, triethylamine, and other reagents, when not specified, were from Sigma or Thermo Scientific. Alexa Fluor 488 carboxylic acid, succinimidyl ester was from Invitrogen. IRDye® 800CW NHS Ester was from LI-COR.

Probe synthesis: (See Supplemental Figures 4 and 5). 7.8 mg solid LC-SPDP (**2**)(18.5 μmol) was added to a solution of 5 mg BG-NH_2 (**1**)(18.5 μmol) in 800 μl dimethlyformamide (DMF) and 5 μl triethylamine (35.8 μmol). The reaction was shaken at 30°C overnight. This generates the BG-amide (**3**) that retains the 2-pyridyl disulfide (determined m/z 580.2). A 10 fold molar excess of cysteamine (185 μmol) in DMF was added and incubated overnight at 4°C. Disulfide exchange results in formation of the BG-amide-NH_2 and retains the disulfide group (**4**) (m/z 546.2). The reaction in the DMF produced greater than 85% yield of the free amine compared with the reaction in aqueous solution, which produced only ~50% yield. To remove excess cysteamine and reduced BG thiol, the reaction was diluted 10 fold in water and the pH was reduced to ~3 with trifluoroacetic acid (TFA) or HCl. Care must be taken not to allow the pH to go below 3.0. We have noticed that, under very low pH conditions, the final dye-labeled product, although susceptible to disulfide reduction *in vitro*, is poorly cleaved when bound to SNAP-tagged proteins in cells. The reaction was bound by gravity flow to an SP-Sepharose Fast flow cation exchange column (GE Healthcare) equilibrated with 5% DMF/0.05% TFA (or 1 mM HCl). The column was prepared by adding 2 ml of a 1:1 slurry SP-Sepharose to a Poly-Prep chromatography column (BioRad). The column was washed once with 5% DMF/0.05% TFA (or 1 mM HCl). Excess cysteamine was eluted from the column with 5% DMF/0.05% TFA/50 mM NaCl. Removal of cysteamine was determined by reaction with Ellman's reagent (Thermo Scientific). The BG-amide-NH_2 product was eluted with PBS and desalted with a reversed phase C18 Sep-Pak cartridge (Waters). The product was eluted in 80% acetonitrile/0.05% TFA, and dried in a Speed-Vac. BG-amide-NH_2 was suspended 0.2 ml DMF and added to Alexa Fluor 488 carboxylic acid, succinimidyl ester in 0.1 ml DMF (1:1 molar ratio) and 4 μl triethylamine, and shaken at 30°C overnight. The final BG-amide-S-S-Alexa 488 ligand (**5**) (m/z 1062.2) can be used directly without further purification. Purity was generally greater than 95%, as determined by HPLC. Under these conditions, the BG-amide-NH_2 should be consumed. If still present, it will compete with the labeled probe for binding with SNAP-tag fusion proteins. In this case, additional NHS ester dye can be added. The concentration of BG compounds were determined by measuring UV absorbance at λ = 280 nm using the extinction coefficient of *O*^{6}-(4-aminomethyl-benzyl)guanine (ε_{280} = 7100 $M^{-1}cm^{-1}$) (*25*). Reverse phase (C18) analyses and molecular weight determinations of BG-conjugated probes were carried out on an Agilent 1100 series high pressure liquid chromatograph with electrospray ionization and detected by a model G1946 mass spectrometer equipped with a time-of-flight detector (Agilent).

Supplemental Figure 1. Loss from surface of SNAP-β2ADR in 293A cells treated with isoproterenol. Cells were treated with 200 μM isoproterenol for indicated times at 37°C, labeled with BG-S-S488 for 30 min at 4°C, then washed and fixed. Surface fluorescence

was then quantified by measuring total integrated intensity per cell area. Error bars, s.e. (n = ~ 50 individual cells for each time point, from 2 independent experiments).

Supplemental Figure 2. Uptake of BG-S-S-800 labeled SNAP-β2ADR in HEK293A cells. Cells were labeled at 4°C then chased for the indicated periods of time in the absence or presence of isoproterenol. Duplicate time points were treated or not with TCEP. Cell lysates were run on non-reducing SDS-PAGE and directly imaged on an infrared scanner (top). Quantitation of internalization. The percentage uptake was determined from background-subtracted TCEP treated/Untreated signal intensities.

Supplemental Figure 3. Colocalization of β-arrestin2-TagRFP (red) with AP2 (blue) in HEK293A cells stably expressing SNAP-β2ADR. BG-S-S-488 (green) labeled cells were incubated with isoproterenol for 10 min at 37°C, treated with TCEP, fixed, permeabilized and stained with a primary antibody to the adaptor AP2. Alexa Fluor 633 labeled goat anti-mouse (Invitrogen) was used to detect AP2. β-arrestin2-TagRFP colocalizes with AP2 after 10 min uptake, but not with SNAP-β2ADR. Scale bar, 10 μm.

Supplemental Figure 4. Schematic of the synthesis of BG-S-S-488.

Supplemental Figure 5. LC-ESI mass spectrometry analyses of BG-S-S-488. (a) Absorbance at 210 nm (top panel), 488 nm (second panel), and 290 nm (third panel). Mass selective detector (MSD) (bottom panel). (b) Absorbance spectrum of BG-S-S-488. (c) MS spectrum. Calculated for $C_{45}H_{47}N_{10}O_{13}S_4^+$ [M^+]: 1063.22, Found: 1063.21.

Supplemental Video 1. BG-S-S-488 labeled COS cells expressing SNAP-β2ADR. Uptake of the receptor was 30 min at 37°C in the presence of isoproterenol, and then imaged. 5 mM TCEP was added at the indicated time point.

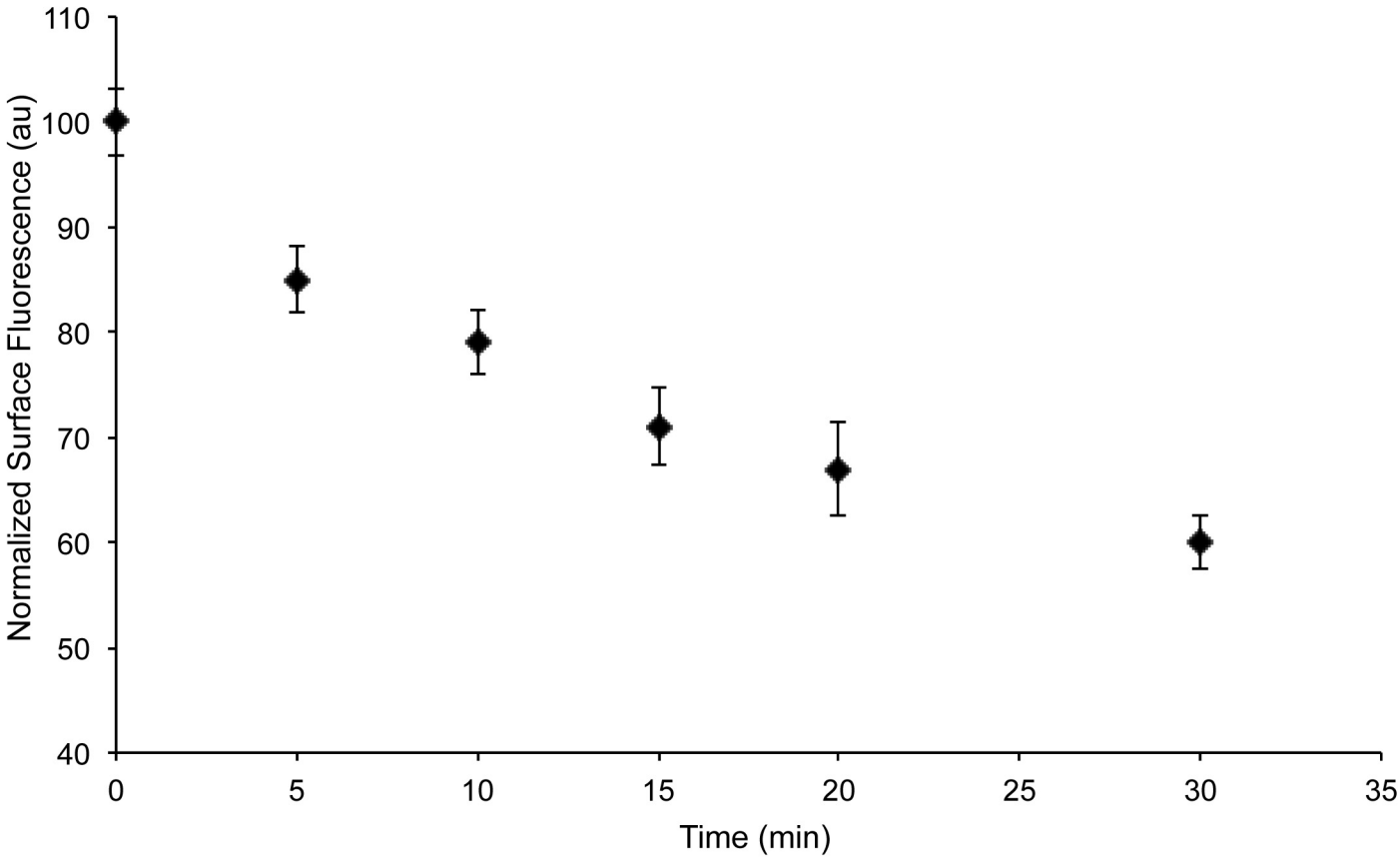
Supplemental Video 2. SNAP-Surface 488 labeled COS cells expressing SNAP-β2ADR. Uptake of the receptor was 30 min at 37°C in the presence of isoproterenol, and then imaged. 5 mM TCEP was added at the indicated time point.

Supplemental Video 3. HEK293A cells stably expressing SNAP-β2ADR transfected with β-arrestin2-TagRFP. Isoproterenol was added to stimulate uptake (“Iso” flash), followed by subsequent addition of 5 mM TCEP (“TCEP” flash). β-arrestin2-TagRFP is rapidly recruited to the plasma membrane but does not colocalize with endocytosed SNAP-β2ADR.

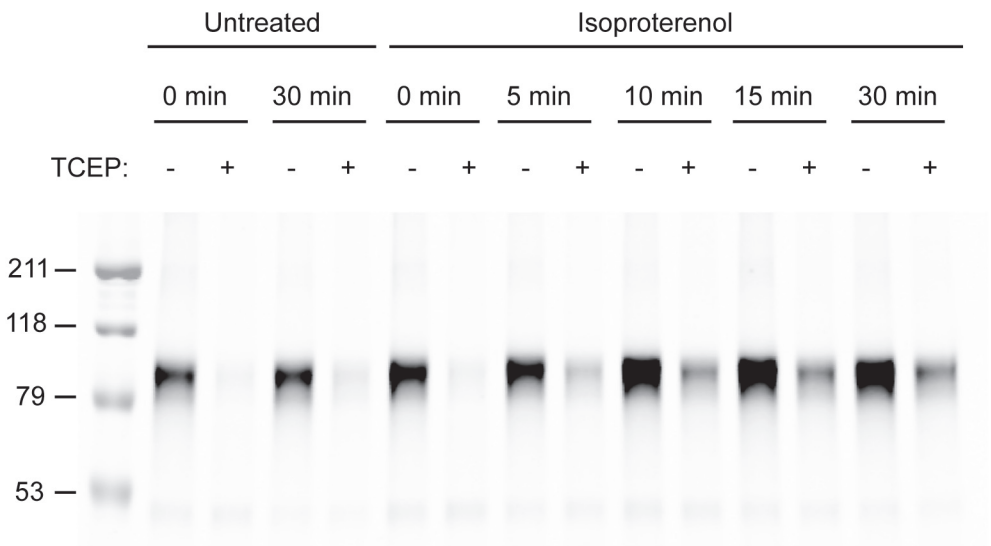
Supplemental Video 4. HEK293A cells stably expressing SNAPNK1R transfected with β-arrestin2-TagRFP. Substance P was added to stimulate uptake (“SubP” flash), followed by subsequent addition of 5 mM TCEP (“TCEP” flash). β-arrestin2-TagRFP is rapidly recruited to the plasma membrane and colocalizes with endocytosed SNAP-NK1R.

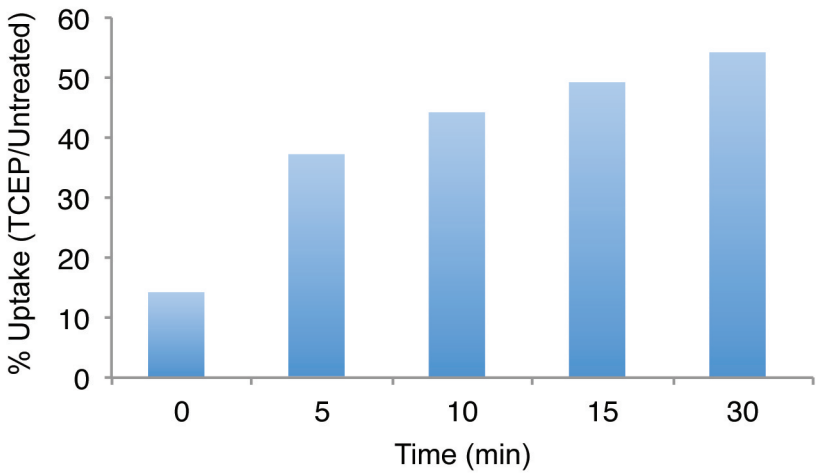
25. Juillerat, A., Gronemeyer, T., Keppler, A., Gendreizig, S., Pick, H., Vogel, H., and Johnsson, K. (2003) Directed evolution of O6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules in vivo, *Chem. Biol. 10*, 313-317.

Supplemental FIgure 1

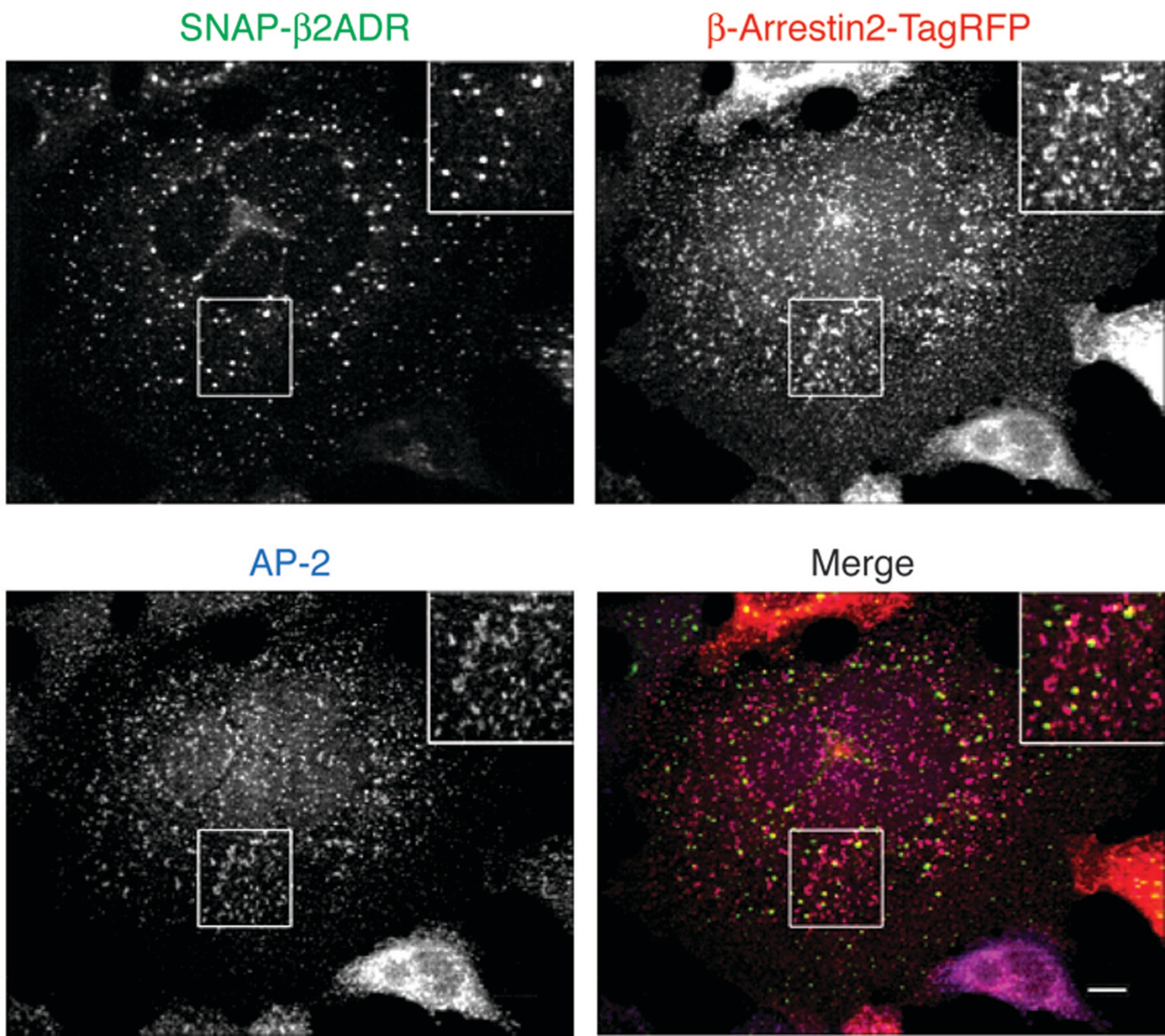


Supplemental Figure 2

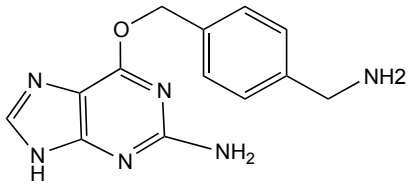




Supplemental Figure 3

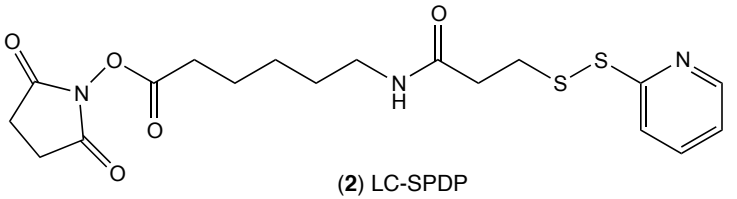


Supplemental Figure 4

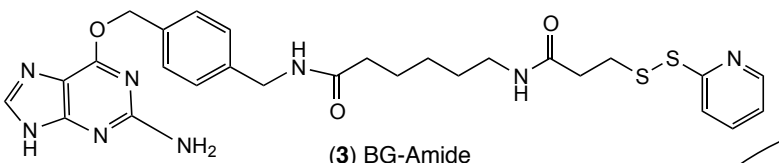


(**1**) BG-NH_2
(O6-(4-Aminomethyl-benzylguanine)
Chemical Formula: $C_{13}H_{14}N_6O$
Molecular Weight: 270.29

1:1
BG-NH_2:LC-SPDP
DMF/TEA
16 h 30°C



(**2**) LC-SPDP
Chemical Formula: $C_{18}H_{23}N_3O_5S_2$
Molecular Weight: 425.52

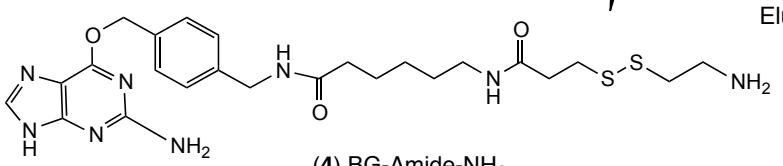


(**3**) BG-Amide
Chemical Formula: $C_{27}H_{32}N_8O_3S_2$
Molecular Weight: 580.72

$HS\text{-}CH_2\text{-}CH_2\text{-}NH_2$

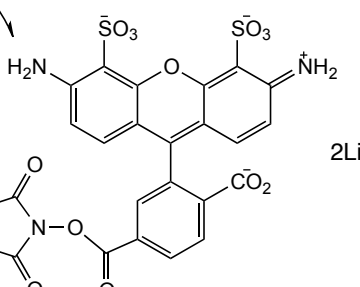
Cysteamine
Chemical Formula: C_2H_7NS
Molecular Weight: 77.15

1:10
DMF
16 h 4°C
Dilute 10 fold H_2O/TFA
SP-Sepharose
Elute PBS



(**4**) BG-Amide-NH_2
Chemical Formula: $C_{24}H_{34}N_8O_3S_2$
Molecular Weight: 546.71

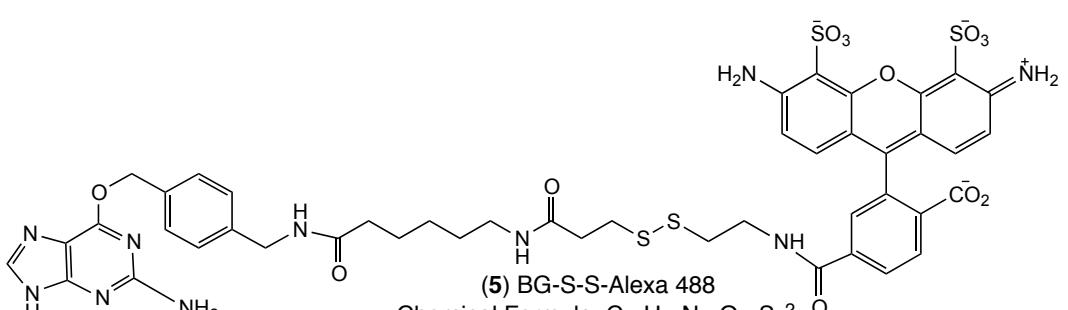
Reversed Phase C18



2Li

Alexa Fluor® 488 carboxylic acid, succinimidyl ester
Chemical Formula: $C_{25}H_{15}Li_2N_3O_{13}S_2^{2-}$
Molecular Weight: 643.41

1:1
DMF/TEA
16 h 30°C



(**5**) BG-S-S-Alexa 488
Chemical Formula: $C_{45}H_{44}N_{10}O_{13}S_4^{2-}$
Molecular Weight: 1061.15

Supplemental Figure 5