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SNAP-tagging Live Cells via Chelation-Assisted Copper-Catalyzed Azide-Alkyne Cycloaddition

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Abstract

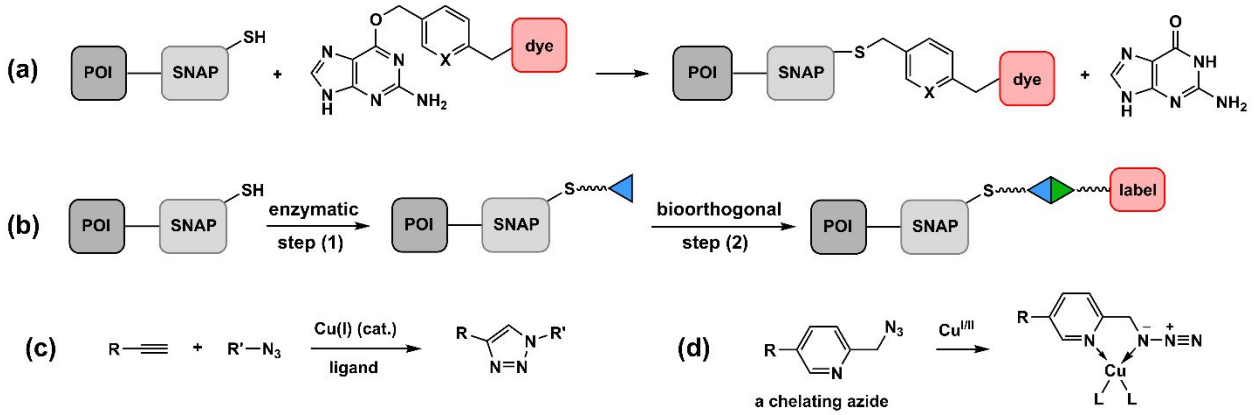
SNAP-tag is a single-turnover enzyme that has become a powerful tool, hence a popular choice, of targeted cellular protein labeling. Three SNAP-tag substrates that carry the copper-chelating 2-picolyl azide moiety are prepared, one of which has an unconventional 5-pyridylmethyl-substituted guanine structure, rather than the usual benzylguanine that is optimized to be accepted by SNAP-tag. All three substrates are effective in transferring a 2-picolyl azide moiety to SNAP-tag in live cells under conventional labeling conditions (30-minute incubation of cells with labeling reagents at 37 °C under 5% CO_2). Live cells that are decorated with chelating azido groups on the extracellular side of membranes undergo copper-catalyzed azide-alkyne cycloaddition (CuAAC) with an ethynyl-functionalized fluorophore to accomplish membrane protein labeling by a fluorescent dye. The chelation-assisted CuAAC labeling step is rapid (< 1 minute) with a relatively low dose of the copper catalyst (20 μM), and consequently exerts no ill effect on the labeled cells. A SNAP-tag substrate that carries a non-chelating azide moiety, on the other hand, fails to produce satisfactory labeling under the same constraints. Considering the wide utility of SNAP-tag in protein labeling, the rapid, live cell-compatible SNAP-tag/chelation-assisted CuAAC two-step labeling method adds to the toolbox of protein labeling technologies.

Introduction

SNAP-tag is a self-labeling protein that was engineered from the human O^6-alkylguanine alkyltransferase.[1] This single-turnover enzyme acts on a cargo-substituted benzylguanine (BG) to selectively transfer the cargo, which commonly is a fluorescent dye, to the cysteine residue in its own active site (Scheme 1a).[2] When SNAP-tag is genetically expressed as a tether to a protein of interest (POI) in cultured cells[2, 3] or a multicellular organism,[4] the POI could be specifically labeled by the subsequent reaction with a BG-conjugated fluorophore.[5] The SNAP-tag technology has proven successful for labeling POIs to study their distributions and dynamics in cellular and other biological settings.[6, 7]

For accommodating an ever-expanding range of molecular structures as payloads, some of which may impede the conjugation reaction due to docking difficulties, a two-step procedure could be adopted where the SNAP-tagging enzymatic step would first attach a small, reactive molecular handle on the protein. In the subsequent chemical step the attached handle reacts with the payload that is equipped with a complementary handle to complete the labeling (Scheme 1b).[8] The two-step protein-tag strategy is akin to the practices in (a) metabolic labeling to monitor the biosynthesis of disease markers[9-13] and (b) incorporation of unnatural amino acids for the purpose of genetic code expansion.[14-16] In the former, a substrate analog that carries a conjugation handle is taken up in a metabolic pathway, while in the latter, an amino acid substitute that is tethered with a small chemical functional group is processed by the protein synthesis machinery. A second, chemical yet biocompatible reaction follows to introduce the intended label. The second step can be selected from the inventory of bioorthogonal reactions, the development of which was arguably motivated by the needs in metabolic labeling and incorporation of unnatural amino acids in proteins.[17-23] The two-step protein labeling strategy has been practiced on the occasions where the one-step alternative is not effective due to either poor membrane permeability of the one-step substrate,[24, 25] or the restrictions placed on the substrate structure by the enzymatic step.[26] The additional benefits of a two-step method may include the increased specificity of labeling, similar to the use of primary and secondary antibodies in the enzyme-linked immunoassay (ELISA). In the time domain, a *rapid* second step reaction may be employed for the selective labeling of newly translated proteins, which could be a useful tool for studying the fate of proteins during different time points of an event of interest. Other unique advantages of two-step labeling methods will be commented on throughout this article.

Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) is a copper(I)-catalyzed conjugation reaction between azido and ethynyl functional groups (Scheme 1c).[27, 28] This reaction is efficient under a variety of conditions, and generally unaffected by the structures that carry the mutually reactive azido and ethynyl groups.[28] For this reason, the now Nobel Prize-winning CuAAC was one of the earliest transformations that were ordained as "click reactions",[29] and is considered bioorthogonal under many circumstances. The use of this reaction under biological settings, however, can be frustrated by the necessity of a copper(I) catalyst,[16] which could, among other things, (1) activate molecular oxygen[30] and consequently lead to a myriad of oxidation processes to compromise the delicate structures of proteins (e.g., oxygenation of imidazole)[31] or nucleic acids (e.g., oxidation of ribose),[32] and (2) be sequestered by cellular transition metal-binding peptides and proteins and therefore rendered unavailable for catalysis.[33]



Scheme 1 (a) SNAP-tag action on its substrate. X = C or N; (b) two-step labeling. The blue and green triangles represent complementary bioorthogonal reaction handles; (c) Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC), the ligand is optional. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, structure shown in Fig. S1)[34, 35] was used in the current study; and (d) a copper-chelating azide. 'L' denotes an arbitrary ligand or coordinating solvent that fills the coordination sphere of Cu (I or II).

Our group has studied a class of organic azides that could act as bi- or multi-dentate ligands for copper.[36-39] These azides, to which we have referred as "chelating azides" (see a drawing in Scheme 1d),[37] possess higher reactivity in CuAAC reactions than non-chelating azides. Other investigators have

validated the elevated reactivity of chelating azides over those of non-chelating azides in biologically relevant settings.[26, 40, 41] Increasing reactivity of a substrate could drive down the catalyst loading (and consequently its adverse effect), should it become necessary. Ting and coworkers demonstrated the utility of chelating azides in labeling plasma membranes of live cells by a two-step PRIME technology.[26, 42] In short, an extracellular membrane POI is genetically fused to a LAP (LplA acceptor peptide) sequence, which is conjugated via the ε-amino group of a lysine residue with a chelating azide-carrying carboxylate substrate under the catalysis of an engineered LplA (lipoate acid ligase). The labeling is done by the subsequent treatment with a CuAAC cocktail of ethynyl-functionalized fluorophore, a low dose of $CuSO_4$ (40 μM or less), sodium ascorbate, and an accelerating ligand.

Comparing to the PRIME technology, the self-labeling SNAP-tag does not require the involvement of a separately expressed enzyme for catalyzing the conjugation. Therefore, although SNAP-tag (20 kDa) is substantially bigger than a LAP-tag (13 AAs), the simpler protocol of labeling, in conjunction with comparable efficiency and selectivity, are the major draws for its use in genetic/synthetic hybrid labeling experiments. Furthermore, the complementary CLIP-tag[43] that acts on an orthogonal benzylcytosine (BC)-based substrate offer additional attractions to users who wish to execute two-color labeling in pulse-chase experiments.[43, 44] With the aim to expand the utilities of SNAP-tag, we report the combined uses of SNAP-tag and chelation-assisted CuAAC reaction – two processes that offer high conjugation rates, biochemically for the former and chemically for the latter – in the rapid two-step labeling of POIs on the extracellular membranes of live human cells.

Results and Discussion

Molecular structures. In this work, four SNAP-tag substrates are appended with an azido group (Chart 1). **5PG-AZIDE**, **BG-PyAz-1**, and **BG-PyAz-2** each contains a chelating 2-picolyl azide moiety,[36, 38] while the previously reported **BG-AZIDE**[45] carries a non-chelating azido group. A notable feature of **5PG-AZIDE** is that rather than being a typical BG substrate, this compound has a slightly different 5-pyridylmethylguanine (5PG) structure that has been shown to be an acceptable substrate of the SNAP-tag.[46] Both **BG-PyAz-1** and **-2** are the more conventional BG substrates. Comparing to **BG-PyAz-1**, **BG-PyAz-2** has an ethylene glycol linker between the 2-picolyl azide and the BG moiety to provide conformational flexibility and to improve aqueous solubility. The syntheses (Schemes 2-3) of new compounds are described in the Experimental. The structures of ethynyl-substituted dyes used in this

study and a CLIP-tag substrate used in two-color labeling experiments, all of which are commercially available, are shown in Chart 1.

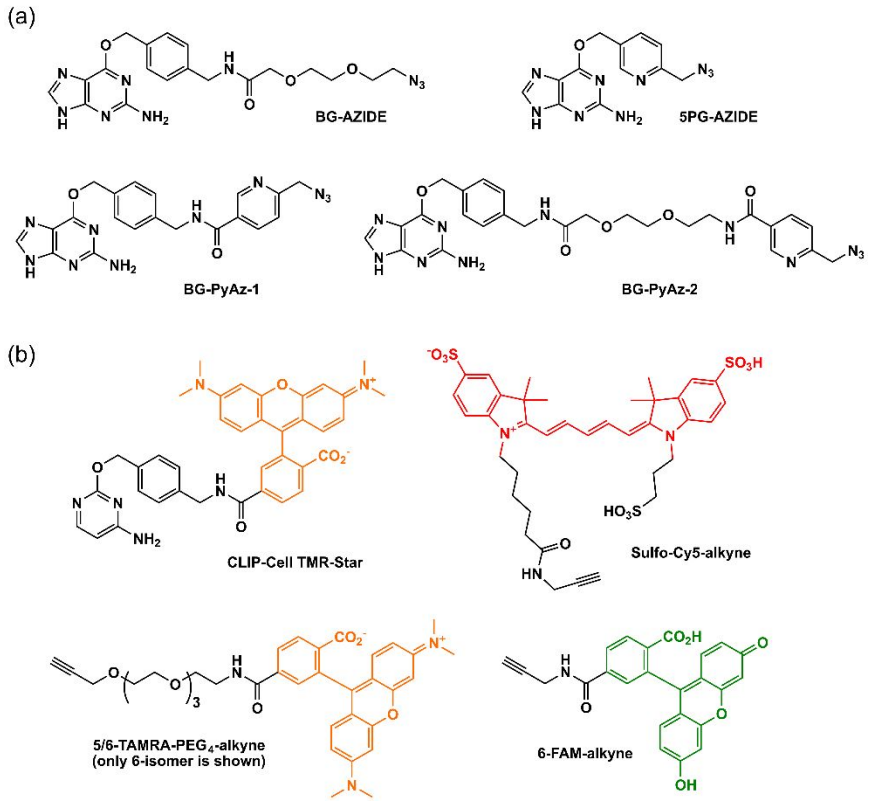


Chart 1 Azido-containing SNAP-tag substrates (a), and the dyes (b) that were used in this study.

Verifying the reactivity using SNAP-tag protein. Commercially available SNAP-tag purified protein (from NEB) was analyzed by ESI-TOF-MS, which revealed the presence of both the intact protein (MW = 19,694) and mono-DTT adduct (MW = 19,829, Fig. S2) as indicated in the datasheet of the product. Both species were found to react with each of the SNAP-tag substrates shown in Chart 1a to form a conjugation product in 1:1 ratio in 30 minutes under the conditions described in Experimental. No unreacted proteins were detected after the first step SNAP-tagging reaction (Figures S3-5). SNAP-tag tolerates the change of benzyl to methylpyridyl group in accepting **5PG-AZIDE** as a substrate. The quantity of the commercial sample was however insufficient for the characterization of the second step labeling. We subcloned the SNAP-tag in a pET28a vector, which was transformed into BL21(DE3) cells to

produce multi-milligrams of SNAP-tag. That proved to be sufficient for the two-step conjugated products to be captured and characterized against various controls in the SDS-PAGE experiments.

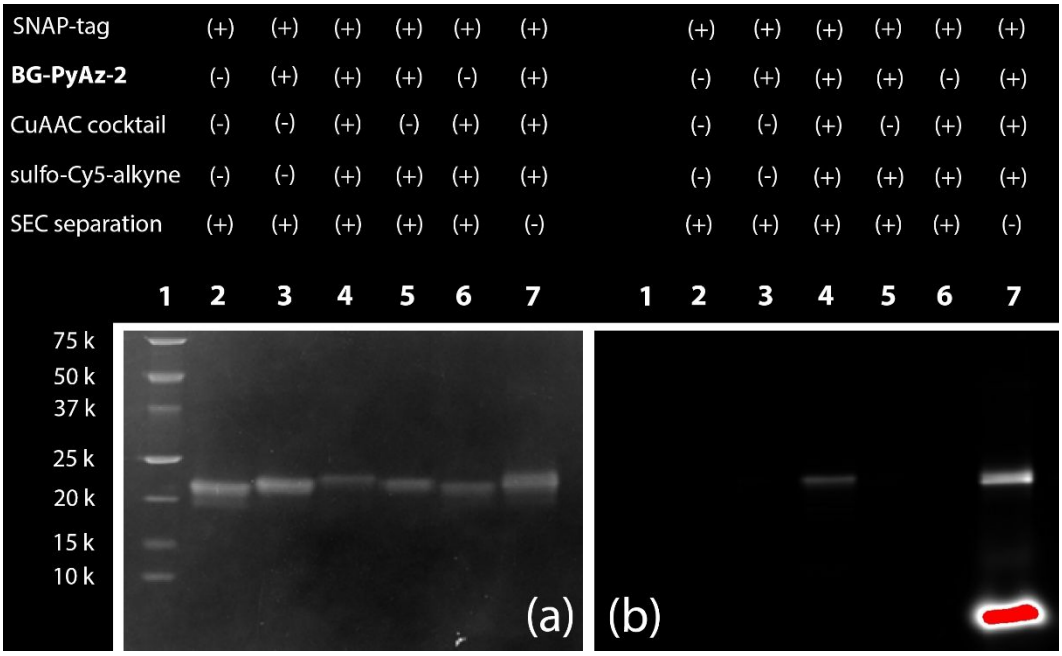


Fig. 1 SDS-PAGE gel imaged after (a) and before (b) Coomassie staining using a Bio-Rad ChemiDoc MP Imaging System under the Cy5 setting. Lane 1 – ladder; Lane 2 – SNAP-tag only; Lane 3 – SNAP-tag and **BG-PyAz-2**, after SEC; Lane 4 – **BG-PyAz-2**-attached SNAP-tag and sulfo-Cy5-alkyne treated with the CuAAC cocktail (see text), after SEC; Lane 5 – **BG-PyAz-2**-attached SNAP-tag and sulfo-Cy5-alkyne without the CuAAC cocktail, after SEC; Lane 6 – SNAP-tag and sulfo-Cy5-alkyne treated with the CuAAC cocktail without **BG-PyAz-2**, after SEC; and Lane 7 - **BG-PyAz-2**-attached SNAP-tag and sulfo-Cy5-alkyne treated with the CuAAC cocktail, without SEC separation.

The SNAP-tag protein and the one-step and two-step conjugation products, using **BG-PyAz-2** as the handle and sulfo-Cy5-alkyne as the dye, were analyzed using SDS-PAGE. The unstained gel was imaged to detect the emission from Cy5 (Fig. 1b). Only the bands in Lanes 4 and 7 were fluorescent, both of which have properly completed the two-step conjugation reaction. The “CuAAC cocktail” was used for the second step conjugation reaction for labeling with Cy5, which contains the following: sodium ascorbate (20 μL of a 25 mM solution), copper(II) acetate (10 μL, 2 mM), THPTA (5 μL, 20 mM), and

sulfo-Cy5-alkyne, 6.4 μL, 3 mM). The protein in Lane 4 was purified by the SEC to remove excess unlabeled Cy5 dye, while that in Lane 7 was not. The two bands in Lanes 4 and 7 in the middle of the gel were the fully assembled two-step conjugation product, while the overexposed band in Lane 7 (in red) with a low molecular weight was attributed to the unreacted sulfo-Cy5-alkyne. The lanes without either the CuAAC cocktail (Lane 5) or the handle **BG-PyAz-2** (Lane 6) did not show fluorescent bands, suggesting that the Cy5 labeling is specific to azido-functionalized protein and reliant on a copper(I) catalyst.

The gel was then stained with Coomassie Blue G250 and destained overnight. Coomassie absorbs and emits in the similar spectral region as Cy5.[47, 48] Therefore, all bands including the ladder were visualized by fluorescence in the Cy5 channel (Fig. 1a) post Coomassie staining (the brightfield image of the gel is shown in Fig. S6). The successive additions of the **BG-PyAz-2** (Lane 3) and CuAAC cocktail (Lane 4) to the SNAP-tag slightly but discernably reduced the mobility of the protein, consistent with the addition of a relatively small amount of mass after each step. The second step did not proceed when the CuAAC cocktail was excluded, leaving the **SNAP-BG-PyAz-2** conjugate behind (Lane 5, which levels with the band in Lane 3). When the handle **BG-PyAz-2** was absent, both conjugation steps failed, leaving the SNAP-tag protein untouched (Lane 6, which levels with the band in Lane 2). The two control experiments shown in Lanes 5 and 6 demonstrated that the labeling by Cy5 is specific to the correct two-step sequence, while the nonspecific protein staining was not detected. In conjunction with the fluorescence image (Fig. 1b) taken prior to the Coomassie staining, it is concluded that the SNAP/CuAAC two-step labeling has produced the anticipated conjugation product with efficiency and specificity.

Effectiveness of two-step SNAP-tag labeling in fixed cells via CuAAC. We have previously shown[45, 49] that **BG-AZIDE** could be accepted by SNAP-tag that is tethered with an intracellular protein and subsequently be conjugated with a cyclooctyne moiety via a strain-promoted azide-alkyne cycloaddition (SPAAC) reaction.[50, 51] In the current work, we wish to add CuAAC as an option of the second chemical step in a two-step labeling procedure. Comparing to SPAAC, CuAAC (1) is faster under most circumstances,[18] and (2) works with ethynyl-substituted dyes which are less expensive to procure than cyclooctyne-substituted fluorophores. The toxicity of Cu(I), which is the catalyst required by CuAAC, is cited as the major drawback of CuAAC in live cell labeling.[52] Chelating azides have shown elevated reactivities in CuAAC,[36, 37] which shall drive down the Cu(I) loading and consequently lessen the toxic effect of Cu imposed on cells subjected to labeling.[26, 40] This idea has been successfully implemented in a two-step PRIME technology by Ting and coworkers.[26, 42] SNAP-tag is arguably more widely applied, which

is one of the motivations for us to develop chelating azide-containing substrates of SNAP-tag and to characterize their utilities in live cell protein labeling.

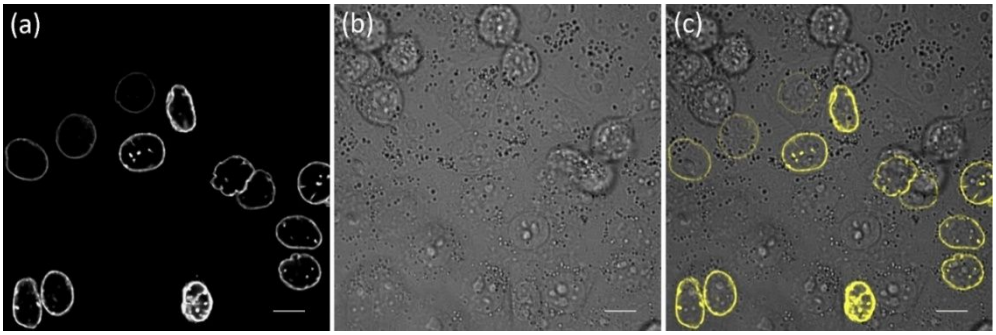


Fig. 2 Fluorescence (a), brightfield (b), and overlay (c) images of HeLa cells transiently expressing SNAP-LaminA labeled by **5PG-AZIDE** (3 μM, 1 h) followed by $CuSO_4$ (4 mM) and 5/6-TAMRA-PEG_4-alkyne (6 μM) as parts of the Click-iT kit for 30 minutes at rt. The cells were fixed using paraformaldehyde (4% in PBSA, which is PBS without Ca^{2+} or Mg^{2+}) and permeated by Triton 100-X (0.2%) after they were tagged with **5PG-AZIDE**, and before the CuAAC step. λ_{ex} = 543 nm, emission window 560−620 nm. Scale bar = 10 μm.

The first step in this process is to demonstrate that the combination of SNAP-tag and CuAAC with any of the four azides listed in Chart 1 is effective in protein labeling in fixed cells. We followed the procedure of labeling DNA of proliferating cells that would incorporate 5-ethynyluridine in newly synthesized DNA and upon fixation would be labeled by an azido-functionalized dye via the CuAAC (Click-iT EdU, see Experimental).[53] The same procedure, which recommends the concentration of $CuSO_4$ at 4 mM, was adopted in this experiment to label SNAP-tag that was expressed on nuclear envelopes and was treated with **5PG-AZIDE** before the cells were fixed. The labeling was successful judging by the abundance of the nuclear envelope labeling and the high signal-to-noise ratio (Fig. 2). The concentration of $CuSO_4$ as low as 40 μM was effective in as short as 10 minutes in labeling under otherwise identical conditions as demonstrated by **BG-PyAz-2** (Fig. S7a,b), albeit with a lower signal-to-noise ratio. Using the non-chelating azide **BG-AZIDE**, which would not enjoy the accelerating effect of chelation, also resulted in nuclear envelope labeling, with further deterioration of the signal-to-noise ratio (Fig. S7c,d).

Live cell extracellular membrane labeling using the SNAP/chelation-assisted CuAAC method. Having established that 40 μM of $CuSO_4$, which is on the same level as Ting and coworkers reported in live cell labeling using the PRIME technology,[26] was effective in labeling azido groups in fixed cells, the attention was turned to examine whether CuAAC at this level of copper dosing (or lower) would (1) be amenable to live cell labeling in conjunction with SNAP-tag, and (2) amplify the difference between reactivities of chelating and non-chelating azides.

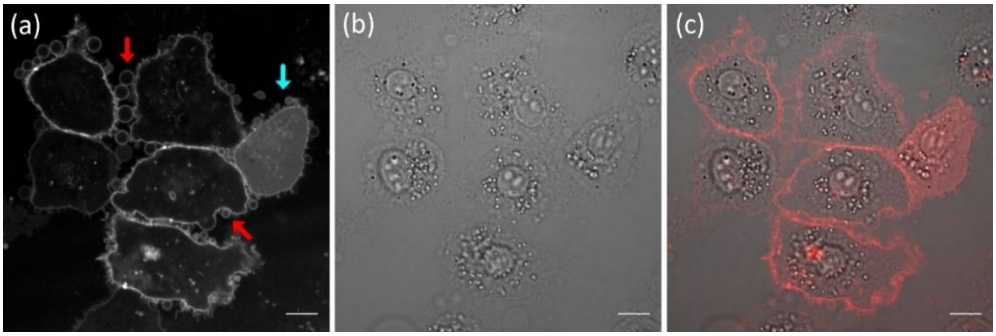


Fig. 3 Fluorescence (a), brightfield (b), and overlay (c) images of $pSNAP_f$-ADRβ2-expressing HeLa cells labeled by **BG-PyAz-2** (3 μM) for 30 minutes at 37 °C, followed by $CuSO_4$ (40 μM) and sulfo-Cy5-alkyne (6 μM, 30 minutes at rt) in PBSA. λ_{ex} = 633 nm, λ_{em} > 650 nm. Scale bar = 10 μm. Red arrows in (a) point to two blebs, while the cyan arrow in (a) points to the cell with a leaky membrane.

Given the extra considerations of intracellular labeling that are unrelated to the reactivity of chelating azides (e.g., the membrane permeability of the reagents, the heterogeneity of the intracellular space, the metal sequestration by the regulatory apparatus, etc.), we opted to first attempt the two-step labeling on the extracellular surfaces. The cells were transfected with $pSNAP_f$-ADRβ2 that upon reacting with any of the four BG substrates in Chart 1 would display azido groups on extracellular surfaces. ADRβ2 stands for β2 adrenergic receptor,[54] which is a transmembrane protein that belongs to the family of G-protein coupled receptors (GPCRs). For achieving optimal outcomes of extracellular membrane labeling, the employed dye needs to be membrane-impermeable (see the contrast of images using membrane-impermeable and permeable dyes in Fig. S8), of which sulfo-Cy5-alkyne (Chart 1) was selected.[55] Using the Click-iT EdU kit with the quantities that were established in the nuclear envelope

labeling of fixed cells, in particular the concentration of $CuSO_4$ at 40 μM, the two-step labeling of extracellular membranes could be done with all four azides (Figures 3 and S9). However, the penetrations of the dye into intracellular spaces and the blebbing[56] – a sign of apoptosis - seen with some of the cells suggested the toxicities of certain components in the kit, which disrupted membrane integrity and/or compromised cell viability. As an effort to exert more control over the functions of the "CuAAC reaction cocktail", we decided to create our own using the known ingredients of the CuAAC reaction.

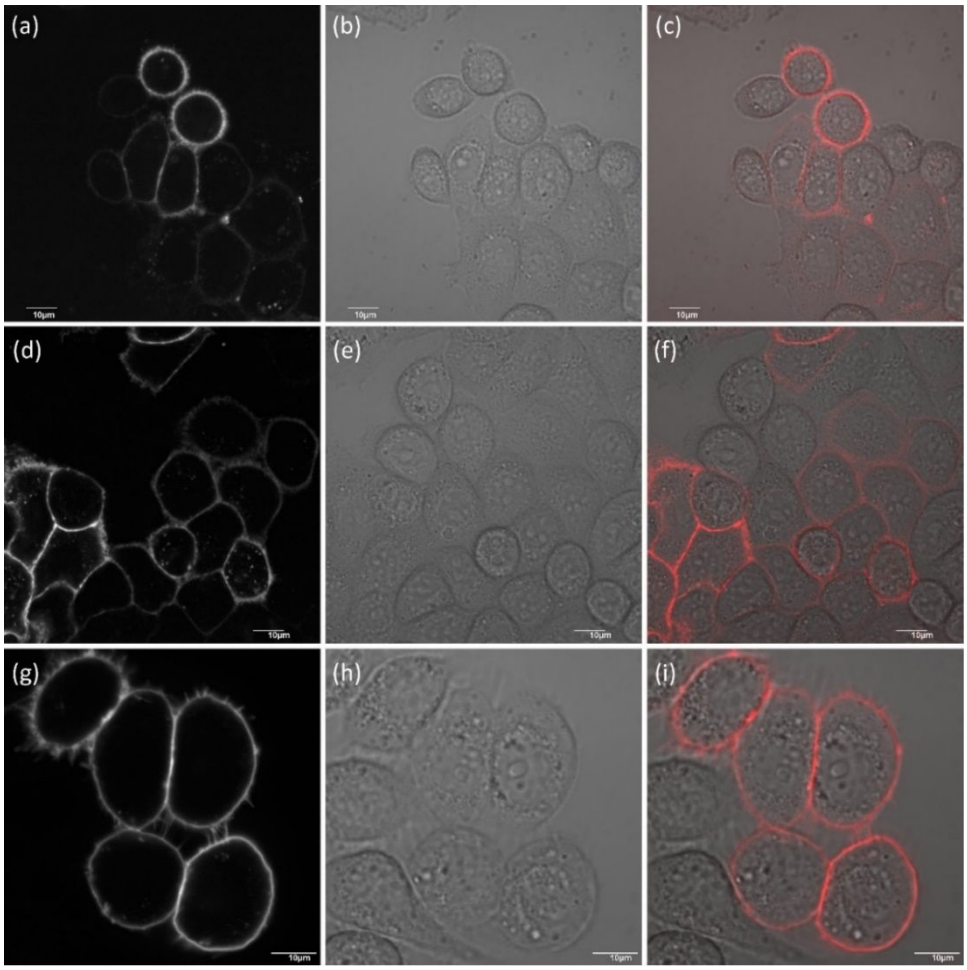


Fig. 4 Fluorescence (left), DIC (middle), and overlay (right) images of $pSNAP_f$-ADRβ2-expressing live HeLa cells labeled by **5PG-AZIDE** (a, b, c), **BG-PyAz-1** (d, e, f,) and **BG-PyAz-2** (g, h, i) at 3 μM each for 30 minutes at 37 °C, followed by sulfo-Cy5-alkyne (6 μM, 10 minutes at rt) using a homemade CuAAC cocktail in PBSA: $[Cu(OAc)_2]$ = 20 μM, [SA] = 0.5 mM, [THPTA] = 0.1 mM. λ_{ex} = 633 nm, λ_{em} > 650 nm.

The CuAAC reaction requires (1) a copper precatalyst – $CuSO_4$ or $Cu(OAc)_2$, (2) a reducing agent – sodium ascorbate (SA) to produce the catalytically active oxidation state of copper (+1), and (3) a copper-binding ligand – THPTA.[35] The ligand may either raise the reactivity of Cu,[57] or protect Cu from deactivation[58] during the reaction, or both. When the copper concentration was kept at 40 μM, while excess amounts of ligand (0.2 mM, 5x of [Cu] as recommended by Finn and coworkers[57]) and SA (1 mM) were used, the two-step labeling using **BG-PyAz-2** was successful without blebbing or cell membrane leakage. This condition appears to be compatible with live cell imaging within at least a short duration (e.g., 10-minute incubation of the second step labeling in the acquisition of images in Fig. 4), which is made possible by the high reactivity of a chelating azide in a CuAAC reaction.

We subsequently found that the concentrations of the reaction cocktail components – Cu catalyst, ligand, and SA can be halved to 20 μM, 0.1 mM, and 0.5 mM, respectively, without lowering the quality of labeling (Fig. 4). All three chelating azide substrates – **5PG-AZIDE**, **BG-PyAz-1**, and **BG-PyAz-2** – worked well, while the non-chelating **BG-AZIDE** resulted in at best inconsistent membrane labeling of much lower signal intensity (see a comparison between **5PG-AZIDE** and **BG-AZIDE** in Fig. S10), suggesting a lower CuAAC reaction rate of non-chelating comparing to chelating azides under live cell compatible conditions. Further reductions of copper catalyst and SA, or the elimination of ligand THPTA, resulted in decreased qualities of labeling. Over the course of this project, **BG-PyAz-2** has consistently resulted in a satisfactory quality of labeling. **BG-PyAz-2** contains an ethylene glycol linker which shall increase the conformational flexiblity and water solubility of the compound, and consequently may have led to a higher efficiency in the CuAAC reaction than **BG-PyAz-1** or **5PG-AZIDE** in the pocket of the SNAP-tag active site. The data listed in this paper from this point on were collected using **BG-PyAz-2**.

One-step labeling is operationally simple and has essentially no restriction placed on the location of the POI – whether it is found in intracellular organelles or on plasma membranes. Comparing to that, two-step labeling appears to be more laborious to execute, and POIs can be restricted by the nature of the bioorthogonal reaction, some of which, including the CuAAC, can only take place effectively in extracellular space (more on this later). The two-step labeling, on the other hand, carries the potential benefits of adapting to a broader range of substrate structures as argued earlier, and provides flexibility in trying different labels quickly. The latter feature has been applied in two-step labeling experiments using HaloTag to evaluate the applicability of various bioorthogonal reactions in live cells.[59] The following experiment highlights the convenience in screening various dyes in SNAP-tag labeling. Using **BG-PyAz-2**, different ethynyl-substituted dyes, which are widely available, can be used depending on the

needs of the labeling experiments without making or purchasing SNAP-tag substrates. The commercially available SNAP-tag substrates are comparably limited in scope, and can be higher in cost.

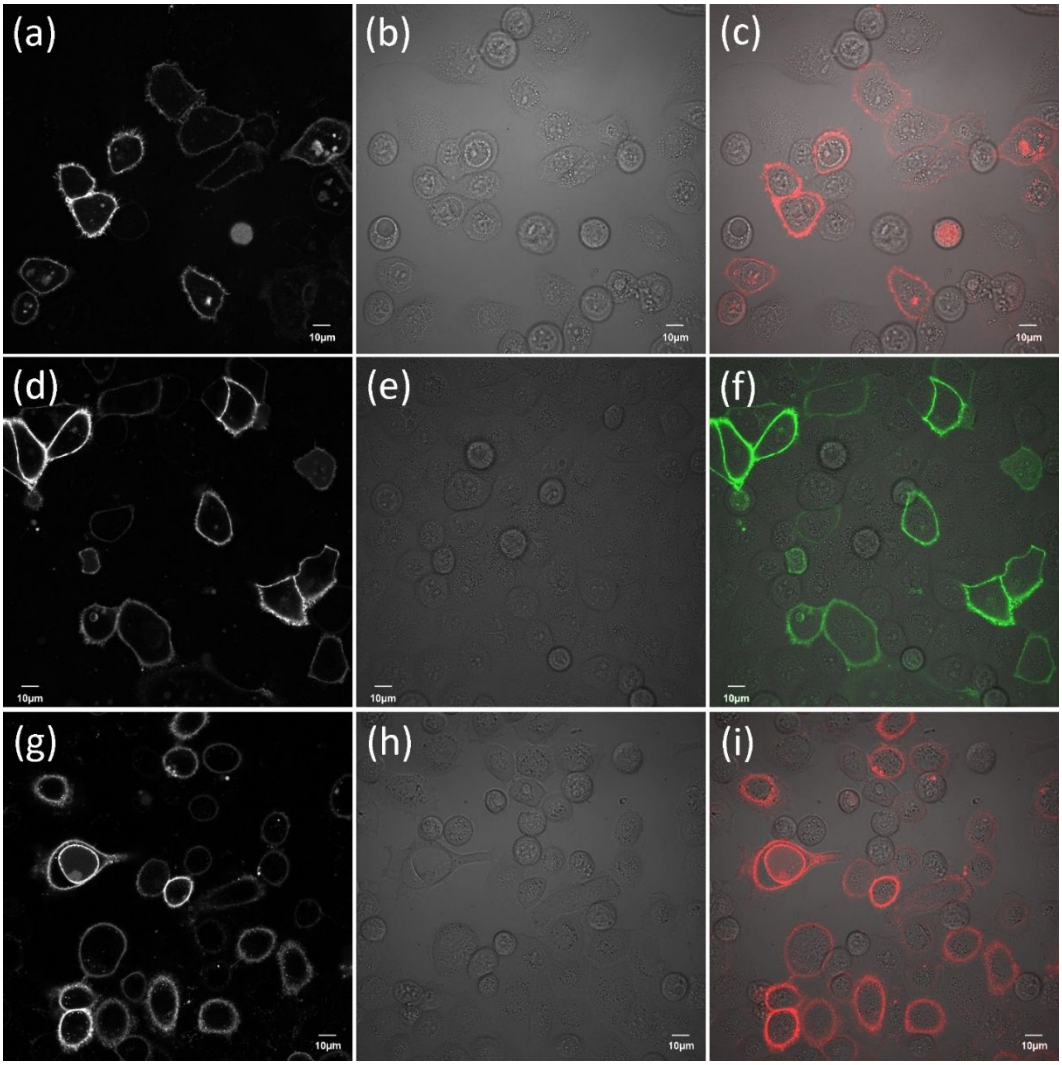


Fig. 5 Fluorescence (left), DIC (middle), and overlay (right) images of pSNAP$_f$-ADRβ2-expressing live HeLa cells labeled by **BG-PyAz-2** (a-f) at 3 μM each for 30 minutes at 37 °C, followed by sulfo-Cy5-alkyne (a-c, 6 μM) or 6-FAM-alkyne (d-f, 6 μM) for 10 minutes at 37 °C using a homemade CuAAC cocktail in PBSA: [$Cu(OAc)_2$] = 20 μM, [SA] = 0.5 mM, [THPTA] = 0.1 mM. Frames g-I were acquired from the experiment in which **BG-PyAz-2** (3 μM) was mixed with the CuAAC cocktail for 30 minutes to produce the labeling media. The pseudo one-step labeling occurred in 10 minutes at 37 °C. For Cy5, λ_{ex} = 633 nm, λ_{em} > 650 nm. For FAM, λ_{ex} = 488 nm, λ_{em} =500-600 nm.

In Fig. 5, images covering the entirely field of view are shown, which shall be more representative of the qualities of the labeling than the selected cell clusters, which are shown in Fig. S11. Figures 5a-c are images using sulfo-Cy5-alkyne as the dye in the second step, while in Figures 5d-f the green-emitting FAM-alkyne (Chart 1) resulted in labeling with similar quality upon visual examination. In the experiment represented by Figures 5g-i, **BG-PyAz-2** and sulfo-Cy5-alkyne were premixed for 30 minutes in PBSA that contains the CuAAC cocktail, followed by incubating the cells in the unprocessed (i.e., no effort to remove copper was made) reaction mixture for 10 minutes. Selective membrane labeling was also achieved, albeit with a slightly higher background. This set of experiments demonstrated the ease of trying more than one labels quickly in the two-step labeling, as well as the tolerance of experimental sequences – either sequential steps bridged by washing, or premixing the handle and dye in a "pseudo" one-step labeling may work. Considering the wide availability of ethynyl-substituted labels and the efficiency of the chelation-assisted CuAAC (10 minutes or less labling time), The SNAP-tag/chelation-assisted CuAAC two-step method could be favored in experiments where a number of labels need to be screened quickly.

One often-cited benefit of CuAAC (in particular the chelation-assisted version) over other bioorthgonal reactions such as SPAAC is its efficiency. The qualities of images of the two-step labeling of the extracellular portion of ADRβ2 using chelation-assisted CuAAC and SPAAC were compared. The cells were transfected using SNAP-ADRβ2, followed by the first step labeling using **BG-PyAz-2** for 30 minutes. The cells were then treated with either DBCO-sulfo-Cy5, which would lead to the SPAAC reaction, or sulfo-Cy5-alkyne along with the CuAAC cocktail, for 30 seconds (the time for mixing the dye-containing media with the cells labeled with **BG-PyAz-2**). The images of labeled cells (Fig. 6a-f) were obtained after the dye was removed by washing. The labeling of extracellular membrane was successful in both cases, while the application of the chelation-assisted CuAAC second step (Fig. 6d-f) resulted in a signal-to-noise ratio ~ 6 times higher than SPAAC. Cysteine has been reported to react effectively under certain circumstances with strained[60] or electrophilic alkynes.[61, 62] Because the labeling was unexpectedly facile, we checked the possibility that cyteine residues on SNAP-ADRβ2 that was unlabeled with **BG-PyAz-2** may have reacted with an alkyne,[63] and thus being unintentionally labeled on the membranes. The answer was negative (Fig. S12). This experiment demonstrated that (1) the labeling time can be reduced further to ~ 30 seconds, and (2) chelation-assisted CuAAC is indeed faster than SPAAC in achieving extracellular labeling with high contrast. The general protocol for one-step SNAP-tag labeling calls for an incubation of 30 minutes during which some labeled proteins may have translocated. In two-step

labeling, the attachment of a fluorescent dye occurs in the second step. Given the high efficiency of the chelation-assisted CuAAC second step, which could be achieved in less than 1 minute, a relatively fast membrane protein transport event could conceivably be visualized with a higher time resolution than what is achievable by the corresponding one-step procedure. The demonstration of such an advantage of the two-step approach in delineating a rapid cellular biological event is the goal in an ongoing study.

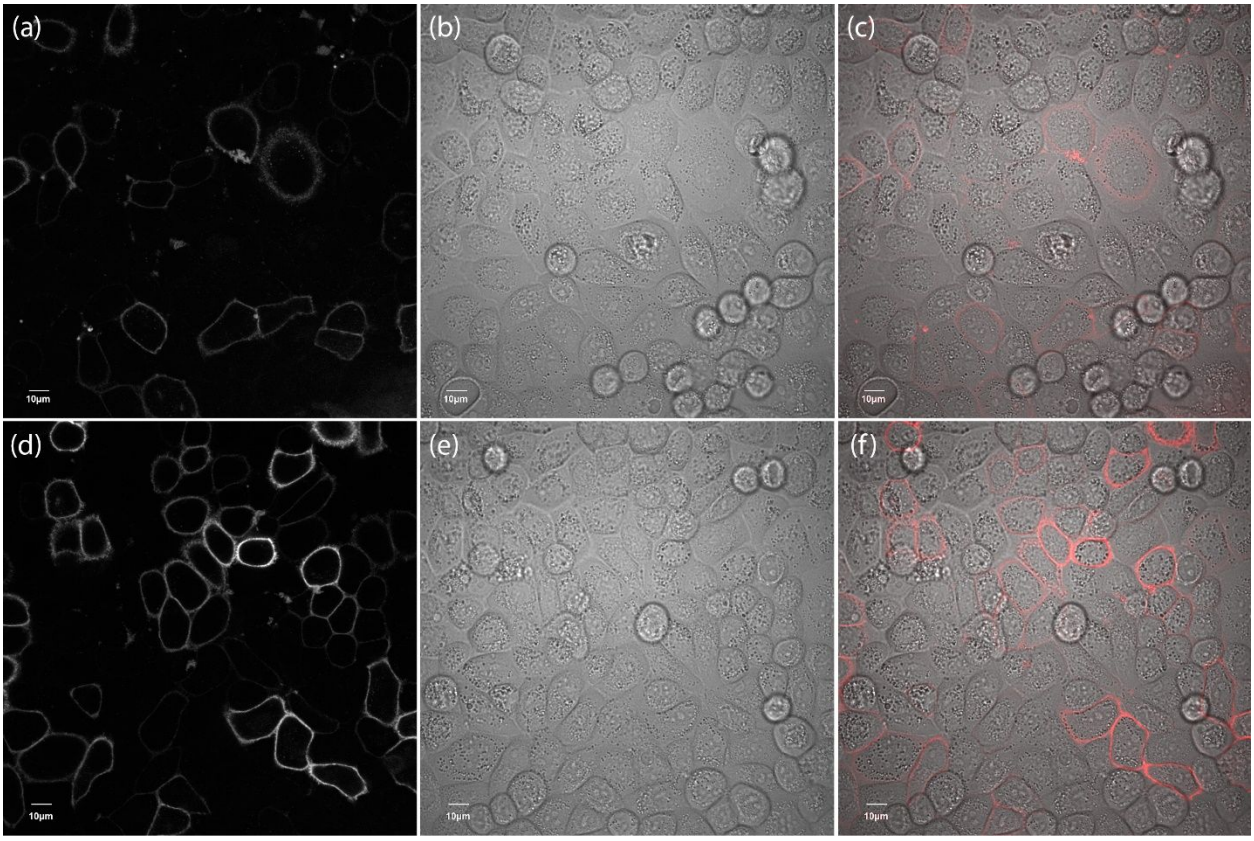


Fig. 6 Fluorescence (left), DIC (middle), and overlay (right) images of pSNAP$_f$-ADRβ2-expressing live HeLa cells labeled by **BG-PyAz-2** (a-f) at 3 μM for 30 minutes at 37 °C, followed by DBCO-sulfo-Cy5 (a-c, 6 μM in PBSA) or sulfo-Cy5-alkyne (d-f, 6 μM in the homemade CuAAC cocktail) for 30 seconds at 37 °C. λ_{ex} = 633 nm, λ_{em} > 650 nm. For d-f, [$Cu(OAc)_2$] = 20 μM, [SA] = 0.5 mM, [THPTA] = 0.1 mM.

Evidence of compatibility of the SNAP/chelation-assisted CuAAC labeling method with live cells. Besides the absence of blebbing or membrane leakage, the cells after the two-step SNAP/chelation-

assisted CuAAC treatment were able to internalize the labeled ADRβ2,[64] which is the correct metabolic consequence of this membrane-spanning receptor protein and is therefore a testimonial for the viability of the labeled cells. After the labeling of cell membrane using the $pSNAP_f$-ADRβ2/**BG-PyAz-2**/sulfo-Cy5-alkyne two-step method, the cells were reincubated at 37 °C in a CO_2 incubator for 24 h, and were again imaged. The labeled proteins were by then internalized into intracellular vesicles (Fig. 7), suggesting that the endocytic pathway was unobstructed after the treatment with the reagents during the two-step labeling process, while the cells appeared to bear no ill effect from the brief treatment (10 minutes) of the CuAAC cocktail and to have since proliferated.

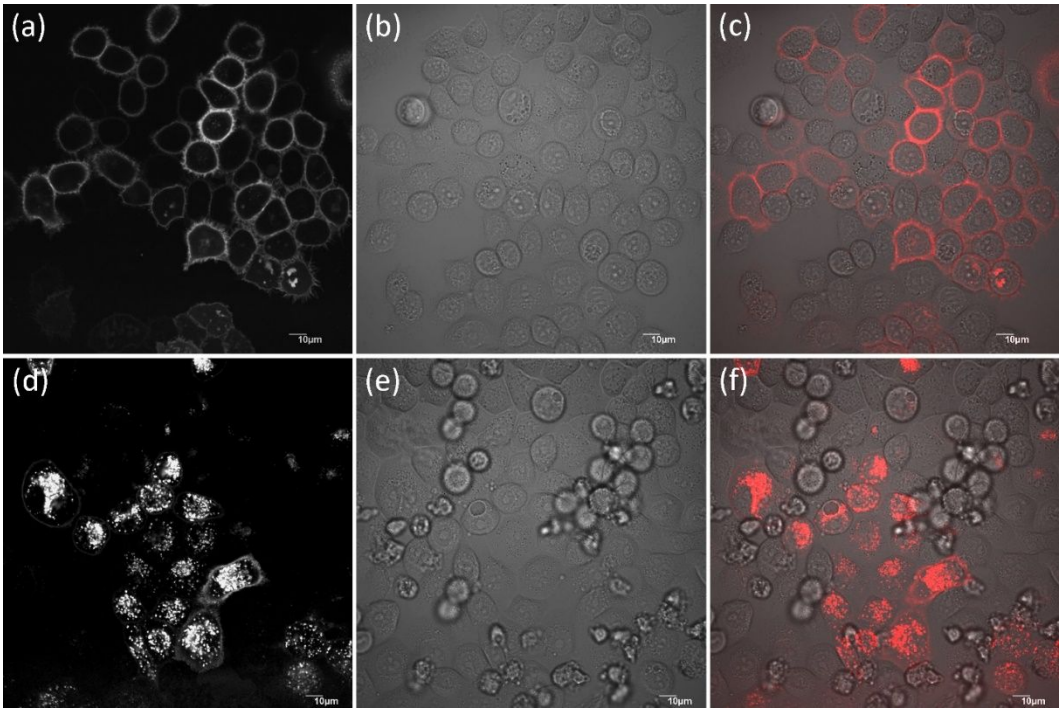


Fig. 7 Fluorescence (left), DIC (middle), and overlay (right) images of $pSNAP_f$-ADRβ2-expressing live HeLa cells labeled by **BG-PyAz-2** at 3 μM for 30 minutes at 37 °C, followed by sulfo-Cy5-alkyne (3 μM, 10 minutes at rt in PBSA) using a homemade CuAAC cocktail: [$Cu(OAc)_2$] = 20 μM, [SA] = 0.5 mM, [THPTA] = 0.1 mM. Frames a-c are images taken immediately after the two-step labeling step, while frame d-f were acquired 24 h after the labeling experiment. An additional layer of cells appeared in (e) due to the proliferation of the cells over the 24-h period. λ_{ex} = 633 nm, λ_{em} > 650 nm.

The cytotoxicity of the CuAAC cocktail was characterized using the CCK-8 cell viability assay,[65] which is a variant of the MTT assay.[66] The tetrazolium salt used in the CCK-8 assay and its metabolically reduced formazan dye are both soluble in the culture media, which make the experiment easy to execute. HeLa S3 cells were seeded in a 96-well plate at a density of 1,000-3,000 cells per well. Following an overnight growth, the cells were treated with the CuAAC reaction cocktail at different concentrations for 30 minutes. Other wells contained ingredients that were intended to serve as either positive or negative controls, and to test factors other than mitochondrial metabolic capacity on the absorption at 450 nm, which is considered characteristic of the formazan dye.

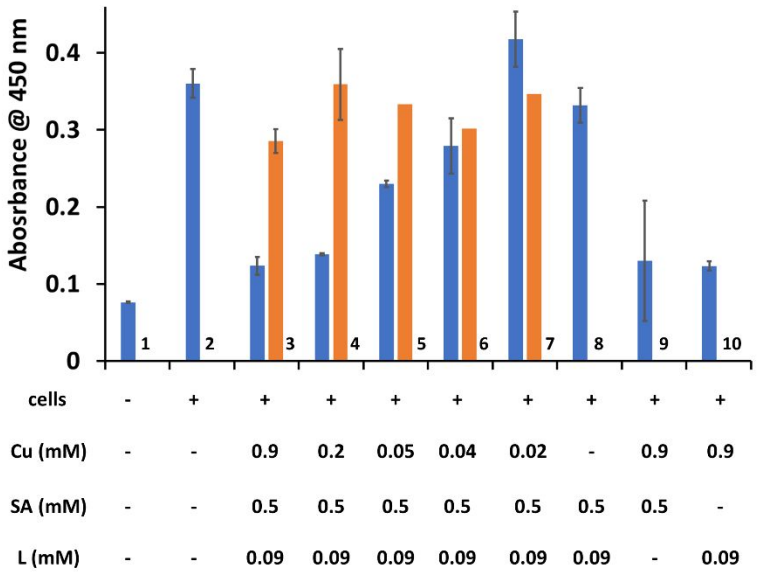


Fig. 8 Toxicity of different conditions as shown by the CCK-8 assay. Cu was administered as an aqueous solution of $Cu(OAc)_2$. SA = sodium ascorbate; L = ligand THPTA. Blue columns: data of the wells where the components were mixed and incubated prior to the addition of the MST-8 dye; orange columns: data of the wells where the CuAAC cocktails (Cu, SA, and L) were added after cells were incubated with the MST-8 dye.

The negative (#1, Fig. 8) and postive (#2) controls that define the high and low values of absorption at 450 nm, which were generated respectively from the wells that contained only the media, and that contained live cells that were treated with the tetrazolium MST-8 dye. The data from the wells where

the cells were treated for 30 minutes with the CuAAC reaction cocktails that contained $Cu(OAc)_2$ from 0.9 mM to 20 μM are shown by columns #3-7. The cell viability increases as the [Cu] decreases. At 20 μM of Cu (#7), the viability is on par with that of the positive control (#2). The absorbance at 450 nm was also recorded for the wells where the CuAAC cocktails were added *after* the cells were treated with WST-8. Those data are shown as orange columns and serve as controls for the effect, or the lack thereof as determined to be the case, of the CuAAC cocktail on the color of the formazan dye. As the last set of controls, Cu, SA, and the ligand THPTA were individually taken away from the CuAAC cocktail (#8-10) to test which one is responsible for the cytotoxicity. The removal of Cu restored the absorbance at 450 nm (#8), while the removal of SA (#10) or THPTA (#9) did not. Therefore, it is the Cu salt, rather than SA or the ligand, that was killing the cells.

Application of the SNAP/chelation-assisted CuAAC two-step labeling method in colabeling. The next set of experiments demonstrated the utility of the two-step SNAP/chelation-assisted CuAAC in conjunction with CLIP-tag[43] or a fluorescent protein for two-color labeling. HeLa S3 cells were co-transfected with $pSNAP_f$-ADRβ2 and CLIP-LaminA,[45] followed by incubation at 37 °C in a CO_2 incubator in a media that contains both **BG-PyAz-2** (3 μM) and CLIP-Cell-TMR-Star, a benzylcytosine-tethered orange fluorescent dye available from the NEB (see Chart 1, 1 μM). After 30 minutes, the cells were washed with media followed by the addition of the CuAAC reaction cocktail – sulfo-Cy5-alkyne (6 μM), $Cu(OAc)_2$ (20 μM), THPTA (0.1 mM), and SA (0.5 mM) in a PBSA buffer. Further incubation at rt for 30 minutes was followed by washing to remove chemical agents before imaging. As shown in Fig. 9a-c, the dually labeled cells exhibited emissions from both nuclear envelopes and plasma membranes, that match the excitation and emission profiles of TMR and Cy5, respectively. The colabeling experiment was repeated using CLIP-LifeAct[45] instead, which targets actin fibers.[67] As shown in Fig. 9d-f, the plasma membranes of the cells were labeled by sulfo-Cy5 while the intracellular actin fibers fluoresced in yellow, which was from the dye TMR. In the next experiment, HeLa cells were cotransfected with $pSNAP_f$-ADRβ2 and mEmerald-Rab5a-7. The latter is a green fluorsecent protein that is tethered to Rab5, a protein found in early endosomes.[68] After SNAP/chelation-assisted CuAAC two-step labeling of the plasma membranes with sulfo-Cy5-alkyne, the plasma membranes were visualized to encapsulate the vesicles that were shown in the green channel (Fig. 9g-i). This set of experiments shows that the SNAP/chelation-assisted CuAAC two-step plasma membrane labeling is effective concurrent with labling of additional POIs in live cells using other protein tags or fluorescent proteins.

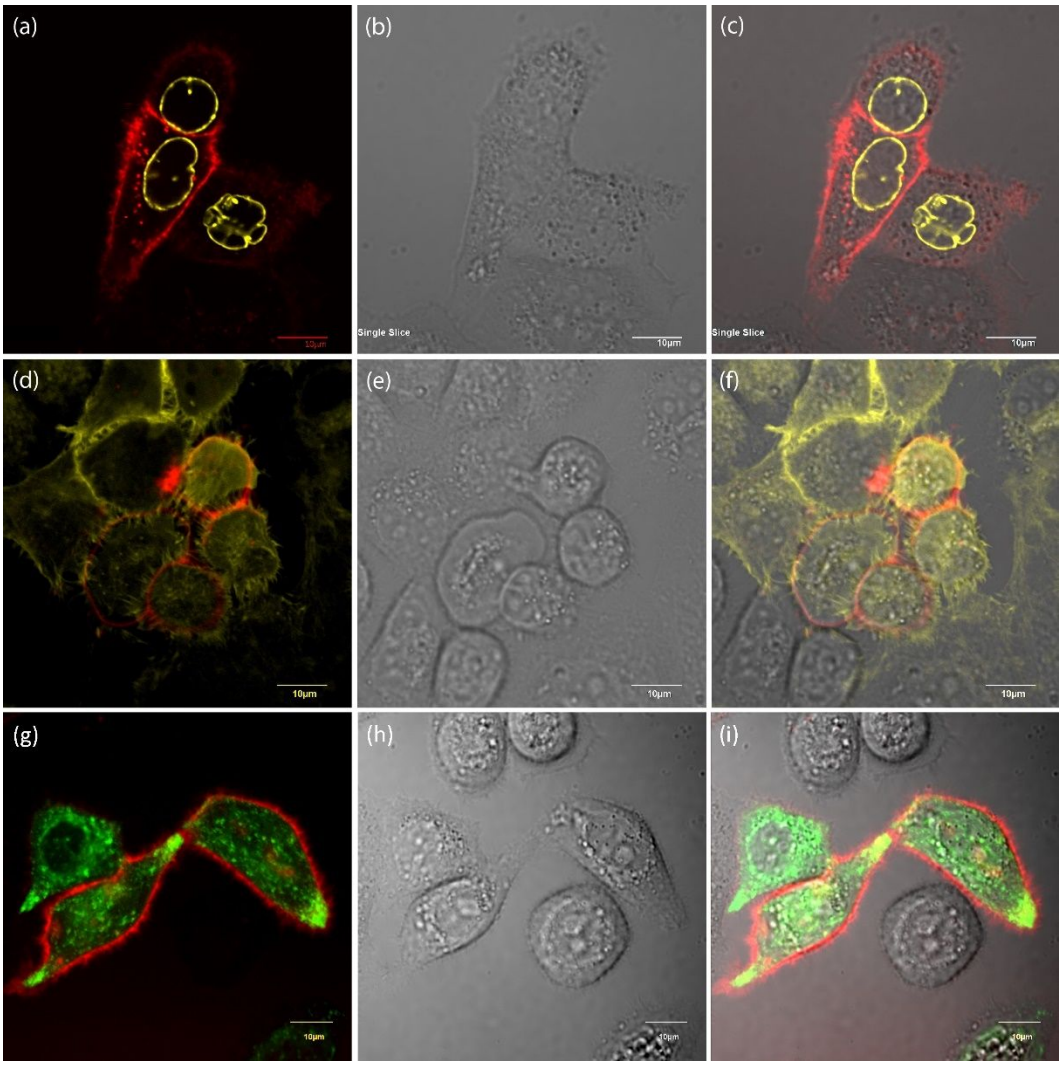


Fig. 9 Fluorescence (a, d, g), brightfield (b, e, h), and overlay (c, f, i) images of live HeLa S3 cells expressing pSNAP$_f$-ADRβ2 and pCLIP$_f$-LaminA (a, b, c) or pCLIP$_f$-LifeAct (d, e, f), or mEmerald-Rab5a-7 (g, h, i). The fluorescence images in individual channels were taken sequentially at slightly different focal planes before overlayed to form the dual-color images. The cells were treated by **BG-PyAz-2** (3 μM), and for the top two rows, CLIP-Cell TMR Star (1 μM, yellow in a and d, λ_{ex} = 543 nm, λ_{em} = 560-620 nm) for 30 minutes. After washing, the cells were labeled with sulfo-Cy5-alkyne (6 μM, red in a, d, and g, λ_{ex} = 633 nm, λ_{em} > 650 nm), $Cu(OAc)_2$ (20 μM), SA (0.5 mM), and THPTA (0.1 mM) for 30 minutes. Rab5 in the bottom row was labeled with mEmerald (green, λ_{ex} = 488 nm, λ_{em} = 500-600 nm).

Intracellular attempt. The chelation-assisted CuAAC second step was attempted for intracellular labeling in live cells. HeLa S3 cells were transfected with SNAP-LaminA, followed by the sequential treatments of **BG-PyAz-2** and the CuAAC cocktail that were applied successfully in plasma membrane labeling. However, no nuclear envelope labeling was observed. Because we know that **BG-PyAz-2** could be accepted by SNAP-LaminA expressed in live cells (Fig. S7a,b), the failure of labeling is attributed to the second, CuAAC step. Intracellular copper, which is an essential trace element that is found in many enzymes, is strictly regulated[69, 70] because of its aforementioned cytotoxicity. It has been clear in bioinorganic literature that the abundance of copper-binding peptides and proteins, such as metallothioneins,[33, 71] renders copper ion virtually unavailable to species that are outside the regulatory and transport apparatus.[33] The efficient and near-complete sequestration of copper inside mammalian cells acts as an inherent barrier for the CuAAC reaction to be effective in intracellular bioconjugation under biologically meaningful conditions.[72] Unsurprisingly, most reported applications of CuAAC in labeling proteins of live cells occur on cell surfaces.[12, 26, 40, 52] The recent development of plasma membrane permeable synthetic ligands with attomolar to zeptomolar affinity to Cu(I) offers tantalizing prospects for developing technology that enables the transport of copper(I) complexes to intracellular space while maintaining structural integrity and catalytic potency.[73-75]

Discussion. Among the four azides in Chart 1, the three chelating azides are shown to be effective in two-step labeling of extracellular membranes. To a modest degree, **BG-PyAz-2** exhibited the most consistent level of labeling, which could be attributed to the presence of the ethylene glycol linker between the chelating azide moiety and the BG substrate. This linker would free the chelating azide from the steric hinderance of the enzyme active site, and at the same time provide a relaxed tether in the aqueous environment due to the amphiphilicity of the ethylene glycol moiety. Comparing to the performances of two other popular bioorthogonal reactions – SPAAC and inverse electron-demand Diels-Alder reaction (IEDDA)[76-78] - in two-step labeling observed from our group,[45] the chelation-assisted CuAAC reaction is undoubtedly faster (IEDDA could be faster than conventional CuAAC with proper pair of substrates).[18, 79] Plasma membrane labeling with a good signal-to-noise ratio could be completed *in less than a minute* using chelation-assisted CuAAC in the second step, while the other two reactions in our hands take longer.[45, 49] CuAAC, on the other hand, have not, yet, been shown consistently workable inside live mammalian cells under physiological conditions, while the other two bioorthogonal reactions are the current tools of choice for labeling intracellular proteins. A more systematic comparison will be

made in the future on stably transfected cell lines so that the SNAP-tag expression level is controlled to reduce the heterogeneity of the labeling, and in consequence to make quantitative analysis possible.

Conclusion

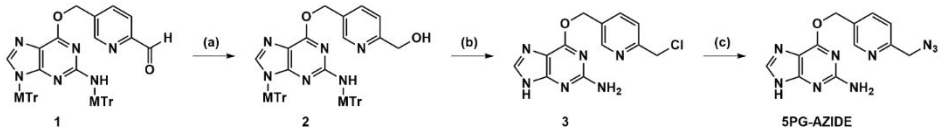
In summary, three chelating azide-tethered SNAP-tag substrates are reported, one of which contains an unconventional methylpyridylguanine, rather than benzylguanine, that is nonetheless accepted by the SNAP-tag protein. The combined use of SNAP-tag and chelation-assisted CuAAC in labeling protein of interest on live cells is described. In addition to the general benefits of two-step labeling,[8] which includes (1) addressing issues of poor one-step labeling substrate acceptance if the payload structure hinders active site docking, and (2) enabling quick screening of multiple labels without laborious synthesis of separate SNAP-tag substrates, this specific enzymatic and bioorthogonal combination offers a method that is rapid – the sequential two steps can be done in well under one hour (30 minutes for SNAP-tagging and less than 1 minute for chelation-assisted CuAAC). The rapidity of the chelation-assisted CuAAC under live cell-compatible conditions is made possible by the elevated reactivity of 2-picolyl azide,[36] a chelating azide that doubles as an activating and protective ligand for the copper catalyst.[37, 38] Hence, a toxic level of copper from a high dosage is no longer needed to maintain an efficient coupling reaction. The dual-color live cell labeling that includes the SNAP/chelation-assisted CuAAC sequence and the complementary CLIP-tag is also demonstrated. Considering the ease of use and therefore the wide utilities of SNAP- and CLIP-tags, the two-step labeling involving the second chelation-assisted CuAAC step provides options to labeling experiments that require flexibilities in the identity of the tags and the timing of labeling.

Experimental

Materials and general methods. Reagents and solvents were used as received from various commercial sources. Thin layer chromatography (TLC) was performed on silica gel 60 F254 plates. Flash column chromatography was performed using 40-63 μM (230-400 mesh) silica gel. ^{1}H and ^{13}C NMR spectra were acquired at 500/600 and 125/150 MHz, respectively. Chemical shifts are reported in δ (ppm) values relative to the residual internal $CHCl_3$ (δ_H 7.26, δ_C 77.2) or DMSO-d_5 (δ_H 2.50, δ_C 39.5). High

resolution mass spectra of reported organic compounds were obtained either at the Mass Spectrometry Laboratory at FSU or at the Mass Spectrometry Research and Education Center at University of Florida. ESI-TOF-MS analyses of the SNAP-tag protein (acquired from NEB) and its first step conjugation products were conducted on an Agilent 6230 TOF-MS instrument at the Mass Spectrometry Laboratory at FSU. Fluorescence microscopy was carried out on an Olympus FV1000 Laser Scanning Fluorescence Microscope that is equipped with a Bioptechs DeltaT Culture Dish Controller for live cell imaging. **CAUTION! Low molecular weight organic and inorganic azides potentially explosive. Appropriate protective measures should always be applied when handling these chemicals.**

Synthesis of the three chelating azides



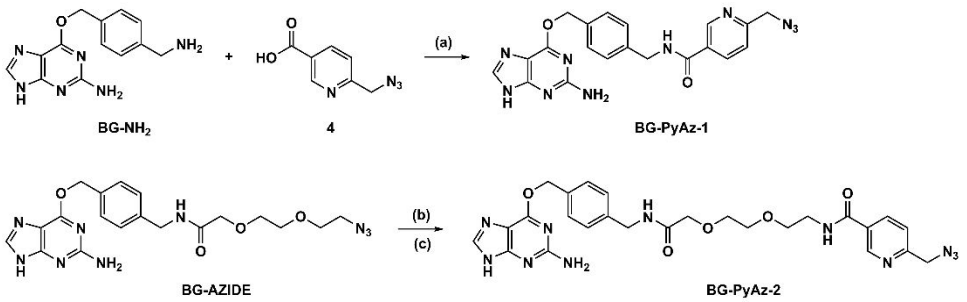
Scheme 2 Synthesis of **5PG-AZIDE**. Reagents and conditions: (a) $NaBH_4$, EtOH, 0-5 °C to rt, 4 h, 81%; (b) $SOCl_2$, DCM, under argon, rt, 4 h, 76%; (c) NaN_3, DMF, 90 °C, overnight, quantitative yield.

Compound 2 (Scheme 2). A solution of compound **1** (0.330 g, 0.40 mmol), the preparation of which was reported in a previous paper,[46] in EtOH (16 mL) was cooled to 0-5 °C in an ice bath. $NaBH_4$ (0.040 g, 1.05 mmol) was then added in 3 portions. The resulting mixture was stirred for 4 h allowing slow warming to rt. The crude mixture was adsorbed on silica and chromatographed (SiO_2, EtOAc to 10% MeOH in EtOAc). The desired compound was obtained as a white solid (0.267 g, 81% yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ/ppm 8.26 (s, 1H), 7.51 (s, 1H), 7.48-7.43 (m, 1H), 7.32-7.26 (m, 6H), 7.19-7.07 (m, 16H), 7.07-6.98 (m, 4H), 6.82-6.77 (m, 2H), 6.69-6.64 (m, 2H), 5.76 (s, 1H), 4.77-4.68 (m, 4H), 3.80 (s, 3H), 3.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ/ppm 159.3, 158.9, 158.7, 158.0, 157.6, 155.2, 148.0, 146.0, 141.7, 141.2, 138.1, 136.8, 133.9, 131.4, 131.0, 130.2, 129.9, 127.9, 127.6, 126.5, 120.1, 116.6, 113.2, 112.8, 75.2, 70.9, 64.9, 64.1, 55.4, 55.3; HRMS (ESI+) calcd from $C_{52}H_{45}N_6O_4$ $[M+H]^+$ 817.3502, found 817.3471.

Compound 3 (Scheme 2). Intermediate **2** (0.100 g, 0.12 mmol) was placed in a Schlenk flask and put under vacuum, then the flask was backfilled with argon. Under the inert atmosphere, dry DCM (2 mL) was added, followed by the dropwise addition of $SOCl_2$ (40 µL, 0.55 mmol). The mixture was stirred at rt

for 4 h. The crude mixture was concentrated in vacuo, the residue was dissolved in MeOH, adsorbed on silica and chromatographed (SiO_2, DCM:MeOH 50:1 to 30:1 to 9:1). Compound **3** was recovered as a white solid (0.027 g, 76% yield). ^{1}H NMR (DMSO-d_6, 600 MHz) δ/ppm 12.57 (bs, 1H), 8.73 (s, 1H), 8.03-7.96 (m, 1H), 7.84 (s, 1H), 7.61-7.54 (s, 1H), 6.33 (s, 2H), 5.52 (s, 2H), 4.79 (s, 2H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ/ppm 159.5, 156.1, 149.6, 137.7, 132.0, 123.0, 64.1, 46.6; HRMS (ESI+) calcd from $C_{12}H_{12}Cl_1N_6O_1$ $[M+H]^+$ 291.0761, found 291.0762.

5PG-AZIDE (Scheme 2). To a solution of compound **3** (0.017 g, 0.06 mmol) in dry DMF (1.5 mL), NaN_3 (0.005 g, 0.07 mmol) was added. The mixture was stirred overnight at 90 °C. The solvent was removed under vacuum and the product was further dried under high vacuum. The desired compound was obtained as a yellow solid (0.017 g, quantitative yield). ^{1}H NMR (DMSO-d_6, 600 MHz) δ/ppm 8.76 (s, 1H), 8.03-7.96 (m, 1H), 7.83 (s, 1H), 7.50-7.41 (m, 2H), 6.33 (s, 2H), 5.52 (s, 2H), 4.52 (s, 2H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ/ppm 159.5, 159.2, 156.3, 155.5, 149.8, 138.7, 137.6, 131.8, 122.2, 112.7, 64.1, 54.2; HRMS (ESI+) calcd from $C_{12}H_{12}N_9O_1$ $[M+H]^+$ 298.1165, found 298.1167.



Scheme 3 Syntheses of **BG-PyAz-1** and **BG-PyAz-2**. Reagents and conditions: (a) HOBt, DCC, DIPEA, DMF, argon protection, rt, overnight, 78%; (b) Ph_3P, THF, water, rt, overnight; (c) **4**, HOBt, DCC, DIPEA, DMF, argon protection, overnight, 48% for two steps.

BG-PyAz-1 (Scheme 3). In a Schlenk flask were placed compound **BG-NH$_2$** (0.050 g, 0.19 mmol),[45] compound **4**[26] (0.033 g, 0.19 mmol), 1-hydroxybenzotriazole (HOBt, 0.025 g, 0.19 mmol), and DCC (0.038 g, 0.19 mmol). The flask was connected to a Schlenk line to draw vacuum, followed by backfilling with argon. The solvent DMF (2 mL) was subsequently added, followed by the addition of DIPEA (35 µL, 0.47

mmol). The mixture was stirred at rt overnight. The crude solution was concentrated in vacuo. The residue was dissolved in MeOH, adsorbed on silica and chromatographed (SiO_2, DCM:MeOH 50:1 to 30:1 to 9:1) to give 0.062 g (78% yield) of a white solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ/ppm 12.43 (bs, 1H), 9.30-9.21 (m, 1H), 9.04 (s, 1H), 8.30-8.22 (m, 1H), 7.82 (s, 1H), 7.56-7.51 (m, 1H), 7.51-7.43 (m, 2H), 7.40-7.32 (m, 2H), 6.25 (s, 2H), 5.46 (s, 2H), 4.59 (s, 2H), 4.51 (s, 2H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ/ppm 164.5, 159.6, 158.3, 148.3, 139.1, 136.2, 135.4, 129.1, 128.6, 127.4, 121.9, 66.5, 54.1, 42.5; HRMS (DART+) calcd from $C_{20}H_{19}N_{10}O_2$ $[M+H]^+$ 431.1687, found 431.1701.

BG-PyAz-2 (Scheme 3). To a solution of **BG-AZIDE** (0.088 g, 0.20 mmol)[45] in THF (1 mL) and water (50 μL), PPh_3 (0.157 g, 0.60 mmol) was added. The mixture was stirred overnight at rt. The crude mixture was diluted with MeOH and passed through a short plug of silica using a mixture DCM:MeOH:Et_3N 50:1:0.1 to 30:1:0.1 to 9:1:0.1. After concentration of the fractions containing the reduced intermediate, a white powder was recovered (0.058 g, 70% crude yield). Without further purification the next step was performed. In a Schlenk flask were placed the crude product obtained in the previous step (0.030 g, 0.07 mmol), compound **4** (0.013 g, 0.07 mmol), HOBt (0.010 g, 0.07 mmol), and DCC (0.015 g, 0.07 mmol). The flask was connected to a Schlenk line to draw vacuum, followed by backfilling with argon. DMF (1 mL) was added, followed by the addition of DIPEA (14 μL, 0.08 mmol). The mixture was stirred at rt overnight. The crude solution was concentrated in vacuo. The residue was dissolved in MeOH, adsorbed on silica and chromatographed (SiO_2, DCM:MeOH 50:1 to 30:1 to 9:1 to 7:1). **BG-PyAz-2** was obtained as a white solid (0.020 g, 48% yield). ^{1}H NMR (DMSO-d_6, 500 MHz) δ/ppm 12.51 (bs, 1H), 9.01-8.97 (m, 1H), 8.80-8.74 (m, 1H), 8.28-8.23 (m, 1H), 8.21 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.82 (s, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.46-7.40 (m, 2H), 7.29-7.24 (m, 2H), 6.27 (s, 2H), 5.75 (s, 1H), 5.44 (s, 2H), 4.58 (s, 2H), 4.30 (d, *J* = 6.5 Hz, 2H), 3.94 (s, 2H), 3.64-3.57 (m, 4H), 3.57-3.52 (m, 2H), 3.47-3.39 (m, 2H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 169.3, 164.7, 159.6, 158.3, 148.3, 139.3, 136.1, 135.2, 129.1, 128.5, 127.3, 121.9, 70.3, 70.0, 69.4, 68.8, 66.5, 54.9, 54.1, 41.5; HRMS (ESI+) calcd from $C_{26}H_{30}N_{11}O_5$ $[M+H]^+$ 576.2426, found 576.2446.

ESI-TOF mass spectrometry. SNAP-tag purified protein (NEB, 5 μL of 50 μM stock solution, 0.25 nmole of protein) was diluted with water (100 μL) and mixed with a SNAP-tag substrate, for example, **5PG-AZIDE** (0.5 μL of 1 mM stock solution in DMSO, 0.5 nmole of **5PG-AZIDE**) in an Eppendorf tube (1.5 mL). After vortexing for 5 sec followed by centrifuging for the mixture to settle, the reaction was left at rt for 30 min before cooled on ice for transporting to the Mass Spectrometer Laboratory for analysis.

The injection sample volume was 5 μL, while the capillary voltage and fragmentor voltage were set at 5,000 V and 150 V, respectively. Protein quasimolecular ions with +14 to +26 charges were usually observed, the molecular weights of the modified proteins listed on Figures S6-9 were converted from the m/z values of the observed ions.

General culture and transfection procedures. HeLa cells were grown in RPMI 1640 (1X) supplemented with 10% cosmic calf serum (Hyclone) and penicillin, streptomycin and amphotericin B (1X). The cultures were kept in an incubator at 37 °C, 5% CO_2 and 95% humidity. Twenty-four hours before transfection, exponentially growing cells were seeded on Bioptechs Delta T4 dishes. The cells were transfected (2 μg DNA/mL media) using Qiagen Effectene® Transfection Reagent following the manufacturer's procedure (https://www.qiagen.com/us/products/discovery-and-translational-research/functional-and-cell-analysis/transfection/effectene-transfection-reagent/). The plasmids used in this study are the following: SNAP-LaminA, SNAP-ADRβ2, CLIP-LaminA, CLIP-LifeAct, and mEmerald-Rab5a-7. Incubation as above was continued overnight before the labeling experiments. SNAP-LaminA, CLIP-LaminA, and CLIP-LifeAct were reported in previous papers,[45, 46] while $pSNAP_f$-ADRβ2 Control Plasmids was acquired from Addgene.

Labeling and imaging procedure

(1) SNAP- or CLIP-tag reaction (the first step). HeLa S3 cells transiently expressing either SNAP- or CLIP-tag (e.g., $pSNAP_f$-ADRβ2) were incubated for 30 minutes in 1 mL of growth media containing an appropriate substrate (e.g., **BG-PyAz-2**, 3 μM, diluted in media from a ~ 3 mM stock solution in DMSO that was stored in a -20 °C freezer). The cells were then washed twice with 1 mL of PBSA.

(2) Fixation. The fixation was done after the first-step (e.g., SNAP-tag phase) labeling *if needed*. After the SNAP-tag reaction, the media was removed and freshly prepared paraformaldehyde (4% in PBSA, which may last for ~ 1 wk in a fridge. PBSA is PBS without calcium and magnesium) was added, followed by incubation for 15 minutes at 37 °C in a dry incubator. After fixation, the paraformaldehyde was aspirated and the cells were washed by adding PBS-G (PBS containing 50 mM glycine) and the dish was placed on a shaker for 5 minutes (repeated once more). Next, the cells were permeated by treating the dish with a Triton 100-X solution (0.2% in PBSA) and placed on a shaker for 5 minutes. The fixed cells can be used immediately or on the next day (while stored in a fridge) for labeling and imaging.

(3) CuAAC reaction (the second step)

(a) Using the Click-iT EdU kit. The SNAP-LaminA-expressing HeLa S3 cells that contained azido groups in a ΔT culture dish were fixed using 4% paraformaldehyde (PFA) and permeabilized using 0.2% Triton 100-X. The fixed cells were washed twice with PBS on a shaker (5 min each). Using the Invitrogen Click-iT EdU imaging kit (https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/protocols/click-it-edu-imaging-protocol.html), a reaction cocktail was created by adding the following reagents to an Eppendorf in this order: 430 μL (or more depending on the quantity of Cu solution used) of 1x reaction buffer, 20 μL (or less) of 1x $CuSO_4$ (final conc. 4 mM, or lower), 1 μL of 3 mM 5/6-TAMRA-PEG_4-alkyne in DMSO (final conc. 6 μM), and 50 μL of freshly prepared buffer additive. The total volume was 501 μL. The PBS in the dish was replaced with the reaction cocktail and covered with aluminum foil to protect the cells from light. The culture dish was incubated for 30 minutes at rt on a shaker. Afterward, the buffer containing the reaction cocktail was aspirated from the dish, and the cells were washed twice with PBS on the shaker for 5 minutes each. The PBS buffer was replaced afresh, and the cells were ready for imaging.

(b) Using the home-made CuAAC cocktail. The following stock solutions were prepared: (1) $Cu(OAc)_2$ at 2 mM in water; (2) THPTA ligand at 20 mM in water; and (3) sodium ascorbate at 25 mM in water. The stock solutions dispensed into aliquots can be stored at -20 °C for up to 2 months, except sodium ascorbate which ought to be prepared fresh prior to a CuAAC reaction. Each aliquot can be frozen and thawed for up to 4 times.

The CuAAC cocktail (500 μL) was produced by first mixing the stock solutions of $Cu(OAc)_2$ (5 μL, 2 mM) and THPTA (2.5 μL, 20 mM) in a 1.5-mL Eppendorf tube, followed by the addition of sodium ascorbate stock solution (20 μL, 25 mM). After mixing on a Vortex, the mixture was incubated at rt for 5 minutes. PBSA buffer (472 μL) was then added to the mixture, followed by the final addition of the alkyne substrate sulfo-Cy5-alkyne (1 μL, 3 mM in DMSO) to complete the 500-μL CuAAC cocktail. Final concentrations: [$Cu(OAc)_2$] = 20 μM; [THPTA] = 0.1 mM; [sodium ascorbate] = 1 mM; and [sulfo-Cy5-alkyne] = 6 μM. The cells that are already labeled by a handle (e.g., **BG-PyAz-2**) were incubated in the CuAAC cocktail for various durations (0.5, 10, or 30 minutes) at 37 °C. The CuAAC cocktail was subsequently removed via aspiration. The cells were washed with PBSA twice before the dish was refilled with warm whole media (1 mL) before imaging.

(4) An example of two-color labeling procedure. HeLa S3 cells were plated in a ΔT culture dish and allowed to grow overnight. The cells were then transfected with $pSNAP_f$-ADRβ2 and CLIP-LaminA using Qiagen Effectene Transfection kit followed by an overnight incubation (~ 24 h). A dual-labeling mix was prepared by including **BG-PyAz-2** (3 μL of 1 mM solution in DMSO) and CLIP-Cell-TMR-Star (1 μL of 1 mM solution in DMSO) in warm whole media (37 °C, 1 mL). The washed cells were incubated in the dual-labeling mix for 30 minutes at 37 °C. The cells were then washed twice with warm whole media, before treated with the CuAAC reaction cocktail (500 μL) that contains the following: sulfo-Cy5-alkyne (6 μM), $Cu(OAc)_2$ (20 μM), THPTA (0.1 mM), sodium ascorbate (0.5 mM), and PBSA buffer. The cells were incubated for 30 minutes at rt, then washed with warm (37 °C) whole media twice before imaging.

(5) Fluorescence Imaging was performed on an Olympus FV1000 Laser Scanning Confocal Microscope. A 60x oil objective and a 488-nm Ar (for mEmerald and FAM), 543-nm He/Ne (for TAMRA), or a 633-nm (for Cy5) He/Ne laser were used. For live cell imaging, the ΔT culture dish was secured in a Delta T Stage Adaptor that is connected to a Bioptechs Delta T Culture Dish Controller. The temperature was maintained at 37 °C during the imaging experiments.

ImageJ was used to quantify the signal-to-noise ratio of each image shown in Fig. 6. Both images were acquired on the same day in back-to-back single session experiments using identical set of instrument and software parameters. To quantify noise, the mean intensity of a section exhibiting no fluorescence was measured and divided by the area in which the measurement took place. The signal was determined in a similar manner by using 6 different cells in the same image. The average of intensity of these cells was used as the signal, resulting in the signal-to-noise values reported (5.7 for CuAAC and 0.97 for SPAAC). This process was repeated twice for both the CuAAC and the SPAAC confocal images.

Two-step conjugation of purified SNAP-tag protein. The pSNAP-tag (T7) vector (Plasmid #101137) was purchased from Addgene. The pSNAP-tag sequence was subcloned into the pET28a vector where restriction enzymes Nde1 and EcoR1 were used to digest both the vector and the SNAP(tag)-T7 clone. The expressed protein has an N-terminal His-Tag. There is a stop codon at the end of the pSNAP-tag so the C-terminal His-tag would not be translated. The newly acquired vector with pSNAP-tag in the pET28a backbone with an N-terminal His-tag was transformed into BL21(DE3) cells.

The transformed *E. coli* BL21(DE3) cells with pET28a (Kanamycin) which encode $(His)_6$-SNAP-tag were grown on a LB agar plate containing 100 μg/mL kanamycin. A single colony was picked to inoculate 20

mL of LB containing 100 μg/mL kanamycin and grown at 37 °C for 16 h with shaking at 200 rpm. The 20 mL culture was then used to inoculate 2 L of LB containing 100 μg/mL kanamycin and grown at 37 °C with shaking at 200 rpm until OD_{600} ~0.6. The culture was induced by the addition of isopropyl-B-D-thiogalactoside (IPTG) to a final concentration 0.5 mM, and then grown at 30 °C for an additional 4 h. Cells were harvested by centrifugation at 4,000 rpm for 30 min, and the pellets were stored at -20 °C until further use. The cell pellet was resuspended in a lysis buffer [1x PBS, 10 mM imidazole, 1 mM DTT]. Cells were lysed by sonication [30% amplitude, 1 min of alternating cycles of 1s ON and 1s OFF, and the crude lysate was centrifuged at 15,000 rpm for 30 min]. The supernatant obtained was filtered through a Whatman filter paper. The filtered supernatant was loaded onto a pre-equilibrated 5 mL nickel beads column. The column was washed with wash buffer [1x PBS, 20 mM imidazole, 1 mM DTT]. The SNAP-tag protein was eluted using the elution buffer [1x PBS, 250 mM imidazole, 1 mM DTT]. The collected fractions were analyzed by SDS-PAGE. The fractions of correct MW were pooled and were concentrated to load the gel filtration column with 1XPBS with 1 mM DTT (Superdex 200 Increase HiScale 16/40, Cytiva). The purified SNAP-tag protein was incubated in the presence or absence of thrombin (10 units per mg protein, Cytiva) at 4 °C overnight. The cleaved SNAP-tag protein was purified with a gel filtration column (Superdex 200 Increase HiScale 16/40) with 1XPBS with 1 mM DTT. Then, His-tag-removed SNAP-tag protein was confirmed by SDS-PAGE.

Conjugation reactions were performed on the purified SNAP-tag protein (~ 0.3 mg/mL in PBSA buffer) to characterize the one- and two-step conjugation products. As an example, the first step, the addition of an azido group, was accomplished by adding **BG-PyAz-2** (33 μL, 3 mM in DMSO) to the purified SNAP-tag protein (2 mL, 0.3 mg/mL). Half of the mixture was transferred to an Eppendorf tube. The reaction vessel was inverted four times. After centrifuging and allowing the sample to react for 30 minutes at rt, the product was then subjected to purification using a size exclusion column (SEC). Purification was accomplished with a Superdex 200 Increase HiScale 16/40, 39-41 cm (Cytiva, former GE) column using a PBS elution buffer containing DTT (1 mM).

The concentration of the purified first step conjugation product (e.g., **SNAP-BG-PyAz-2**) was measured using a Thermo Nanodrop ND-1000 UV/Vis Spectrophotometer. By obtaining the absorption at 280 nm and using the general reference setting, the concentration was estimated to be 0.1 mg/mL. To initiate the second CuAAC conjugation step, the purified **SNAP-BG-PyAz-2** (1 mL, 0.1 mg/mL) was reacted by first adding sodium ascorbate (20 μL, 25 mM), followed by the addition copper(II) acetate (10 μL, 2 mM) and THPTA (5 μL, 20 mM). Following the addition of these reactants, the reaction vessel was inverted

four times and centrifuged. Finally, Sulfo-Cy5-alkyne (6.4 μL, 3 mM in DMSO, final conc. 19 μM) was added to the reaction mixture and allowed to react for 30 min at rt. Final concentrations: [$Cu(OAc)_2$] = 20 μM, [THPTA] = 0.1 mM, [sodium ascorbate] = 0.5 mM. After the completion of the second CuAAC step, the product was subjected to purification using the same SEC procedure previously described to yield the final labeled protein (0.06 mg/mL). Other samples were prepared by omitting one key ingredient (handle or CuAAC cocktail) under otherwise identical conditions. Samples obtained after each successive reaction were stored at -80 °C for future analysis.

The protein samples were subjected to SDS-PAGE on 12% polyacrylamide gel that was constructed using the Sigma-Aldrich Bis-Tris Polyacrylamide Gel Casting Kit. Using the PAGE data shown in Figure 7 as an example, samples were loaded into the gel in combination with NuPAGE LDS Sample Buffer, distilled water, and DTT (500 mM): Lanes: 1, molecular weight marker (5 μL); 2, unreacted SNAP-tag (2.2 μg); 3, SNAP-tag that was reacted with **BG-PyAz-2** (1.4 μg); 4, SNAP-tag containing the **BG-PyAz-2** handle, after the addition of Sulfo-Cy5-alkyne treated with the CuAAC cocktail (1.2 μg); 5, SNAP-tag containing the **BG-PyAz-2** handle, and Sulof-Cy5-alkyne without the treatment with the CuAAC cocktail (0.9 μg); 6, SNAP-tag deprived of any handle, and Sulfo-Cy5-alkyne treated with the CuAAC cocktail (1.0 μg); 7, SNAP-tag containing the **BG-PyAz-2** handle, after the addition of Sulfo-Cy5-alkyne treated with the CuAAC cocktail without SEC (1.7 μg). The gel was run for 1.5 h at 50 V, after which the voltage was increased to 150 V and the gel was allowed to run to completion for an additional 37 min. Prior to Coomassie staining, the finished gel was imaged using a Bio-Rad ChemiDoc MP Imaging System to overcome the similar absorption properties of Coomassie Blue G250 and Sulfo-Cy5. The Cy5-containing bands were visualized (Figure 7a) by irradiating the gel using the Cy5 excitation setting (625-650 nm). Afterward, the gel was stained using Coomassie Blue G250 (0.5 g) dissolved in methanol (20%, 80 mL) acetic acid (10%, 40 mL) and distilled water (280 mL). Images were then acquired after staining using both a white and blot/UV/stain free sample trays compatible with the imaging system. The image obtained on the white sample tray was acquired using the Coomassie Blue setting on the imaging system (Figure S10). The image obtained on the blot/UV/stain free sample tray (Figure 7b) was acquired to highlight each band on the gel using the Cy5 excitation setting on the imaging system (625-650 nm).

The CCK-8 cell viability assay. A 96-well plate was warmed at 37 °C, 5% CO_2 for 24 h before usage. On the next day, HeLa S3 cells were trypsinized and removed from a 75-cm^2 flask. A hemocytometer was used to determine the density of cells. The trypsinized cell stock was diluted to about 10,000-30,000 cells/mL in RPMI media. This diluted cell suspension (100 μL, 1,000-3,000 cells) was added to each well,

with blank wells containing only RPMI media. The plate was again incubated at 37 °C, 5% CO_2 for overnight (~ 20 h).

The CuAAC reaction mixtures of different Cu concentrations were made by first mixing (1) 25 μL of a 40 mM $Cu(OAc)_2$ stock solution, (2) 50 μL of a 4 mM $Cu(OAc)_2$ stock solution, and (3) 30, 20, 10 μL of a 2 mM $Cu(OAc)_2$ stock solution, respectively, with THPTA (20 mM, 5 μL). Sodium ascorbate (25 mM, 20 μL) was then added into each mixture. Following a 5-min wait, PBSA buffer was added to bring the solution to 100 μL each. Of these mixtures, [Cu] = 10, 2, 0.6, 0.4, and 0.2 mM, [THPTA] = 1 mM, and [sodium ascorbate] = 5 mM. These solutions were made 15 min before mixing with the cells.

A CuAAC reaction mixture (10 μL) that contained 1 mM THPTA, 5 mM sodium ascorbate, and varying concentrations of $Cu(OAc)_2$ (10 mM to 0.2 mM) was added to each well that contained 100 μL of HeLa S3 cell suspension that was plated on the previous day (final [Cu]s are 0.9, 0.2, 0.05, 0.04, 0.02 mM, respectively; final [THPTA] = 0.09 mM, and final [sodium ascorbate] = 0.5 mM in each well). Each reaction mixture was tested in triplicate. After a 30-minute incubation, each well was treated with the WST-8 dye that was provided as a ready-to-use solution in Cell Counting Kit-8 (CCK-8, 10 μL), followed by incubation at 37 °C, 5% CO_2 for 3 h. Care was taken to avoid creating bubbles in the wells, which might interfere with the reading of absorption. The absorbance values of the wells at 450 nm were recorded after 3 h on a SpectraMax iD5 microplate reader. These values, which are linearly correlated with the concentrations of the formazan dye as the metabolic product, were plotted in Figure 5 against various concoctions of the CuAAC reaction cocktail.

Author contributions

Conceptualization - LZ and MMC; Investigation – DJS (cell culture, imaging, mass spectrometry, toxicity assays), MMC (synthesis, cell culture, and preliminary imaging), SMC (SDS-PAGE), CFTB (cell culture, imaging, mass spectrometry), GS (protein expression and purification); Formal Analysis – LZ, DJS, MMC, CFTB, and SMC; Writing – all have participated in writing and editing; Supervision – LZ.

Conflicts of Interest

There are no conflicts to declare.

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Electronic supplementary information (ESI) available: Additional fluorescence images of dye-labeled cells, mass spectrometric data, SDS-PAGE image, and NMR spectra of new compounds. See DOI: 10.1039/x0xx00000x

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