Published in final edited form as:

Chembiochem. 2017 March 02; 18(5): 470–474. doi:10.1002/cbic.201600627.

Versatile interacting peptide (VIP) tags for labeling proteins with bright chemical reporters

Dr. Hannah K. Zane[§], **Ms. Julia K. Doh**[§], **Prof. Caroline A. Enns**[b], and **Prof. Kimberly E. Beatty**[a]

[a]Department of Biomedical Engineering, Oregon Health & Science University, 2730 Moody Avenue (CL3B), Portland, OR, USA

[b]Department of Cell, Developmental, and Cancer Biology, Oregon Health & Science University

Abstract

Fluorescence microscopy is an essential tool for the biosciences, enabling the direct observation of proteins in their cellular environment. New methods that enable attachment of photostable synthetic fluorophores with genetic specificity are needed to advance the frontiers of biological imaging. Here we describe a new set of small, selective, genetically-encoded tags for proteins based on a heterodimeric coiled-coil interaction between two peptides: CoilY and CoilZ. Proteins expressed as a fusion to CoilZ were selectively labeled with the complementary CoilY fluorescent probe peptide. Fluorophore-labeled target proteins were readily detected in cell lysates with high specificity and sensitivity. We found that these versatile interacting peptide (VIP) tags allowed rapid and specific delivery of bright organic dyes or quantum dots to proteins displayed on living cells. Additionally, we validated that either CoilY or CoilZ could serve as the VIP tag, which enabled us to observe two distinct cell-surface protein targets with this one heterodimeric pair.

Keywords

fluorescent probes; coiled coils; protein engineering; conjugation; microscopy

Fluorescent labeling of proteins enables the evaluation of protein function, interactions, dynamics, and sub-cellular localization[1]. The fluorescent proteins (e.g., GFP and mCherry) are useful tools for microscopy, but their large sizes (~28 kDa) can disrupt protein function, trafficking, stability, and sub-cellular morphology[2]. The tripeptide-derived fluorescent protein chromophores are formed spontaneously, but have a limited range of photophysical properties. Faced with these limitations, chemical biologists designed genetically-encoded protein tags that bind synthetic fluorophore ligands[3]. These include tags based on DNA alkyl transferases (SNAP[4] and CLIP[5] tags), dehalogenases (HaloTag[6]), dihydrofolate reductase (TMP tag[7]), and antibody fragments (e.g., fluorogen-activating proteins[8]). The introduction of synthetic chemical reporters improves the palette of fluorophores and

Correspondence to: Kimberly E. Beatty.

[§]Authors contributed equally.

Supporting information for this article is given via a link at the end of the document.

enables new applications in cellular imaging such as multi-color super-resolution imaging[3, 9]. Still, these protein tags remain relatively large, adding 18 to 33 kDa to the protein of interest.

In contrast to the protein tags, short peptide tags facilitate fluorophore labeling with a minimal increase in the target protein's molecular weight. The tetracysteine tag[10] was the first peptide tag but its fluorescent biarsenical reporter is toxic and prone to labeling cysteine-rich cellular proteins[11]. Another approach exploits ligase variants to append biotin[12], coumarin[13], or resorufin[14] to short (<2 kDa) peptide tags. These peptide tags utilize the specificity and speed of enzymatic catalysis but are relatively complicated and limited in scope. Thus, despite over 15 years of community development, there are few versatile peptide tags and those tags have little spectral diversity compared to fluorescent proteins.

An ideal genetically-encoded peptide tag would be small, target-specific, easy to use, and compatible with diverse chemical reporters. We report herein two peptide tags, CoilY and CoilZ, with all of these features. These peptides heterodimerize to form a structured motif called an α-helical coiled-coil. We used the strong interaction between CoilY and CoilZ to fluorescently label proteins *in vitro* and on cells (Scheme 1). Our approach achieves spectral diversity through the delivery of a range of biophysical probes, including bright organic fluorophores and quantum dots (Qdots).

We initiated this project by identifying candidate heterodimers from the literature. We considered the E3/K3 pair described by Litowski and Hodges[15], and adapted for imaging by Matsuzaki and coworkers[16]. But this pair lacked bidirectionality—only the basic peptide could be used to deliver a fluorescent label. Therefore we sought a heterodimeric pair with strong affinity and better isoelectric properties. We were inspired by a report from Keating and coworkers, which described 27 coiled-coils designed for synthetic biology applications[17]. From among those peptides, we selected SYNZIP-5 and SYNZIP-6, a high affinity coiled-coil pair (K_D < 15 nM) that does not homodimerize. These peptides are small (5–6 kDa), biocompatible, and have a good balance of basic (K/R) and acidic (E/D) residues[17–18].

We used this pair to create our CoilY and CoilZ peptide tags. We added a short linker adjacent to each coil to create our genetically-encoded peptide tags (see Table S1 for sequences and properties). Further modifications were needed to make the CoilY and CoilZ probe peptides. We used gene assembly PCR to enable recombinant expression of the peptides in *E. coli*. We included a short linker (GGGAAA) before a cysteine residue, which we introduced for site-specific conjugation to fluorescent reporters. A C-terminal hexahistidine tag was included for affinity purification of the peptides. We analyzed the solution phase structures of the peptides by circular dichroism (CD) spectroscopy and found that both had α-helical structures. A CoilY/CoilZ mixture also had a coiled-coil structure (Figure S1), consistent with the crystal structure of SYNZIP-5/SYNZIP-6 published by Keating *et al.* (structure available: 3HE4.pdb)[17].

The CoilY and CoilZ probe peptides were assessed for their ability to label tagged proteins in cell lysates (Figure 1). Peptide-tagged proteins were genetically-encoded in a pDisplay vector[12], which anchored model proteins on the cell surface via the transmembrane (TM) domain of platelet-derived growth factor receptor. The CoilY tag was fused near the N-terminus of mCherry (CoilY-mCherry-TM) and the CoilZ tag was fused near the N-terminus of enhanced GFP (CoilZ-EGFP-TM). Sequences are described in the ESI (Table S2). Transfected human embryonic kidney (HEK) 293FT cell lysates contained tagged (lanes 2 and 4) or untagged proteins (lanes 1 and 3). Cell extracts were denatured, subjected to polyacrylamide gel electrophoresis (PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was incubated sequentially with CoilZ-fluorescein and then CoilY-rhodamine. Fluorescence imaging demonstrated that only CoilY-mCherry-TM and CoilZ-EGFP-TM were labeled by their corresponding probe peptides (lanes 2 and 4). Homodimerization, which would appear as red fluorescence in lane 2 or green fluorescence in lane 4, was not observed. The probe peptides did not label untagged EGFP-TM, mCherry-TM, or other cellular proteins, demonstrating their specificity and selectivity. Additionally, our results indicated that either coil could be used as the genetically-encoded peptide tag for protein labeling *in vitro*.

This membrane assay enabled us to compare our probe peptide labeling as an alternative to fluorescent immunoblotting (i.e., Western blotting). For this experiment we used purified His_6-CoilY-mCherry (400 ng to 0.8 ng). The sensitivity of direct detection using CoilZ-fluorescein was compared with an AlexaFluor (AF) 488-labeled anti-His antibody (Figure S3). CoilZ-fluorescein could detect as little as 3 ng of His_6-CoilY-mCherry, while detection by immunolabeling required 8-fold more protein. Anti-His antibodies often exhibit low sensitivity and selectivity[19]. However we used a penta-His antibody from Qiagen, which is one of the best available commercially. Many proteins lack good antibodies, and we posit that our detection method offers a sensitive alternative to immunolabeling.

We translated our *in vitro* results to living cells. Flow cytometry was used to evaluate protein labeling in live human osteosarcoma (U-2 OS) cells expressing CoilZ-EGFP-TM, CoilY-EGFP-TM, or untagged EGFP-TM. Cells were treated with 500 nM AF647-conjugated probe peptide before analysis, and we gated for EGFP-expressing cells. We found that both peptide tags, CoilY and CoilZ, enabled selective protein labeling via heterodimerization (Figure 2). Treatment of cells expressing CoilZ-EGFP-TM with CoilY-AF647 resulted in bright, selective protein labeling with a greater than 40-fold enhancement. Non- specific labeling of untagged EGFP-TM and homodimerization with CoilY-EGFP-TM were minimal for cells exposed to CoilY-AF647. Cells expressing CoilY-EGFP-TM were labeled with CoilZ-AF647, but with only a 4-fold enhancement. Non-specific labeling was slightly higher for cells treated with CoilZ-AF647, and we suspect that this positively charged probe might interact with the negatively charged cell surface resulting in a higher non-specific signal.

We assessed protein labeling as a function of CoilY-AF647 concentration. Live cells expressing CoilZ-EGFP-TM or untagged EGFP-TM were treated with a range of CoilY-AF647 concentrations (50 nM to 1000 nM for 30 min at room temperature). Flow cytometry showed that the median AF647 signal increased with increasing concentration of probe peptide (Figure S4). Cells had 21- to 44-fold higher median fluorescence compared to cells

expressing untagged protein. Treatment with 300 nM AF647-CoilY gave optimal labeling, with 98% of cells labeled and a 35-fold increase in AF647 fluorescence. Treatment with an excess of probe peptide (i.e., 1000 nM) enhanced the AF647 signal, but at the cost of a small increase in non-specific labeling. These results demonstrate that a range of concentrations could be used successfully to label tagged proteins.

We used confocal fluorescence microscopy to assess protein labeling in transfected U-2 OS cells (Figure 2). Again, we relied on our CoilZ-EGFP-TM, CoilY-EGFP-TM, and untagged EGFP-TM constructs, which allowed us to track protein targets based on their green fluorescence. After transfection, EGFP fluorescence was observed both within cells and at the cell surface, where the peptide tag would be accessible to extracellular labeling by probe peptides. Live cells were blocked to reduce non-specific labeling, and nuclei were stained with Hoechst 33342. Cells were then chilled on ice to minimize endocytosis and treated with a cold solution of probe peptide (300 nM). Unbound probe was removed by washing. We acquired optically-sectioned images at room temperature within 10 min of probe peptide labeling. Fluorescent micrographs showed that labeling was tag-dependent and AF647 signal colocalized with tagged EGFP at the cell surface. Again, we observed no homodimerization and untagged EGFP-TM was not labeled. Protein-specific labeling was also observed with cells that were fixed prior to imaging (Figure S6).

The probe peptides were membrane-impermeant towards living cells, therefore AF647-labeling was limited to the subset of EGFP-TM localized to the cell surface at the time of treatment. We anticipate that this property will make the VIP tags useful for monitoring endocytosis or recycling of cell-surface receptors. While we have not yet undertaken such studies, we did find that tagged proteins could be observed by time-lapse imaging (Figure S7).

In prior work, Matsuzaki and coworkers used an E3-K3 heterodimer for protein labeling and reported that the acidic (i.e., negatively charged) E3 peptide (pI = 4.5) could not be used to label K3-tagged proteins. The lysine-rich K3 peptide had a pI of 9.7[16]. In contrast, we found that both our basic CoilZ (pI = 8.0) and acidic CoilY (pI = 6.4) enabled selective labeling. We attribute this bi-directionality to the better overall charge balance of CoilY and CoilZ compared to E3 and K3. Although our data suggest that CoilZ is a better genetically-encoded tag than CoilY, it is notable that either peptide could be used to fluorophore-label proteins.

We took advantage of CoilY and CoilZ's bi-directionality to observe two different protein targets simultaneously. Cells expressing CoilY-mCherry-TM were combined with cells expressing CoilZ-EGFP-TM to demonstrate the target-specific fluorophore labeling of distinct cell populations (Figure 3). Live cells were blocked and then cooled to halt endocytosis. Cells were labeled sequentially with CoilZ-biotin (500 nM; 15 min) and CoilY-AF647 (500 nM; 15 min). After fixation, cells were treated with Qdot565-conjugated streptavidin to Qdot-label CoilZ-biotin. To assess specificity, we used the same protocol to label cells expressing untagged EGFP-TM or mCherry-TM.

Four-color fluorescence imaging revealed that CoilY and CoilZ could be used concurrently for tagging two distinct cell populations. When exposed to a mixture of cells displaying either CoilY or CoilZ tags, CoilY-AF647 and CoilZ-biotin correctly heterodimerized with their respective targets at the cell surface. We observed the expected colocalization of AF647 with EGFP and Qdot565 with mCherry, indicative of highly specific protein labeling. There was no cross-reactivity or labeling of untagged proteins (Figure S8). Therefore, this one heterodimeric pair unambiguously distinguished between two distinct protein targets at once.

In summary, we demonstrated that CoilY and CoilZ are two new genetically-encoded peptide tags that enable the selective fluorescent labeling of target proteins *in vitro* and on the cell-surface. Fluorescent labeling of proteins in cells was analyzed by flow cytometry and confocal fluorescence microscopy, and we confirmed that this one heterodimeric pair could be used to detect two proteins simultaneously in a mixed cell population. These two genetically-encoded tags offer significant advantages over extant protein and peptide tags. Labeling was rapid (15 to 30 min) and cells could be imaged either live or post-fixation. CoilY and CoilZ are small, target-specific, easy to use, and compatible with diverse chemical reporters. In the current work, we labeled proteins with organic fluorophores (fluorescein, rhodamine, and AF647) and Qdots (Qdot565). The reporter chemistry can be selected and optimized for different applications, which makes this labeling strategy highly versatile. We look forward to developing other selective heterodimeric coiled-coils into new VIP tags in future work.

Experimental Section

Details of experimental procedures are provided in the Supporting Information. The ESI also includes supplementary tables and figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Drs. L. Lavis, J. Gray, S. Kaech-Petrie, J. Garay, and G. Sahay for helpful discussions. We thank Dr. U. Shinde for the use of his CD spectrometer and Dr. S. Gibbs for the use of her inverted light microscope. We are grateful to Drs. S. Levine and K. Tallman for her advice and assistance. J.K.D. was partially supported by the Oregon chapter of the Achievement Rewards for College Scientists (ARCS) Foundation. Plasmids were provided by Dr. A. Ting (Addgene # 20861) and Drs. R. Tsien and M. Davidson (Addgene # 54630). Funding for K.E.B. was generously provided by the OHSU School of Medicine and the OHSU Knight Cancer Institute.

References

1. a) Dean KM, Palmer AE. Nat Chem Biol. 2014; 10:512–523. [PubMed: 24937069] b) Liu Z, Lavis Luke D, Betzig E. Mol Cell. 2015; 58:644–659. [PubMed: 26000849] c) Giepmans BNG, Adams SR, Ellisman MH, Tsien RY. Science. 2006; 312:217–224. [PubMed: 16614209]
2. a) Brock R, Hamelers IHL, Jovin TM. Cytometry. 1999; 35:353–362. [PubMed: 10213201] b) Huang L, Pike D, Sleat DE, Nanda V, Lobel P. PLoS ONE. 2014; 9:e88893. [PubMed: 24586430] c) Costantini LM, Snapp EL. DNA and cell biology. 2013; 32:622–627. [PubMed: 23971632]

3. a) Xue L, Karpenko IA, Hiblot J, Johnsson K. Nat Chem Biol. 2015; 11:917–923. [PubMed: 26575238] b) Sunbul M, Yin J. Org Biomol Chem. 2009; 7:3361–3371. [PubMed: 19675886]
4. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. Nature Biotech. 2003; 21:86–89.
5. Heinis C, Schmitt S, Kindermann M, Godin G, Johnsson K. ACS Chem Biol. 2006; 1:575–584. [PubMed: 17168553]
6. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. ACS Chem Biol. 2008; 3:373–382. [PubMed: 18533659]
7. Miller LW, Cai Y, Sheetz MP, Cornish VW. Nat Meth. 2005; 2:255–257.
8. Szent-Gyorgyi C, Schmidt BF, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JAJ, Woolford CA, Yan Q, Vasilev KV, Berget PB, Bruchez MP, Jarvik JW, Waggoner A. Nat Biotech. 2008; 26:235–240.
9. a) Dempsey GT, Vaughan JC, Chen KH, Bates M, Zhuang X. Nat Meth. 2011; 8:1027–1036.b) Lukinavičius G, Umezawa K, Olivier N, Honigmann A, Yang G, Plass T, Mueller V, Reymond L, Corrêa IR Jr, Luo Z-G, Schultz C, Lemke EA, Heppenstall P, Eggeling C, Manley S, Johnsson K. Nat Chem. 2013; 5:132–139. [PubMed: 23344448] c) Grimm JB, Klein T, Kopek BG, Shtengel G, Hess HF, Sauer M, Lavis LD. Angewandte Chemie (International ed in English). 2016; 55:1723–1727. [PubMed: 26661345] d) Grimm JB, English BP, Choi H, Muthusamy AK, Mehl BP, Dong P, Brown TA, Lippincott-Schwartz J, Liu Z, Lionnet T, Lavis LD. Nat Meth. 2016 advance online publication. e) Yan Q, Schwartz SL, Maji S, Huang F, Szent-Gyorgyi C, Lidke DS, Lidke KA, Bruchez MP. Chemphyschem. 2014; 15:687–695. [PubMed: 24194371]
10. a) Griffin BA, Adams SR, Tsien RY. Science. 1998; 281:269–272. [PubMed: 9657724] b) Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH. Science. 2002; 296:503–507. [PubMed: 11964472]
11. a) Langhorst M, Genisyuerek S, Stuermer CO. Histochem Cell Biol. 2006; 125:743–747. [PubMed: 16395611] b) Crivat G, Tokumasu F, Sa JM, Hwang J, Wellems TE. PLoS ONE. 2011; 6:e22975. [PubMed: 21860664]
12. Howarth M, Takao K, Hayashi Y, Ting AY. Proc Natl Acad Sci USA. 2005; 102:7583–7588. [PubMed: 15897449]
13. a) Cohen JD, Thompson S, Ting AY. Biochemistry. 2011; 50:8221–8225. [PubMed: 21859157] b) Uttamapinant C, White KA, Baruah H, Thompson S, Fernández-Suárez M, Puthenveetil S, Ting AY. Proc Natl Acad Sci USA. 2010; 107:10914–10919. [PubMed: 20534555]
14. Liu DS, Nivón LG, Richter F, Goldman PJ, Deerinck TJ, Yao JZ, Richardson D, Phipps WS, Ye AZ, Ellisman MH, Drennan CL, Baker D, Ting AY. Proc Natl Acad Sci USA. 2014; 111:E4551–E4559. [PubMed: 25313043]
15. Litowski JR, Hodges RS. J Biol Chem. 2002; 277:37272–37279. [PubMed: 12138097]
16. Yano Y, Yano A, Oishi S, Sugimoto Y, Tsujimoto G, Fujii N, Matsuzaki K. ACS Chem Biol. 2008; 3:341–345. [PubMed: 18533657]
17. Reinke AW, Grant RA, Keating AE. J Am Chem Soc. 2010; 132:6025–6031. [PubMed: 20387835]
18. Thompson KE, Bashor CJ, Lim WA, Keating AE. ACS Synthetic Biol. 2012; 1:118–129.
19. Debeljak N, Feldman L, Davis KL, Komel R, Sytkowski AJ. Anal Biochem. 2006; 359:216–223. [PubMed: 17081490]

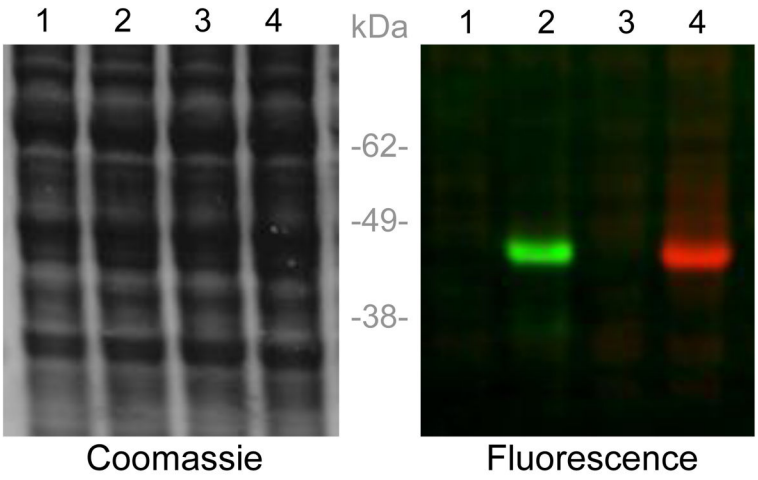


Figure 1.

Selective fluorophore labeling of peptide-tagged proteins. Lysates were resolved by SDS-PAGE, transferred to a membrane, and then incubated with CoilZ-fluorescein (green) and then CoilY-rhodamine (red). Fluorescence imaging revealed green-fluorescent CoilY-mCherry-TM (lane 2) and red-fluorescent CoilZ-EGFP-TM (lane 4). GFP-TM (lane 3) and mCherry-TM (lane 1) were not labeled by either probe peptide. See Figure S2 for individual green and red channels.

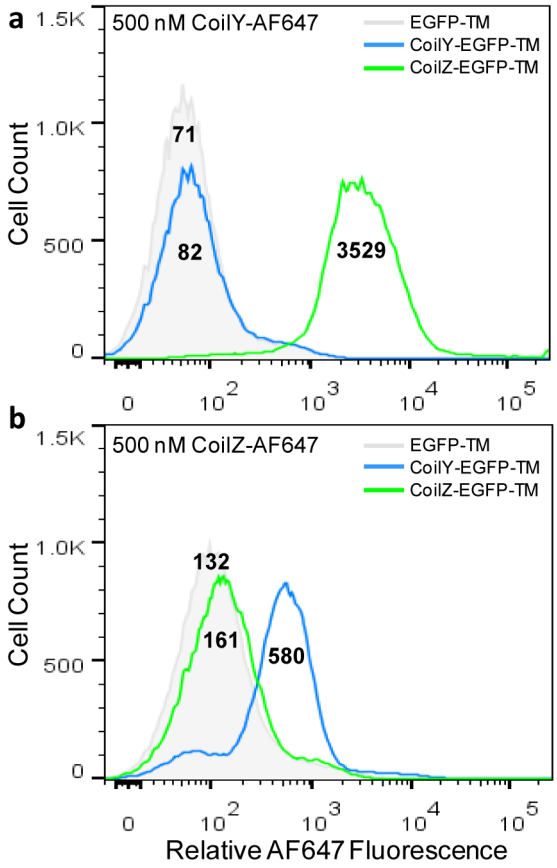


Figure 2.

Histograms of AF647 fluorescence from flow cytometry. Cells were gated for green fluorescence and analyzed for labeling with Coil5-AF647 (A) or Coil6-AF647 (B). Transfected cells expressed Coil6-EGFP-TM (green), Coil5-EGFP-TM (blue), or untagged EGFP-TM (gray). Values in bold indicate the median AF647 fluorescence for each cell population.

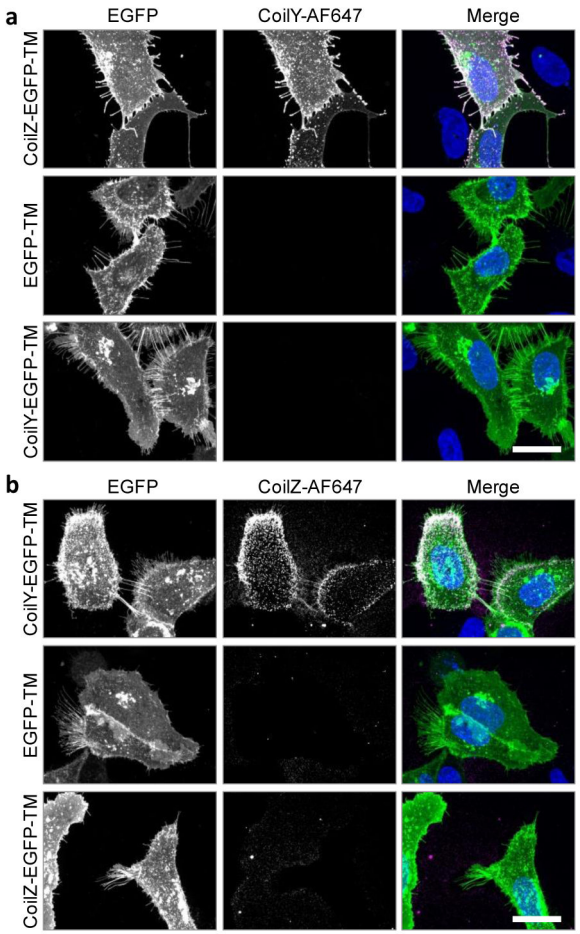


Figure 3.

Selective fluorescent labeling of cell-surface EGFP using CoilY and CoilZ. (a) Cells treated with CoilY-AF647. (b) Cells treated with CoilZ-AF647. In both A and B, labeling was only observed upon heterodimer formation with peptide tagged EGFP-TM. The merged images include EGFP (green), AF647 (magenta), and nuclear stain (blue), and the scale bar represents 25 μm. Individual slices from the Z-projections are available in the ESI (Figure S5).

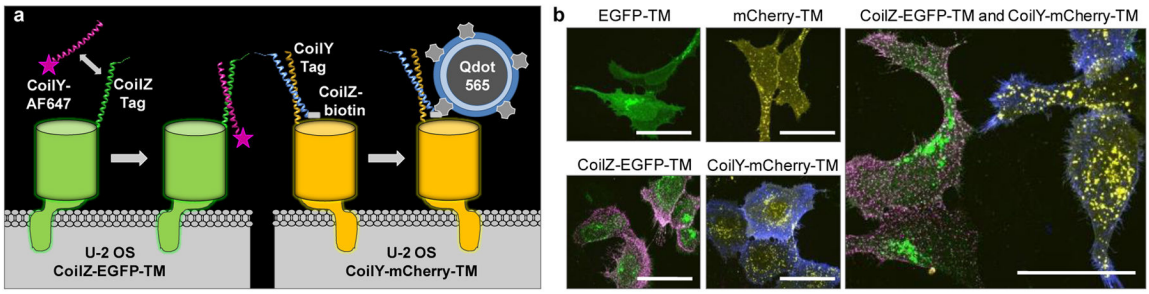
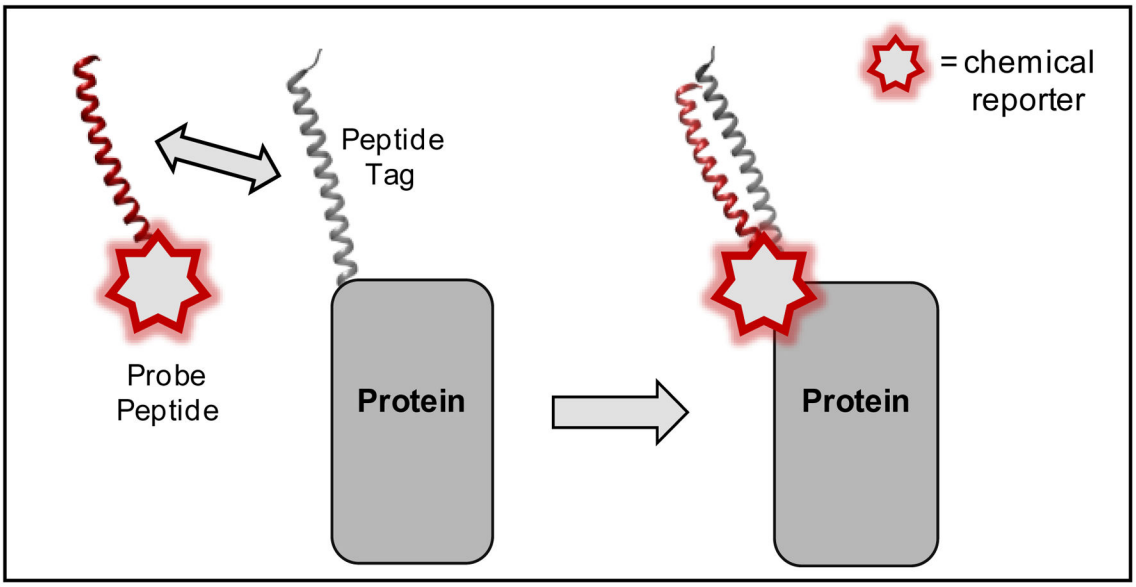


Figure 4.

Selective fluorescent labeling of cell-surface EGFP using CoilY and CoilZ. (a) Cells treated with CoilY-AF647. (b) Cells treated with CoilZ-AF647. In both A and B, labeling was only observed upon heterodimer formation with peptide tagged EGFP-TM. The merged images include EGFP (green), AF647 (magenta), and nuclear stain (blue), and the scale bar represents 25 μm. Individual slices from the Z-projections are available in the ESI (Figure S5).



Scheme 1.

CoilY and CoilZ facilitate the fluorescent labeling of cellular proteins through heterodimerization..