ORGANIC LETTERS

2011
Vol. 13, No. 16
4160–4163

One- and Two-Photon Live Cell Imaging Using a Mutant SNAP-Tag Protein and Its FRET Substrate Pairs

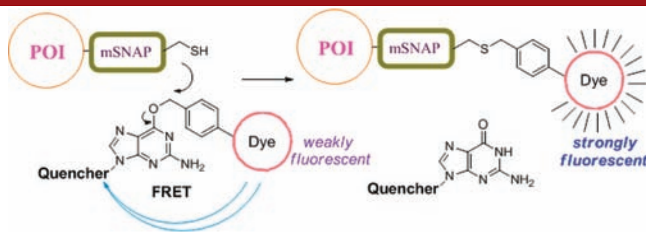
Chong-Jing Zhang,[‡,§] Lin Li,[‡] Grace Y. J. Chen,[†,‡] Qing-Hua Xu,[‡] and Shao Q. Yao*,[†,‡,§]

Departments of Biological Sciences and Chemistry and NUS MedChem Program of the Life Sciences Institute, National University of Singapore, Singapore 117543

chmyaosq@nus.edu.sg

Received May 26, 2011

ABSTRACT



A small molecule-assisted protein labeling strategy based on a mutant SNAP-Tag (mSNAP) and its FRET substrate pairs has been developed. Both one- and two-photon fluorescence microscopic experiments were successfully demonstrated in living cells.

Fluorescence imaging provides an indispensable way to locate and monitor biological targets within complex and dynamic intracellular environments.[1] The revolutionary discovery of genetically encoded fluorescent proteins (FPs) has made it possible to directly visualize proteins and various biochemical activities, with unprecedented resolutions, in the living system.[2] The invention of two-photon microscopy (TPM), which employs two near-infrared photons as the excitation source, provides additional advantages of increased penetration depth (~500 μm), localized excitation, and prolonged observation time, thereby allowing examination of fluorophores present even in deep living samples.[3,4] Unfortunately, because of the various undesirable photophysical properties of most FPs, they have not been widely adopted in TPM applications.[4]

Chemistry-based protein-labeling approaches complementary to FPs have emerged in recent years, most of which make use of a highly specific chemical or enzymatic reaction between a fusion protein and a chemically tractable organic fluorophore/small molecule probe.[5−7] They aim to address two intrinsic shortcomings of FP-based imaging techniques, the size of FPs (> 27 kDa) and their strict confinement to only genetically encoded fluorophores.[8] For example, the FlAsH approach, developed by Tsien et al., uses a small molecule capable of binding to a six-amino acid tetracysteine tag fused to the target protein,[5] and the SNAP-tag technology, developed by Johnsson et al., makes use of a highly efficient enzymatic reaction between O^6-alkylguanine-DNA-alkyltransferase (hAGT) and a variety of O^6-benzylguanine (BG)-modified probes.[9] Other conceptually similar approaches, such as HaloTag,[10] D_4-tag,[11] ACP/PCP-tag,[12,13] and others,[7] are also available. Few of these methods, with the exception of FlAsH, make use of fluorogenic probes for protein labeling (that is, the probe is rendered fluorescent only upon protein labeling), which is an important feature for real-time bioimaging in live cells.[5] In fact,

[†] Department of Biological Sciences.
[‡] Department of Chemistry.
[§] NUS MedChem Program of the Life Sciences Institute.

(1) Lichtman, J. W.; Conchello, J.-A. *Nat. Methods.* **2005**, *2*, 910–919.
(2) Tsien, R. Y. *Angew. Chem., Int. Ed.* **2009**, *48*, 5612–5626.
(3) Kim, H. M.; Cho, B. R. *Chem. Commun.* **2009**, 153–164.
(4) Helmchen, F.; Denk, W. *Nat. Methods* **2005**, *2*, 932–940.
(5) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269–272.
(6) Johnsson, K. *Nat. Chem. Biol.* **2009**, *5*, 63–65.
(7) Chen, I.; Ting, A. Y. *Curr. Opin. Biotechnol.* **2007**, *25*, 1483–1487.
(8) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906–918.

10.1021/ol201430x
Published on Web 07/25/2011

this issue is already being addressed in several recent reports.[14−16] Herein, we report a small molecule-assisted protein labeling strategy, based on a mutant SNAP-tag (mSNAP) and its Fröster Resonance Energy Transfer (FRET) substrate pairs (Figure 1). Our design principle was based on the well-known fact that, in the SNAP-tag technology, in which the SNAP-tag forms a covalent bond with BG derivatives by nucleophilic attack at the active site cysteine (Figure 1a), a guanine moiety is released. Furthermore, this reaction is independent of the dye attached to the BG derivative. An elegant study by Johnsson et al. provided further evidence that SNAP-tag (an extensively mutated form of hAGT) and its suitable mutants may also accept N^9-substituded BG derivatives (Figure 1b).[17] In our strategy, quenched probes such as **BGQFL-9** and **BGQNP-9** (a two-photon probe; Figure 1c), due to introduction of a fluorescence quencher, Disperse Red 1 (DR1), would be effectively nonfluorescent. Upon covalent labeling with mSNAP, the quencher-containing guanine is released, resulting in transfer of the dye onto the tag protein (and fluorescence enhancement). Similar concepts had been proposed previously,[18,19] but successful and general implementation had not yet been realized in the literature. It should be noted that while our manuscript was in preparation, Urano et al. introduced what they call a fluorescence activation-coupled protein labeling (FAPL) method, in which BG derivatives modified with a quencher at the C-8 position were used together with a SNAP-tag.[20] Our present study offers a complementary method while providing the first expansion of the SNAP-tag technology into the realm of TPM.

Details of probe synthesis are presented in the Supporting Information (Scheme S1). In addition to **BGQFL-9** and **BGQNP-9**, we also synthesized N^7-substituted probes **BGQFL-7** and **BGQNP-7** (Figure 1c) as well as the cell-permeable **BGQAF-9**, which is the diacetylated version of **BGQFL-9**. The two-photon dye 8-oxoacenaphthopyrrole (NP) was used because of its desirable photophysical properties for in vivo imaging.[21] Disperse Red 1 was chosen as the fluorescence quencher since the absorption spectrum of DR1 overlaps substantially with the emission spectra of both fluorescein (FL) and NP. All probes were conveniently synthesized using the highly efficient and modular click chemistry and fully characterized by LC–MS and NMR (Supporting Information). Optical properties of the final probes were spectroscopically measured (Figure S2, Supporting Information).

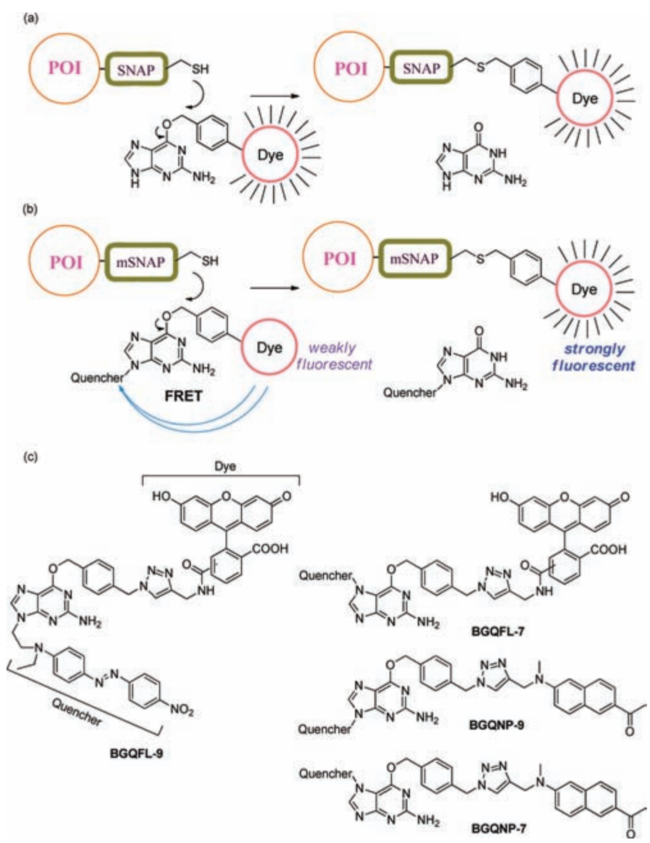


Figure 1. (a) SNAP-tag protein labeling with BG derivatives. (b) Modified strategy based on mSNAP with quenched probes. (c) Quenched probes (N^7- and N^9-substituted BG derivatives) used.

We carried out fluorescence measurement of the above compounds (Table S1 and Figure S2 in the Supporting Information); both **BGFL** and **BGNP** (quencher-free versions of **BGQFL-9** and **BGQNP-9**, respectively) exhibited excellent one- and two-photon fluorescence properties as expected. **BGQFL-9**/**-7** and **BGQNP-9**/**-7**, on the other hand, showed almost no fluorescence, demonstrating high intramolecular FRET efficiency upon the addition of DR1.

We next assessed the labeling efficiency of these probes toward SNAP-tag and its mutants. SNAP-tag is a significantly improved and truncated version of wildtype hAGT with key mutations of G^{131}→K^{131} and G^{132}→T^{132}.[17] Previous structural and mechanistic studies have revealed that, while bulky residues (such as K^{131} and T^{132} in

(9) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86–89.
(10) www.promega.com
(11) Ojida, A.; Honda, K.; Shinmi, D.; Kiyonaka, S.; Mori, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2006**, *128*, 10452–10459.
(12) George, N.; Pick, H.; Vogel, H.; Johnsson, N.; Johnsson, K. *J. Am. Chem. Soc.* **2004**, *126*, 8896–8897.
(13) Yin, J.; Lin, A. J.; Buckett, P. D.; Wessling-Resnick, M.; Golan, D. E.; Walsh, C. T. *Chem. Biol.* **2005**, *12*, 999–1006.
(14) Blum, G.; Mullins, S. R.; Keren, K.; Fonovic, M.; Jedeszko, C.; Rice, M. J.; Sloane, B. F.; Bogyo, M. *Nat. Chem. Biol.* **2005**, *1*, 203–209.
(15) Komatsu, T.; Kikuchi, K.; Takakusa, H.; Hanaoka, K.; Ueno, T.; Kamiya, M.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 15946–15947.
(16) Mizukami, S.; Watanabe, S.; Hori, Y.; Kikuchi, K. *J. Am. Chem. Soc.* **2009**, *131*, 5016–5017.
(17) Juillerat, A.; Heinis, C.; Sielaff, I.; Barnikow, J.; Jaccard, H.; Kunz, B.; Terskikh, A.; Johnsson, K. *ChemBioChem* **2005**, *6*, 1263–1269.
(18) Jaccard, H.; Johnsson, K.; Kindermann, M. WO Pat, 2005/085470 A1, 2005.
(19) Stohr, K.; Siegberg, D.; Ehrhard, T.; Lymperopoulos, K.; Oz, S.; Schulmeister, S.; Pfeifer, A. C.; Bachmann, J.; Klingmuller, U.; Sourjik, V.; Herten, D. *Anal. Chem.* **2010**, *82*, 8186–8193.
(20) Komatsu, T.; Johnsson, K.; Okuno, H.; Bito, H.; Inoue, T.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2011**, *133*, 6745–6751.
(21) Lee, J. H.; Lim, C. S.; Tian, Y. S.; Han, J. H.; Cho, B. R. *J. Am. Chem. Soc.* **2010**, *132*, 1216–1217.

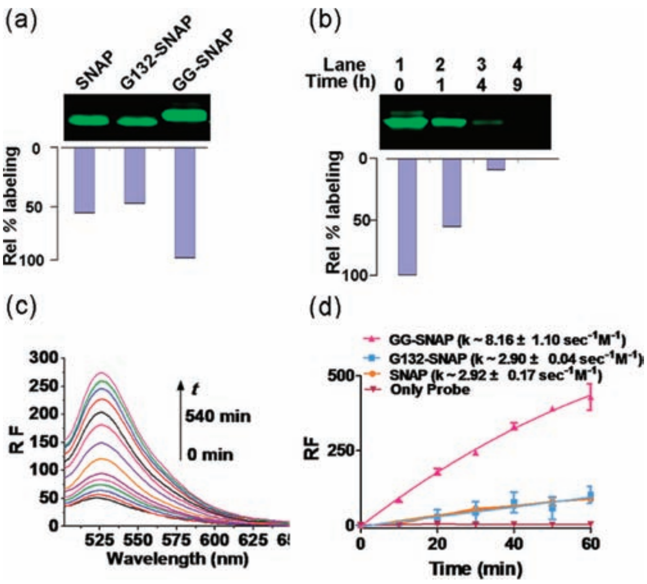


Figure 2. (a) In-gel fluorescence scanning of labeling reactions between different SNAP proteins (1 μM) and **BGQFL-9** (1 μM) after 4-h incubation at room temperature. Fluorescent bands were quantified and plotted (bottom). (b) Indirect competition assay of **BGQNP-9**/GG-SNAP labeling reactions (for 0, 1, 4, and 9 h), followed by subsequent addition of **BGFL** (1 μL, 100 μM; 15 min), SDS-PAGE and in-gel fluorescence scanning. Fluorescent bands were quantified and plotted (bottom). (c) Time-dependent emission spectra (λ_{ex} = 470 nm) of **BGQFL-9** (20 μM) in the presence of GG-SNAP (10 μM) in PBS (pH 7.4) at 25 °C. (d) Time-dependent fluorescence intensity of **BGQFL-9** in the presence of different SNAP mutants (λ_{ex} = 490 nm, λ_{em} = 522 nm). Assay conditions were the same as in (c). The fitted cuves and corresponding rate constants were obtained by fitting the data to a first-order reaction model,[9] giving rise to the resulting second-order rate constants.

SNAP-tag) favor BG derivatives, structurally less demanding residues (such as Gly at positions 131 and 132) prefer N^9-substituted BG compounds (see Figure S11, Supporting Information).[17] We therefore generated the corresponding protein mutants of SNAP-tag (mSNAP)-G132-SNAP (T^{132} in SNAP-tag was replaced by G^{132}) and GG-SNAP (K^{131} and T^{132} in SNAP-tag were replaced by $G^{131}G^{132}$). The bacterial expression construct G132-SNAP was obtained from the corresponding SNAP-tag template (His-SNAP)[22] using a Quick Change site-directed mutagenesis kit (Stratagene). The GG-SNAP construct was generated by Gateway cloning (Invitrogen). The mammalian expression constructs Flag-GG-SNAP and Flag-H2B-GG-SNAP, in which a nuclear localization sequence H2B was fused to the SNAP-tag fusion, were obtained from the corresponding Flag-SNAP and Flag-H2B-SNAP vectors, respectively.[22] All plasmid DNAs were sequence verified. The recombinant proteins (His-SNAP, G132-SNAP, and GG-SNAP) were expressed in BL21(Ai) cells and purified to homogeneity with Ni-NTA beads (Figure S1, Supporting Information). Because of the difference in the vector's backbone (i.e., Gateway destination vector pDEST17 introduces several extra linker residues in GG-SNAP), His-SNAP/G132-SNAP and GG-SNAP migrated slightly differently on the SDS-PAGE gel (Figure 2a). As expected, **BGFL** labeled all three proteins with reasonable efficiency. **BGQFL-7**, on the other hand, could not label any of the proteins (Figure S3, Supporting Information). To our delight, **BGQFL-9** was shown to successfully label all three proteins, with GG-SNAP producing the most intense fluorescence band (Figure 2a). This indicates the $K^{131}\rightarrow G^{131}$ and $T^{132}\rightarrow G^{132}$ mutations in SNAP-tag, giving GG-SNAP, have indeed improved this protein's reactivity toward our quenched probe **BGQFL-9**. The labeling efficiency of SNAP-tag by **BGQFL-9** decreased significantly when compared to the original **BGFL**/SNAP labeling (Figure S3, Supporting Information), indicating the steric bulk of the quencher group in **BGQFL-9** had predictably hindered its enzymatic attachment to SNAP-tag. To test whether the two-photon quenched probe, **BGQNP-9**, could also label GG-SNAP efficiently, we used an indirect competition assay since NP could not be detected with our fluorescence gel scanner. As shown in Figure 2b, preincubation of GG-SNAP with **BGQNP-9** effectively blocked the subsequent fluorescence labeling of **BGFL** in a time-dependent manner, indicative of successful labeling between GG-SNAP and **BGQNP-9**. We next evaluated whether the enhancement of **BGQFL-9** fluorescence upon labeling by GG-SNAP could be monitored spectroscopically (Figure 2c,d); a progressive increase in fluorescence (with max λ_{em} = 522 nm) over time was observed, indicating successful release of the quencher (Figure 2c). In comparison, both SNAP-tag and G132-SNAP produced significantly lower fluorescence increases under identical conditions (Figure 2d). Kinetic data of the labeling reaction between **BGQFL-9** and various SNAP mutants were obtained, indicating GG-SNAP was indeed the most efficient partner of our N^9-substituted quenched probe; with a second-order rate constant of 8.16 ± 1.10 M^{-1} s^{-1}, our labeling system is not as efficient as the original **BGFL**/SNAP combination (k = ~2×10^4 M^{-1} s^{-1})[17] or the newly reported C^8-substituted quenched probe/SNAP pair (k = ~4×10^2 M^{-1} s^{-1})[20] but should offer a good starting point for further improvement using directed protein evolution approaches.[17] Finally, the labeled product of the **BGQFL-9**/GG-SNAP reaction was further analyzed by MALDI-TOF MS (Figure S4, Supporting Information); an expected molecular weight increase of 558 Da was observed, further confirming the success of the labeling reaction.

Lastly, we examined whether the **BGQFL-9**/GG-SNAP protein labeling system can be used for bioimaging applications in live cells. We first applied **BGQFL-9** to mammalian cell lysates (Figure 3a); a single fluorescent band was detected only in mammalian cell lysates obtained from CHO-9 cells transiently transfected with the Flag-GG-SNAP plasmid, thus demonstrating the successful and highly specific labeling reaction of our newly developed

(22) Srikum, D.; Albers, A. E.; Nam, C. I.; Iavaron, A. T.; Chang, C. J. *J. Am. Chem. Soc.* **2010**, *132*, 4455–4465.

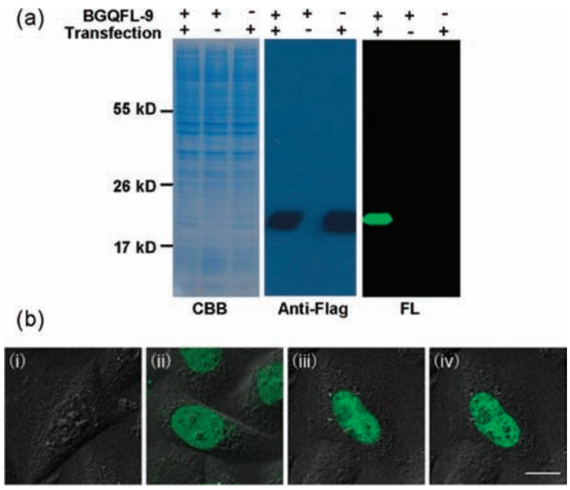


Figure 3. (a) Coomassie brilliant blue (CBB)-stained (left), Western blot (center; with anti-Flag antibody), and fluorescence (right) gels of Flag-GG-SNAP transfected CHO-9 cell lysates incubated with **BGQFL-9**. (b) Live CHO-9 cells transfected with Flag-H2B-GG-SNAP and then labeled with either **BGQFL-9** or **BGQNP-9** (20 μM). Key: (i) nontransfected cells labeled with **BGQNP-9**; (ii) transfected cells labeled with **BGQFL-9**, then imaged by one-photon microscope (λ_{ex} = 488 nm; λ_{em} = 522 nm); (iii) Transfected cells labeled with **BGQNP-9**, then imaged by one-photon microscope (λex =405 nm; λ_{em} = 470 nm); (iv) same as iii, except the image was acquired by a two-photon microscope (λ_{ex} = 800 nm; λ_{em} = 470 nm). Scale bar = 10 μm.

system even in complex biological systems. To label proteins in live cells, we had initially intended to use the cell-permeable **BGQAF-9** (see the Supporting Information for structure), but it was subsequently discovered that, to our pleasant surprise, both **BGQFL-9** and **BGQNP-9** were equally permeant in CHO-9 cells, probably due to their overall enhanced hydrophobicity. These two probes were therefore used to carry out all live cell labeling/imaging experiments. The plasmid Flag-H2B-GG-SNAP, which expresses GG-SNAP in the nuclei, was transfected into CHO-9 cells. After 3 h, to the growth media was directly added either **BGQFL-9** or **BGQNP-9**. Following further incubation, cells were imaged, using one- and two-photon fluorescence microscopes, respectively (Figure 3b); only transfected cells exhibited strong fluorescence signals in their nuclei, indicating successful labeling of GG-SNAP in live cells.

In conclusion, by modifying the existing SNAP-tag protein labeling approach, we have successfully developed both one-and two-photon quenched probes, **BGQFL-9** and **BGQNP-9**, respectively, that could covalently label a SNAP-tag mutant protein (mSNAP) with moderate efficiency. We showed that, in addition to C-8,[20] the N-9 position in BG is also suitable for quencher attachment. Our results indicate real-time detection of both the labeling reaction and live cell imaging could be achieved using this system. To our knowledge, the only other small molecule-based protein labeling approach for two-photon microscopic applications was done using the DHFR/Mtx noncovalent strategy.[23] Our current system thus represents the first covalent protein labeling approach using small molecule probes for TPM applications. This, togeher with other recenlty developed enzyme-detecting TPM probes,[24] will open up new opportunities for bioimaging of important enzymes and their activities in deep tissues, where conventional fluorescence microscopy has very limited utilities.[3,4]

Acknowledgment. Funding support was provided by the Agency for Science, Technology and Research (R-143-000-391-305), and Ministry of Education (R-143-000-394-112). We thank Prof. Christopher J. Chang at the University of California for kindly providing His-SNAP, Flag-SNAP, and Flag-H2B-SNAP plasmids.

Supporting Information Available. Experimental procedures, characterization of new compounds, and biological experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

(23) Gallagher, S. S.; Jing, C.; Peterka, D. S.; Konate, M.; Wombacher, R.; Kaufman, L. J.; Yuste, R.; Cornish, V. W. *ChemBioChem* **2010**, *11*, 782–784.

(24) Hu, M.; Li, L.; Wu, H.; Su, Y.; Yang, P. -Y.; Uttamchandani, M.; Xu, Q. H.; Yao, S. Q. *J. Am. Chem. Soc.*, 2011, DOI: 10.1021/ja200808y.