A wash-free SNAP-tag fluorogenic probe based on the additive effects of quencher release and environmental sensitivity†

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A 1,8-naphthalimide-derived fluorogenic probe was reported to label SNAP-tag fusion proteins in living cells. The probe can rapidly label a SNAP-tag and exhibit a fluorescence increase of 36-fold due to the additive effects of environment sensitivity of fluorophores and inhibition of photo-induced electron transfer from O6-benzylguanine to the fluorophore. The labeling of intracellular proteins has been successfully achieved without a wash-out procedure.

Protein labeling plays an increasingly important role in visualizing and manipulating proteins in living cells. Numerous technologies to label proteins with chemical probes have been validated, including genetically encoded fluorescent proteins (FPs), site-specific incorporation of non-natural amino acids and self-labeling protein tags. However, the application of FPs in vivo is sometimes limited by their relatively large size and the lack of near-infrared (NIR) fluorescence. Non-natural amino acid technology suffers from both the complex preparation of the SNAP-tag, experimental procedures, supplementary schemes and figures. See DOI: 10.1039/c7cc01483j

Among them, the SNAP-tag is one of the most prominent fusion tags, as the HaloTag,3,4 PYP-tag,5–7 SNAP-tag,8,9 TMP-tag10 and BL-tag.11,12 Among them, the SNAP-tag is one of the most prominent fusion tags, which specifically reacts with O6-benzylguanine (BG) derivatives.

Various fluorescent probes have been designed to rapidly and irreversibly react with the SNAP-tag and widely applied in the fields of drug monitoring,13 protein–protein interactions,14 fluorescent sensors15 and super-resolution fluorescence imaging.16

While these probes are very useful and well established, it is required to wash out the unreacted or nonspecifically bound probes before imaging. The washout process is tedious and time-consuming, and may potentially limit their applications in real-time monitoring of molecular events such as receptor–ligand binding, endocytosis, trafficking and so on. In addition, the complete washout is difficult because of the uncontrollable accumulation of synthetic probes in various organelles. To overcome this limitation, fluorogenic probes for SNAP tags based on different mechanisms have been developed.17 The early reported probes DRBG-48818 and CBG-549-QSY719 consist of a fluorescence quencher attached to the C-8 position of the guanine ring. Upon reaction with the tagged protein, the quencher group is dissociated to release the fluorescence intensity of the fluorophore. However, these fluorogenic probes need a complex synthesis procedure and the large molecule size often results in low cell permeability. Notably, the external quenchers reduce the reaction rate and cause a big concern of the toxicity. Tan et al. reported a fluorogenic probe BGSBD based on another mechanism of the fluorophore's environmental sensitivity.20 The attractive feature of this mechanism is to retain the integrity of the BG group and then defend the reaction rate. Even though this probe exhibits a dramatic fluorescence turn-on of 280-fold upon labeling the SNAP-tag, the brightness of this probe after binding to the SNAP-tag was weak (f = 0.143), which was not ideal for living cell imaging. Therefore, fluorogenic probes based on the environmental sensitivity mechanism with bright fluorophores are urgently required.

In this communication, we reported a new fluorogenic probe BGAN-2C based on these two mechanisms of quencher release and the fluorophore’s environmental sensitivity. Among environment-sensitive fluorophores, 1,8-naphthalimide is bright (ε ~ 15 000-40 000, τ = 0.4–0.8 in a nonpolar environment) and has been proved to be a good candidate21,22 to sense protein dynamics and interactions with targets due to its small size to be
embedded into protein pockets and high polarity-sensitivity with significant fluorescence responses.\textsuperscript{23,24} According to the analysis of the crystal structure of the SNAP protein, the BG-binding activity center of the SNAP protein is a hydrophobic narrow cavity.\textsuperscript{25} In addition, guanine itself is an electron-rich group and well-known to quench the fluorescence of organic fluorophores by photo-induced electron transfer (PET). Thus, we hypothesized that a short linker between the BG group and the 1,8-naphthalimide fluorophore would take 1,8-naphthalimide into the hydrophobic environment and simultaneously inhibit BG-induced fluorescence quenching after 1,8-naphthalimide-BG conjugates labeled the SNAP-tag (Fig. 1). An obvious fluorescence turn-on response can then be expected due to the combinational effects of quencher (BG group) release and the environmental sensitivity of 1,8-naphthalimide. Notably, the release of guanine can be considered to be biologically safe, since guanine is a natural constituent of the diet.

Based on the above hypothesis, we designed and synthesized several compounds (BGAN-R) with the BG group directly connected to 1,8-naphthalimide by an amide group (Fig. 1). 4-N,N-Dimethylamino-1,8-naphthalimide (BGAN-DM) was expected to act as a molecular rotor and sense the environmental changes by means of inhibiting the twisted intramolecular charge transfer (TICT) process. Secondary amino group substituted 1,8-naphthalimide derivatives (BGAN-2C, BGAN-8C and BGAN-12C) were anticipated to sense environmental changes only undergoing intramolecular charge transfer (ICT). In order to optimize the environmental sensitivity of 1,8-naphthalimide, alkyl chains of different lengths were introduced into the electron-donor groups as shown in compounds BGAN-2C, BGAN-8C and BGAN-12C. The results of computer simulations showed that, after covalently binding to the SNAP-tag, 1,8-naphthalimide existed in the hydrophobic pocket of the protein which was positioned mainly by Pro 138 and Pro 140,\textsuperscript{25} and the C-4 position of 1,8-naphthalimide exposed at the outside of the cavity (the inset of Fig. 1 and Fig. S1, ESI\textsuperscript{†}). The alkyl chains of different lengths oriented outside and may contribute differently to environmental sensitivity. To evaluate the PET-induced fluorescence quenching from the BG group to 1,8-naphthalimide, compounds AN-DM, AN-2C, AN-8C and AN-12C without BG ligands were synthesized as control compounds (Scheme S1, ESI\textsuperscript{†}).

In order to evaluate environment-sensitive properties, the fluorescence of these compounds in various solvents was firstly studied. 4-Amino-1,8-naphthalimide is a typical push–pull fluorophore. This type of fluorophore ensures that after absorbing light the charge is transferred from the electron donor to the electron acceptor, which creates a highly dipolar excited state. The latter relaxes through interaction with the dipoles of solvent and thus its emission shifts to shorter wavelengths in less polar solvents. As shown in Fig. S2 (ESI\textsuperscript{†}), with decreasing solvent polarity, significant blue-shifts in emissions (from 550 nm to 505 nm, H\textsubscript{2}O/DCM) and fluorescence increases (\textasciitilde 100 fold, Table S1, ESI\textsuperscript{†}) were observed for all of these four compounds. It demonstrated that all of the probes displayed typical solvatochromic properties.

To evaluate the fluorescence quenching effects of a guanine group, the fluorescence intensities of BGAN-2C, BGAN-8C, BGAN-12C and BGAN-DM in PBS buffer solutions were compared with those of their control compounds, respectively. As shown in Table 1, the fluorescence intensities of compounds without BG groups were 19, 5, 2 and 1.3 fold stronger than those with BG groups, respectively, which demonstrated that guanine acted as a quencher in compounds BGAN-2C, BGAN-8C, BGAN-12C and BGAN-DM.

Then, these compounds were examined to label the SNAP-tag. Compounds BGAN-2C, BGAN-8C, BGAN-12C and BGAN-DM all show weak fluorescence in PBS buffer solutions. After the SNAP-tag was labeled with these fluorophores, different degrees of fluorescence enhancement were observed (Fig. 2a). The fluorescence of BGAN-2C, BGAN-8C and BGAN-12C was increased after 2 h with a turn-on ratio of 36, 23 and 9 fold, respectively. We noticed that after much longer time the fluorescence increases of BGAN-8C and BGAN-12C were all over 30 fold while the fluorescence of BGAN-DM exhibited only 2 fold enhancements after covalently binding with the SNAP-tag. These results demonstrated that the fluorescence enhancement is a result of both the inhibition of guanine quenching and 1,8-naphthalimide’s environmental sensitivity. For a compound with a longer alkyl chain, the fluorescence enhancement contributed from the environmental sensitivity becomes more significant. After labeling the SNAP-tag,

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Structure of compounds BGAN-R and the reaction with the SNAP-tag. After covalently binding to the SNAP-tag protein, 1,8-naphthalimide displayed increased fluorescence due to the localization into the hydrophobic binding environment of the SNAP tag and the release of the fluorescence quencher (guanine). Inset: Computer simulations of the hydrophobic SNAP-tag pocket binding with BGAN-R.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>( \lambda_{\text{em}}^a ) [nm]</th>
<th>( \lambda_{\text{em}}^b ) [nm]</th>
<th>Tracking ( \Phi )</th>
<th>( k_2 \times 10^5 ) [M(^{-1}) s(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGAN-DM</td>
<td>548</td>
<td>521</td>
<td>0.017 ± 0.003</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td>AN-DM</td>
<td>548</td>
<td>521</td>
<td>0.021 ± 0.004</td>
<td>-</td>
</tr>
<tr>
<td>BGAN-2C</td>
<td>551</td>
<td>538</td>
<td>0.015 ± 0.005</td>
<td>2031.7 ± 63.7</td>
</tr>
<tr>
<td>AN-2C</td>
<td>550</td>
<td>538</td>
<td>0.286 ± 0.015</td>
<td>-</td>
</tr>
<tr>
<td>BGAN-8C</td>
<td>548</td>
<td>531</td>
<td>0.018 ± 0.005</td>
<td>2031.7 ± 63.7</td>
</tr>
<tr>
<td>AN-8C</td>
<td>555</td>
<td>531</td>
<td>0.087 ± 0.012</td>
<td>-</td>
</tr>
<tr>
<td>BGAN-12C</td>
<td>531</td>
<td>514</td>
<td>0.012 ± 0.007</td>
<td>105.2 ± 6.0</td>
</tr>
<tr>
<td>AN-12C</td>
<td>548</td>
<td>514</td>
<td>0.025 ± 0.007</td>
<td>-</td>
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</tbody>
</table>

\( a \) Fluorescence emissions of compounds before. \( b \) After binding to the SNAP-tag. \( ^* \) Quantum yields of compounds in PBS buffer before. \( ^* \) After incubating with the SNAP-tag for 2 h. \( k_2 \) second-order rate constant.
the long alkyl chain of the probe may increase the hydrophobicity and decrease the polarity of the binding cavity. Unfortunately, BGAN-8C and BGAN-12C displayed a much lower reaction rate.

Fluorescence enhancement contributed from the environmental sensitivity can be further explained based on the polarity-sensitivity of different compounds as shown in Fig. 2b. BGAN-DM displays a fluorescence increase only in low polarity environments, but still weak intensity in a medium polarity environment where TICT dominates fluorescence, while the other three compounds BGAN-2C, BGAN-8C and BGAN-12C, only governed by ICT, were much more sensitive to medium polarity. It can be concluded that the interaction of BGAN-DM with the BG-binding cavity of the SNAP-tag was not so tight to inhibit intramolecular twist, and the polarity of this cavity is medium and suitable for ICT fluorophores. Then molecular rotors based on other fluorophores with larger size may have a chance to be designed as fluorogenic probes for the SNAP-tag.

The binding characteristics of the probes with the SNAP-tag were further investigated by SDS-PAGE (Fig. 2c). A purified SNAP-tag was incubated with these four compounds and analysed. Based on bands appeared in the gel and mass analysis (Fig. S3, ESIF), these compounds all reacted with the SNAP-tag. But only two strong fluorescence bands in accordance with BGAN-2C and BGAN-8C were observed. These fluorescence responses were consistent with those in solutions. Then the labeling reaction of the SNAP-tag-expression cell lysate with BGAN-2C was analyzed by SDS-PAGE (Fig. 2d). The results showed that the reaction between BGAN-2C and the SNAP-tag is highly selective and produced only one single product in the SNAP-tag-expression cell lysate.

The detailed kinetic analysis of the protein labeling was carried out by monitoring the fluorescence intensity of the probes, in which the concentration of probes and the protein was 5 μM (Fig. 2e). The time required for 50% labeling of the SNAP-tag was about 1.1 min for BGAN-2C, 46 min for BGAN-8C and a much longer time for BGAN-12C (Fig. S4, ESIF). None of the probes displayed a time-dependent alteration of the fluorescence intensity in the absence of the SNAP-tag. In further kinetic analysis, the second-order rate constant ($k_2$) for reaction between BGAN-2C and the SNAP-tag was determined to be 2031.7 ± 63.7 M$^{-1}$ s$^{-1}$ (Table 1), which was 20-fold higher than that for BGAN-8C (105.2 ± 6.0 M$^{-1}$ s$^{-1}$, Fig. S5, ESIF). The labeling rate of BGAN-2C is much faster than those of quencher-based SNAP-tag fluorogenic probes, such as DBRGG-488 (396 ± 32 M$^{-1}$ s$^{-1}$) and CBG-549-QSY7 (1027 ± 457 M$^{-1}$ s$^{-1}$), and comparable with that of BGSBD (7200 ± 1600 M$^{-1}$ s$^{-1}$). These results demonstrated that the longer the hydrophobic carbon chains of the probes, the slower the reaction rate with the SNAP-tag. The reason may be that most of the hydrophobic cavities of proteins were easily occupied by hydrophobic alkyl chains which restrained the covalent binding between the SNAP-tag and the BG ligand. It suggests that fluorogenic probes for proteins should be delicately designed. Above all, BGAN-2C was a much better probe than others that we designed for no-wash SNAP-tag labeling.

Subsequently, no-wash live-cell fluorescence labeling of intracellular proteins was carried out using BGAN-2C. HEK 293 cells which expressed SNAP-tag fusion proteins in the cytosol, mitochondria, and nucleus were investigated. First, 5 μM BGAN-2C was added to the live-cells with non-specific SNAP-tag expression and incubated for 20 min. The fluorescence imaging was taken without washing the cells. An obvious fluorescence was observed in the cytosol (Fig. 3a and b), which demonstrates the high cell-permeability of the probe that benefits intracellular protein labeling. In contrast, fluorescence was hardly detected in the non-transfected cells (Fig. 3a and b). The toxicity test indicated that the probe is nontoxic to the HEK 293 cells (Fig. S6, ESIF). In the further investigation, commercialized pSNAP-Cox8A and SNAP-H2B (a gift from Prof. Qingkai Yang) fusing SNAP-tags to cytochrome c oxidase subunit 8 (Cox8A) and human histone H2B were transiently transfected to cells. After 20 min incubation with 5 μM BGAN-2C, the cells were observed without washout of the probe. And clear green fluorescence was observed without obvious background fluorescence. This is in sharp contrast to a conventional green SNAP-tag SNAP-Cell® 505-Star, which required a lengthy post incubation washout period before effective visualization (Fig. S7, ESIF). Co-staining of the probe with red
emission from Mitotracker Deep Red confirmed that the probe localized in a mitochondrial area. Meanwhile, specific labeling of SNAP-H2B fusion proteins in the nucleus was also observed. These results demonstrated that BGAN-2C can be applied in the visualization of target proteins at a specific location with no-wash procedure required.

In conclusion, a 1,8-naphthalimide-derived fluorogenic probe BGAN-2C was designed based on the additive effects of quencher release and the fluorophore’s environmental sensitivity. BGAN-2C displayed 36 fold fluorescence increases after labeling the SNAP-tag. The high signal-to-noise ratio and the reaction rate ensure that BGAN-2C is a good probe capable of labelling proteins in living cells without a washout procedure. Different substituents in the C-4 position of 1,8-naphthalimide were introduced to examine the protein labelling properties. It is concluded that ICT-governed environment-sensitive fluorophores are more suitable to design fluorogenic probes for the SNAP-tag. For molecular rotor type fluorophores, it is promising to develop fluorogenic probes with dedicated modifications to these fluorophores.

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Notes and references