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A Fluorogenic Probe for SNAP-Tagged Plasma Membrane Proteins Based on the Solvatochromic Molecule Nile Red

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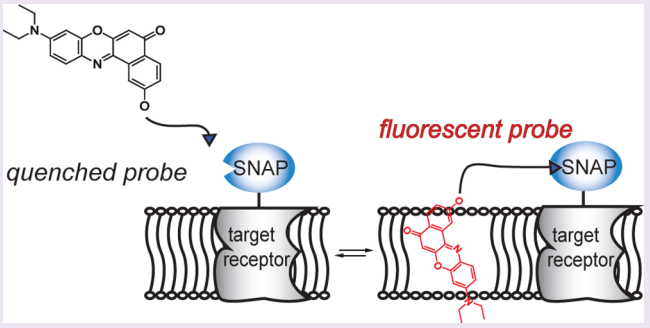
Supporting Information

ABSTRACT: A fluorogenic probe for plasma membrane proteins based on the dye Nile Red and SNAP-tag is introduced. It takes advantage of Nile Red, a solvatochromic molecule highly fluorescent in an apolar environment, such as cellular membranes, but almost dark in a polar aqueous environment. The probe possesses a tuned affinity for membranes allowing its Nile Red moiety to insert into the lipid bilayer of the plasma membrane, becoming fluorescent, only after its conjugation to a SNAP-tagged plasma membrane protein. The fluorogenic character of the probe was demonstrated for different SNAP-tag fusion proteins, including the human insulin receptor. This work introduces a new approach for generating a powerful turn-on probe for "no-wash" labeling of plasma membrane proteins with numerous applications in bioimaging.



Fluorescent probes for the visualization of cell surface receptors are of great importance in medicine as well as in basic research.[1] The ideal probe would be a molecule that is selective and fluorescent only upon binding to its target molecule. To our knowledge, there is no generally applicable approach for the generation of such a probe for cell surface receptors.

The approach presented here is based on the self-labeling tag SNAP-tag, which has been previously introduced by our lab, and the solvatochromic dye Nile Red (NR). SNAP-tag is a mutant of O^6-alkylguanine-DNA alkyltransferase (AGT) that reacts specifically with O^6-benzylguanine (BG) derivatives.[2] NR is a solvatochromic dye; its absorption and emission shifts strongly toward the red part of the spectrum as the solvent becomes more polar.[3] This solvatochromic behavior is due to photoinduced electron transfer (PET) quenching between the diethylamino group that acts as an electron donor and the aromatic acceptor system in the excited state.[3−6] PET quenching is more favored in a polar environment due to solvation.[4,7] Furthermore, the quantum yield (QY) of NR is also strongly dependent on its environment: in low polarity solvents, the QY is high, while polar solvents induce a low QY. As a consequence, NR is a fluorophore that is almost non-fluorescent in aqueous solution, whereas with decreasing polarity of the environment its QY increases significantly to reach values close to unity.[5] The low fluorescence properties of the dye in a polar environment are believed to be due to the formation of aggregates that causes self-quenching[3] and to hydrogen bonding that has been reported to provoke radiationless deactivation for anthraquinone and fluorenone derivatives.[8−12] As a rather hydrophobic molecule, NR spontaneously inserts into membranes, rapidly diffuses inside cells, and tends to accumulate in membranes and lipid droplets where it becomes highly fluorescent due to the low polarity of these structures. It has therefore become popular for the staining of membranes and lipid droplets.[13−15]

However, the nonspecific character of the localization of NR inside cells forbids its use to visualize only a subset of membranes or proteins. We hypothesized that tuning the membrane affinity of NR through chemical derivatization so that its insertion into membranes becomes conditional of its binding to an object sitting close to or on the membrane (e.g., a receptor protein) would enable us to generate a specific and fluorogenic probe for this given object (Figure 1). Only NR molecules bound to the object of interest would display a high effective concentration nearby the membrane, and therefore they would be able to insert into the membrane and become fluorescent. The unbound probe experiencing a polar hydrophilic environment in solution would remain dark, thus allowing "no-wash" experiments. Imaging without the require-

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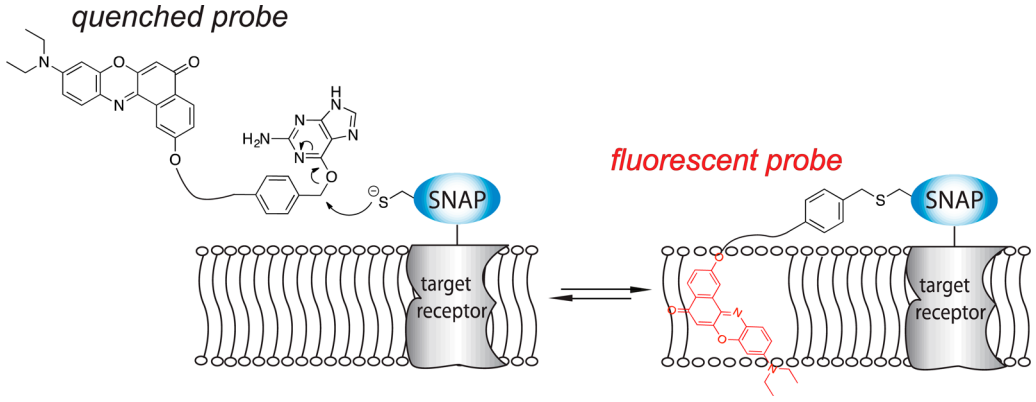

Figure 1. A fluorogenic probe for cell surface proteins. A BG-derivative of Nile Red targeting SNAP is synthesized. The probe on its own does not insert into membranes and therefore is quenched. Upon reaction with SNAP, Nile Red is localized to the plasma membrane, resulting in its subsequent membrane insertion. In the apolar environment the probe becomes fluorescent.

ment of additional washing steps is an attractive property for a probe and therefore has been attempted by others.[16,17] "No-wash" imaging avoids the time-consuming additional washing steps but most importantly allows performing real time measurements and monitoring of molecular events directly after labeling as well as the use of the probe in cases where a washing procedure is not possible, such as in *in vivo* experiments. We chose SNAP-tag to specifically anchor our NR derivatives since a variety of receptor proteins have already been successfully expressed as fully functional SNAP-tag fusion proteins.[18] This offers the advantage that once the ligand has been optimized, it could be applied to a large variety of existing SNAP-tag plasma membrane fusion proteins.

According to our hypothesis, our derivatives must allow the NR part of the molecule to reach the lipid bilayer upon conjugation to the SNAP-tag. Therefore, a long tether between the BG moiety and the NR is required. In addition, the molecule should be hydrophilic enough to avoid a spontaneous insertion of NR in the membrane. We chose poly(ethylene glycol) (PEG) linkers since their length can be varied easily and they possess good water solubility. We therefore synthesized compounds **1**−**4** that consist of (i) a benzylguanine part to enable conjugation to SNAP-tag;, (ii) a PEG linker to allow the dye to reach the lipid bilayer, and (iii) a Nile Red derivative. Compounds **1** and **2** differ from compounds **3** and **4** by the insertion of the negatively charged cysteic acid (Cya) moiety close to the NR to further reduce the hydrophobicity of the compounds and thereby further reduce nonspecific insertion in membranes (Figure 2A). Two different PEG linkers were used: PEG5 (compounds **1** and **3**) and PEG11 (compounds **2** and **4**) (Figure 2A). The synthesis of these compounds is presented in Supporting Information (Figures S1−S5).

We tested whether the solvatochomic properties of NR were retained in compounds **1**−**4**. The excitation and emission spectra, as well as the fluorescence QY of the latter were recorded in solvents of different polarity, such as dioxane, Dubelcco's modified eagle medium (DMEM) + 10% fetal bovine serum (FBS), and phosphate buffered saline (PBS). As shown in Figure 2B and C and Supplementary Table S9, there is a clear red shift of the λ_{abs} and of the λ_{fluor} of compound **4** as the solvent becomes more polar, which is a characteristic for NR.[5] In addition, similar to NR (Supplementary Figure S6), compound **4** shows much higher fluorescence emission and excitation intensity in the apolar solvent dioxane compared to PBS where it is almost non-fluorescent (Figure 2C and Supplementary Table S9). Compounds **1**−**3** showed very similar behaviors (Supplementary Figure S6 and Table S9). Moreover, compounds **1**−**4** react with SNAP-tag with similar rate constants (Supplementary Table S10). These values are in the range of values that have been reported for other BG-substrates for SNAP.[16] Reaction of compound **4** with soluble SNAP-tag in DMEM + 10% FBS resulted in an approximate 9-fold increase in fluorescence (Supplementary Table S10). We believe that this increase is mainly due to removal of guanine after binding of **4** to SNAP, as guanine is known to quench fluorescence through PET.[25] It should be kept in mind that in this experiment NR cannot insert in a membrane and that the spectroscopic properties of NR conjugated to either soluble or membrane proteins are very different (Supplementary Table S10 and vide infra).

Next, we assessed whether probes **1**−**4** were suitable for live cell imaging. HEK 293T cells were transiently transfected with a previously described SNAP-tag fusion protein in which SNAP is fused via CLIP-tag and human carbonic anhydrase (HCA) to a growth factor receptor transmembrane domain, resulting in the display of SNAP-tag on the extracellular side of the plasma membrane.[19] We chose this construct for proof-of-principle experiments because it allows co-staining of CLIP-tag with a separate fluorophore conjugated to a benzylcytosine (BC) moiety[20] to unambiguously identify transfected cells. The cells were labeled first with BC-Alexa488, washed, and treated with compounds **1**−**4** (500 nM) for 1 h at 37 °C. Live cells were then washed three times and subsequently imaged with a wide-field microscope. All four compounds gave a clear co-localization of NR and Alexa488 fluorescence after the cells were washed (Figure 3A), indicating that the conjugation to SNAP-tag was efficient and that the NR was turned-on by experiencing a hydrophobic environment. We observed a moderate nonspecific signal from the membrane of untransfected cells with compounds **1** and **2**. We speculate that this nonspecific signal results from residual affinity of these NR derivatives for cellular membranes. We then characterized the performance of the probes in no-wash experiments (Figure 3B). Here a significant difference between compounds **1** and **2** and compounds **3** and **4**, which structurally differ only by the presence of negatively charged Cya in the linker part becomes apparent: While with **3** and **4** it is clearly possible to visualize transfected cells in the NR channel with acceptable (compound **3**) to low (compound **4**) background, compounds **1** and **2** displayed strong uniform fluorescence throughout the whole

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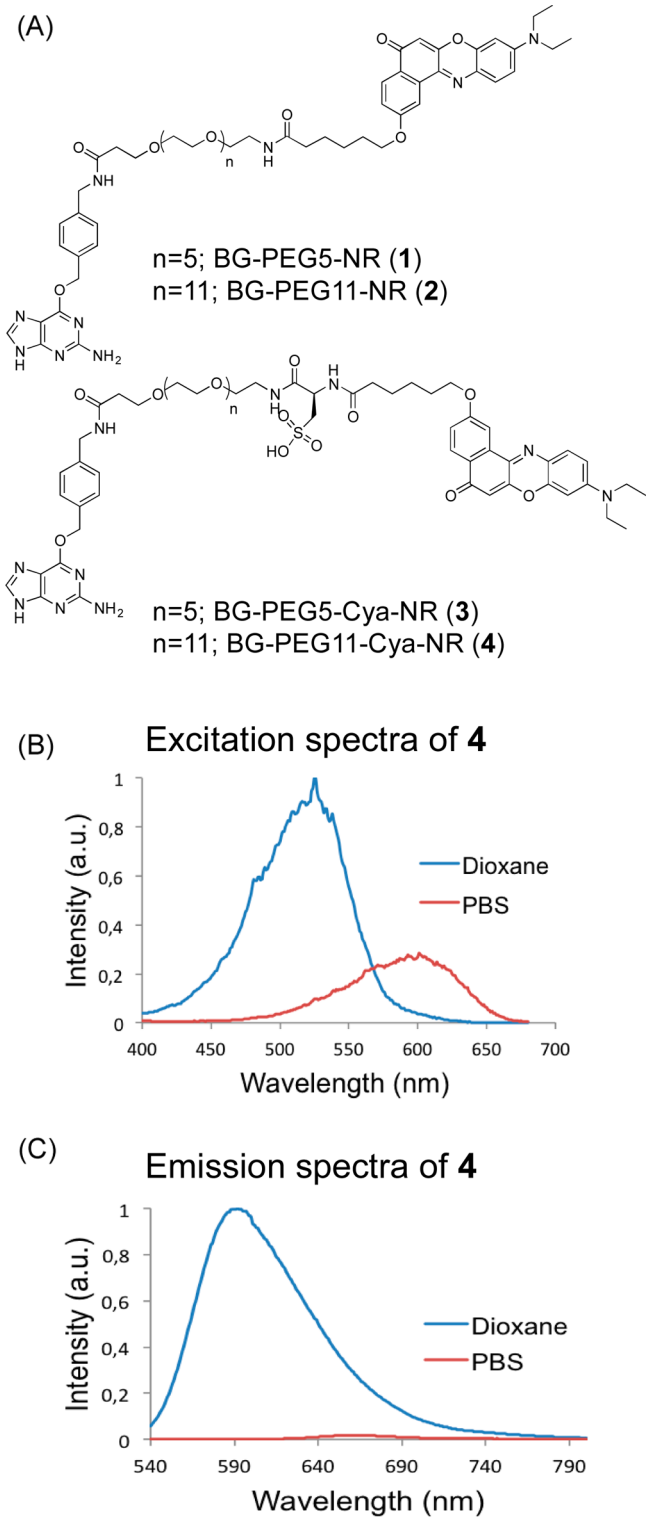

Figure 2. (A) Structures of compounds **1**–**4**. (B) Excitation and (C) emission spectra of the BG-derivative **4** in dioxane and PBS showing typical spectroscopic properties of Nile Red. The intensities have been normalized to those of dioxane.

imaging field. This strong background fluorescence might be due to nonspecific insertion of **1** and **2** into the membrane and nonspecific binding to bovine serum albumin (BSA), present in the imaging medium. It has been previously reported that NR binds specifically to hydrophobic sites of BSA, resulting in a strong increase of its fluorescence.[21] The presence of the charged Cya close to the NR apparently prevents nonspecific binding to membranes and BSA, keeping the probe in a fluorescence-off state and permitting no-wash imaging. Compound **4** showed less background fluorescence in no-wash experiments than compound **3**. Presumably, the longer hydrophilic PEG linker further reduces nonspecific binding to BSA and membranes. This hypothesis is supported by the stronger fluorescence intensity of compound **3** compared to compound **4** in DMEM + 10% FBS (Supplementary Figure S7), as well as by the absorbance and emission maxima of compound **3**, which are 20 nm blue-shifted compared to **4** in DMEM + 10% FBS (Supplementary Table S9). Moreover, a cytotoxicity test was performed for compound **4**. As it can be seen in Supplementary Figure S8, incubation of cells at 37 °C for 24 h with **4** (1 μM) had no effect on their growth when compared to nontreated cells. This is in contrast to what is observed after incubation with the known cytotoxic compound puromycin (used at 3 μg mL^{-1}).

We decided to validate our concept with the best performing compound **4** with a pharmacologically relevant receptor: the human insulin receptor (HIR). HIR belongs to a subfamily of receptor tyrosine kinases. It is activated by the binding of insulin[22] and has a very important role in regulating glucose homeostasis. Diseases such as diabetes are associated with HIR malfunction.[23] CHO cells stably expressing a HIR-SNAP-tag fusion protein were treated with **4** for 30 min at RT in F-12 HAM medium. Live cells were imaged for fluorescence using a confocal microscope after the labeling with and without washing steps (Figure 4A). As with the artificial construct SNAP-CLIP-HCA, compound **4** allowed the specific staining of an extracellularly SNAP-tagged insulin receptor, demonstrating that our strategy can be easily applied to other receptors. In addition, a time-dependent internalization of labeled SNAP-HIR was observed (Figure 4A, after 3 washing steps). Receptor internalization can significantly complicate receptor imaging when long washing steps are needed to reduce background fluorescence. This observation underscores the fact that our "no-wash" approach should facilitate the real time monitoring of receptor trafficking.

Moreover, the fluorescence spectrum of **4** conjugated to SNAP-HIR was recorded on fixed (Figure 4B) and live (Figure 4C) cells in order to obtain more information regarding the behavior of NR when inserted in the plasma membrane. As shown in Figure 4B and C, the emission spectrum of **4** is blue-shifted when compared to the spectrum recorded in PBS. Its maximum emission peak is found to be between those measured for dimethyl sulfoxide (relative dielectric constant ε_r = 46.7) and dioxane (ε_r = 2.3), suggesting indeed that NR inserts into the membrane and experiences an environment much less polar than water (ε_r = 80.1). In order to provide further evidence for the insertion of NR in the membrane, we labeled a purified SNAP-tag protein containing a terminal hexahistidine tag (His-SNAP) with **4**. We then applied His-SNAP to 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) lipid vesicles containing Ni-NTA lipids.[24] As a control, vesicles of the same composition but lacking Ni-NTA lipids were used. His-SNAP should therefore bind to the Ni-NTA moiety at the surface of the vesicles, thereby anchoring it to the membrane while it remains in solution with the control vesicles. The fluorescence spectrum of **4** showed a maximum at 626 nm in the case of the liposomes lacking Ni-NTA lipids, whereas the liposomes containing Ni-NTA lipids showed a maximum at 614 nm (Figure 4D, Supplementary Table S10), a

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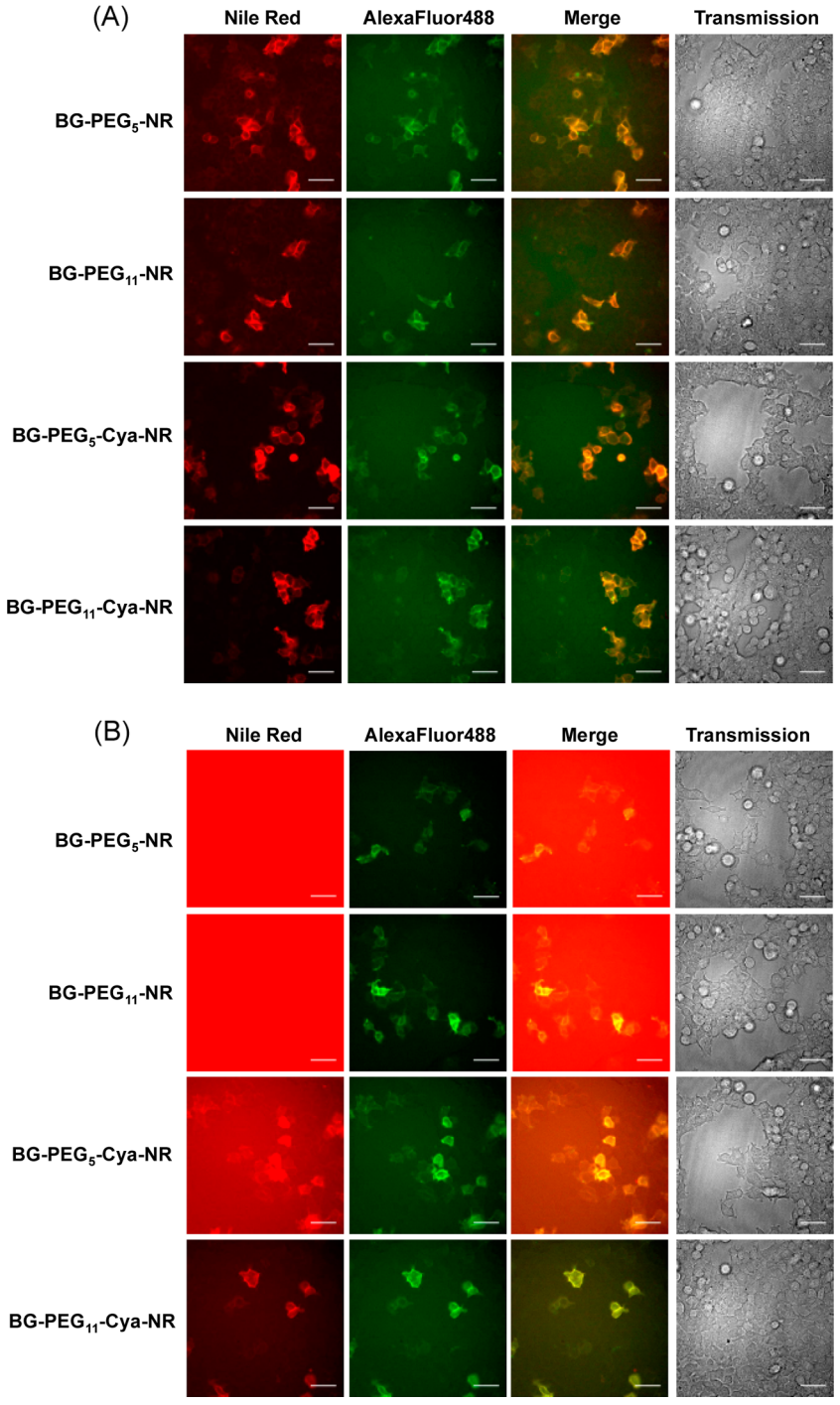

Figure 3. HEK 293T cells, expressing p-display SNAP-CLIP-HCA, labeled with BC-Alexa488 (10 μM, 10 min incubation at 37 °C, three washes with DMEM + 10% FBS) and compounds **1**−**4** (500 nM, 1 h incubation at 37 °C, DMEM + 10% FBS) imaged after three washes (A) or without wash (B). Scale bar, 50 μm.

value which is coherent with insertion of NR in the membrane, as previously reported by Kucherak et al.[13] As a comparison, **4** in PBS showed a maximum of fluorescence at 660 nm. These results provide evidence that upon conjugation to SNAP-tag **4** experiences a more hydrophobic environment witnessed by the observed blue shift from 660 to 626 nm. We attribute this shift to a possible interaction of NR with the surface of SNAP-tag. On top of the blue shift due to conjugation to SNAP-tag, a further blue shift from 626 to 614 nm was observed when SNAP-tag was immobilized on the membrane of liposomes. This accounts for the insertion of Nile red into the liposome membrane.

We have introduced a new turn-on fluorogenic probe for "no-wash" labeling of SNAP-tagged plasma membrane proteins based on the solvatochromic molecule NR. Our strategy is based on the identification of a modified NR derivative that only inserts into membranes when coupled to plasma membrane proteins. This probe proved to efficiently label SNAP-tagged plasma membrane proteins with minimal background in no-wash experiments with live cells and should thus

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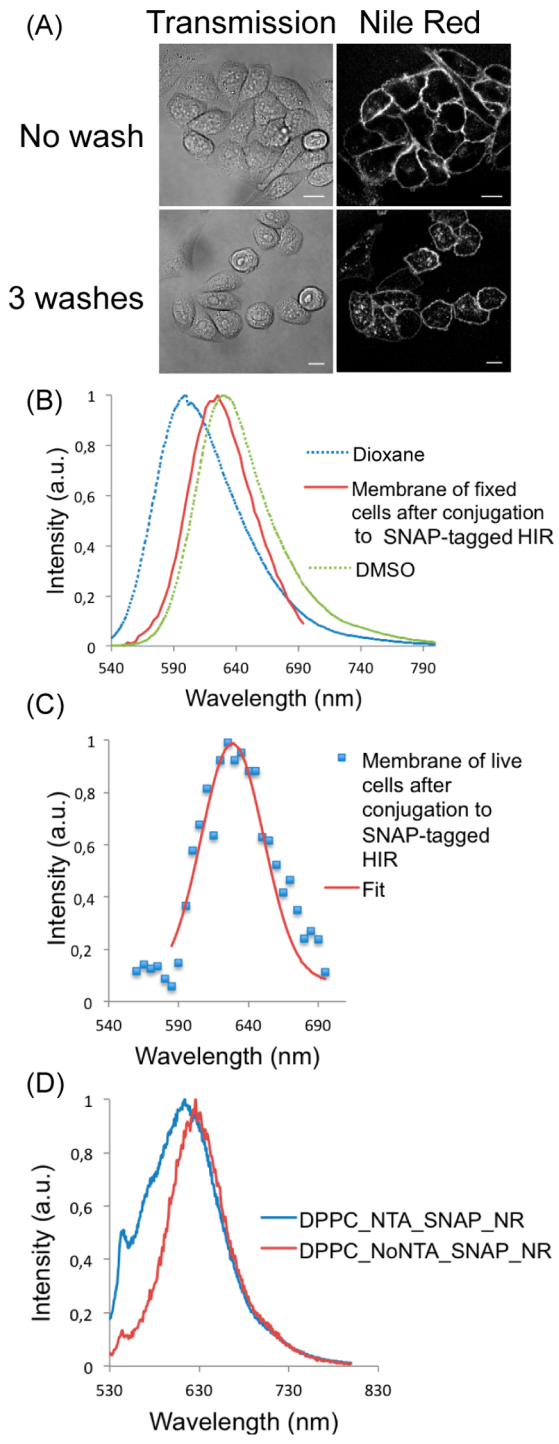

Figure 4. (A) CHO cells stably expressing extracellularly SNAP-tagged HIR labeled with compound **4** (2 μM, 30 min incubation at 37 °C in F-12 HAM medium, w/o FBS) imaged before (no wash) and after 3 washes. Scale bar, 10 μm. (B) Emission spectrum of compound **4** upon conjugation to SNAP-tagged HIR stably expressed in CHO cells (fixed) and comparison to the emission spectra of **4** recorded in dioxane and DMSO. (C) Emission spectrum of compound **4** upon conjugation to SNAP-tagged HIR stably expressed in CHO cells (live). The spectrum was fitted using a Gaussian fit (yielding a maximum at 629 ± 1 nm) as a guide for the eye. (D) Emission spectra of **4** after conjugation to His-tagged SNAP applied on DPPC lipid vesicles containing Ni-NTA lipids and on control vesicles lacking the Ni-NTA lipids. The intensities have been normalized.

become a powerful tool for bioimaging of plasma membrane proteins.

METHODS

Synthesis. The detailed synthetic procedures and compound characterizations are mentioned in the Supporting Information.

Characterization of Fluorescent Properties of Nile Red Derivatives. The samples of the appropriate substrates were prepared in 1.5-mL tubes (Eppendorf) (1 μM) in dioxane, Dubelcco's modified eagle medium (DMEM) + 10% fetal bovine serum (FBS), phosphate buffered saline (PBS), or dimethyl sulfoxide (DMSO). Fluorescence was measured in a 96-well plate on an Infinite M1000 spectrofluorometer (TECAN). Fluorescence emission was detected while exciting at 520 nm, and the excitation spectrum was recorded at 700 nm emission wavelength. Both the excitation and emission bandwidth for all measurements were set to 10 nm.

Determination of Relative Fluorescence Quantum Yield (QY). Fluorescence QY of compounds **1**−**4** were measured in PBS, DMEM + 10% FBS, and dioxane using a solution of Nile Red in methanol (MeOH) as a reference. For the determination of QY of **4** after conjugation to SNAP in PBS and DMEM + 10% FBS, a 3-fold excess of SNAP was added to compound **4**, and the measurement started after 1 h of incubation at RT. Absorbance spectra of Nile Red solutions of different concentrations were recorded using a SHIMADZU UV spectrophotometer UV-1800 in the 400−800 nm interval with 1 nm step and 1 nm fixed bandwidth. Afterward fluorescence of the same samples was measured in 96-well plates on an Infinite M1000 spectrofluorometer (TECAN). Both the excitation and emission bandwidth for all measurements were set to 10 nm. The spectra were recorded with a step size of 1 nm. The excitation wavelength was set to 520 nm, and the emission spectra were recorded in the 540−800 nm interval. Absorbance at 520 nm versus integrated fluorescence intensity was plotted. The ratio of the obtained slope values of each compound to the slope value of NR in MeOH multiplied by measured QY of NR in MeOH (38%)[26] and corrected by the refractive indexes of the solvents gave relative quantum yields reported in Tables S9 and S10, Supporting Information.

***In Vitro* Kinetic Experiments.** SNAP was diluted in PBS buffer with 0.1 mg mL^{-1} BSA to the desired concentration (400 nM). The reaction was started by the addition of a 3-fold molar excess of a substrate (as a 1.2 μM solution in DMSO). At each time point, 10 μL of reaction mixture was withdrawn and immediately mixed with 10 μL of stop buffer (2x SDS loading buffer supplemented with 150 μM O^6-benzylguanine) preheated to 95 °C. The samples were analyzed on a 12% SDS-PAGE gel, and in-gel fluorescence was recorded on a Pharos-FX (Biorad) scanner. Fluorescent band intensity was quantified using Quantity one (Biorad) and plotted against time. Data were fitted to a single exponential equation, and labeling rate constant k was calculated using the formula $k = \ln 2/(t_{1/2}c)$ M^{-1}·s^{-1} where $t_{1/2}$ is the half-life time of labeling and c is the substrate concentration.

Cell Culture and Transfection. HEK 293T cells were grown on polyornithine-coated glass bottom culture Petri dishes (35 mm) in DMEM Glutamax medium (Lonza) supplemented with 10% FBS (Lonza) and transiently transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. CHO cells stably expressing extracellular SNAP-tagged human insulin receptor (HIR) were maintained in HAM's F-12 medium (Invitrogen) supplemented with 10% FBS and 600 μg mL^{-1} G418 at 37 °C. CHO cells stably expressing extracellular SNAP-tagged HIR were grown on 35 mm uncoated glass bottom dishes in medium for 2 days. Starvation of cells followed by washing with F-12 HAM medium (w/o FBS) and incubating for 2 h at 37 °C.

Stable CHO Cell Line Expressing SNAP-Tagged Human Insulin Receptor. *Homo sapiens* HIR (NCBI Reference Sequence: NM_001079817), with the *Hin*dIII site before the start codon and *Xba*I after the stop codon, was subcloned by replacing the EGFP sequence of pEGFP-N1 (Clontech Takara). The SphI site was created by a silent mutation at Ala^{812} (GCT to GCA). The SNAP-tag was inserted at the C-terminus of the α-subunit, between Ser^{746} and Arg^{747}, by replacing the *Bam*HI-SphI fragment in the coding sequence. The

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HIR and the SNAP sequences were synthesized by GeneArt (life technologies). CHO-K1 cells were transfected with the SNAP-HIR plasmid using Lipofectamine LTX (Invitrogen). Twenty-four hours after transfection, the cells were selected in 600 μg mL^{-1} G418, and stably expressing clones were isolated by limiting dilution method. The clones were screened with SNAP-Surface Alexa Fluor 488 (NEB).

SNAP-Tag and CLIP-Tag Labeling on the Cell Surface of HEK 293T Cells Expressing p-Display SNAP-CLIP-HCA and Extracellular SNAP-Tagged HIR Stably Expressing CHO Cells. At 24−48 h after transfection, HEK 293T cells were labeled by incubation with a solution of 10 μM O^2-benzylcytosine (BC) derivative in DMEM (without Phenol Red) supplemented with 10% FBS for 10 min at 37 °C and washed three times with the labeling (and culture) medium. Then, the cells were labeled with the corresponding O^6-benzylguanine (BG) derivative (500 nM) in DMEM (without Phenol Red) + 10% FBS for 1 h at 37 °C. After labeling, cells were imaged directly or after three washes with the labeling (and culture) medium in RT. CHO cells stably expressing extracellular SNAP-tagged HIR were stained with 2 μM BG-PEG11-Cya-NR (**4**) in medium supplemented with 15 mM HEPES for 30 min at RT and imaged directly or after three washes.

Wide-Field Microscopy and Live Cell Imaging. Imaging of the labeled HEK 293T cells was performed using a Leica LAS AF 6000 wide-field microscope equipped with a 40x plan Apochromat 1.25 NA oil immersion objective lens and a xenon arc lamp. The filter sets used are the following: for Nile Red excitation at 572 nm (bandwidth 35 nm) and emission at 632 nm (bandwidth 60 nm), and for AlexaFluor 488 excitation at 470 nm (bandwidth 40 nm) and emission at 520 nm (bandwidth 40 nm).

Confocal Microscopy. Imaging of the labeled SNAP-tagged HIR was performed using a Leica SP5 confocal microscope equipped with an HCX PL Apochromat CS 63X (1.40 NA) oil immersion objective. All of the fluorescent images were obtained under the same acquisition conditions, excitation at 514 nm (Ar laser) and detection at 560−645 nm using HyD detector. The images were deconvolved using the Huygens Essentials package.

Fluorescent Spectral Measurement in Fixed Cells under Confocal Microscope. CHO cells stably expressing extracellularly SNAP-tagged HIR were cultured on coverslips for 2 days and labeled with 2 μM BG-PEG11-Cya-NR (**4**). The coverslips were rinsed in ice-cold PBS and fixed with 3% paraformaldehyde for 10 min at RT, followed by treatment with 50 mM NH_4Cl in PBS for 10 min, and were mounted with Mowiol (Sigma-Aldrich). The specimens were observed under Zeiss 710 confocal microscope equipped with plan-APOCHROMAT 63X oil immersion objective (1.4 NA) and the emission spectrum of BG-PEG11-Cya-NR (**4**) was measured using lambda mode, excitation at 514 nm (Ar laser) and detection at 550−695 nm (step: 3 nm). The spectrum was plotted by Image J (http://rsbweb.nih.gov/ij/) and the spectrum was an average of eight different image areas.

Fluorescent Spectral Measurement in Live Cells under Confocal Microscope. CHO cells stably expressing extracellular SNAP-tagged HIR were cultured on glass bottom dishes for 2 days, and labeled with 2 μM **4** at RTs, followed by washing to remove excess dye. Imaging of the labeled SNAP-tagged HIR was performed using a Leica SP5 confocal microscope equipped with an HCX PL Apochromat CS 63X (1.40 NA) oil immersion objective. The emission spectrum of **4** was measured using lambda mode, excitation at 514 nm (Ar laser) and detection at 560−695 nm (step: 5 nm).

Fluorescent Spectral Measurement of 4 in DPPC Lipid Vesicles. Lipids used were 1,2-dipalmytoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG), and 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl)] (NTA-lipid) from Avanti, USA. Small unilamellar vesicles were made by tip-sonication after hydration of a lipid film in 15 mM NaCl, 0.5 mM imidazole, 5 mM K_2HPO_4 pH 8.0 to 5 mg lipid mL^{-1}. The following lipid compositions (expressed in mass %) were used for the NTA-containing vesicles: 80% PC, 10% PG, 10% NTA-lipid, and control vesicles without NTA: 90% PC, 10% PG. Compound **4** was diluted in PBS buffer with 0.1 mg mL^{-1} BSA to the desired concentration (1 μM). The addition of a 10-fold molar excess of SNAP (10 μM) followed. The reaction mixture was incubated for 1 h at RT and then was applied to the lipid vesicles. An incubation of 15 min followed, and then the fluorescence emission spectra of the samples were measured upon excitation at 520 nm on a Cary Eclipse spectrofluorometer (Varian). The spectra were smoothed using a Savitzky-Golay procedure (5 points, second order).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of the compounds, excitation and emission spectra, tables with spectroscopic properties, QY and kinetics of the labeling reaction with SNAP for all the compounds, cytotoxicity test of compound **4** and comparison of the maximum intensities of compounds **3** and **4** in PBS and DMEM + FBS 10%. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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