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Site-Specific Protein Labeling with SNAP-Tags

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Abstract

Site-specific labeling of cellular proteins with chemical probes is a powerful tool for studying proteins function in living cells. A number of small peptide and protein tags have been developed that can be labeled with synthetic probes with high efficiencies and specificities and provide flexibility not available with fluorescent proteins (FPs). The SNAP-tag is a modified form of the DNA repair enzyme, human O⁶-alkylguanine-DNA-alkyltransferase (AGT), and undergoes a self-labeling reaction to form a covalent bond with O⁶-benzylguanine (BG) derivatives. BG can be modified with a wide variety of fluorophores and other reporter compounds generally without affecting the reaction with the SNAP-tag. In this unit, basic strategies for labeling SNAP-tag fusion proteins, both for live cell imaging and for *in vitro* analysis, will be described. This includes a description of a releasable SNAP-tag probe that allows the user to chemically cleave the fluorophore from the labeled SNAP-tag fusion. *In vitro* labeling of purified SNAP-tag fusions is briefly described.

Keywords

SNAP-tag; chemical labeling; cell biology; endocytosis; imaging

Introduction

Chemical methods for site-specific labeling of peptide tags and fusion proteins with organic fluorophores and other small molecule probes represent a powerful approach to the study and manipulation of protein function in living cells. These methods offer high labeling efficiencies and specificities, and flexibility not available with fluorescent proteins, including temporal control over labeling and, in several cases, the ability to label the same protein fusion with a variety of reporter groups (Hinner and Johnsson, 2010; Jing and Cornish, 2011).

One popular method is based on the self-labeling SNAP-tag. In this approach, the protein of interest is expressed as a fusion with a modified form of the 20-kDa monomeric DNA repair enzyme, human O⁶-alkylguanine-DNA-alkyltransferase (AGT), or SNAP-tag. The SNAP-tag can be specifically labeled with synthetic O⁶-benzylguanine (BG) derivatives, resulting in a stable thioether bond between a reactive cysteine residue in the tag and the probe (see

Internet Resources

For an extensive description of the SNAP-tag labeling system, building blocks for custom probe synthesis, vectors and probes, see the New England Biolabs website: www.neb.com.

For information on infrared scanning using the Odyssey system as well as reagents for custom infrared probes, see the LI-COR website: www.licor.com.

For a number of NHS esters and crosslinking agents for custom probe synthesis, see the Thermo Scientific website: www.thermoscientific.com.

Fig. 1)(Keppler et al., 2004a; Keppler et al., 2004b). The SNAP-tag can be appended onto the N- or C-terminus of proteins without affecting the function of a large number of fusion proteins (Keppler et al., 2004b; Srikun et al., 2010). The advantages of self-labeling proteins over current small peptide tags are their high reaction rate and specificity of labeling, the large number of commercially available BG-conjugated substrates, and simple synthetic chemistries for custom probe synthesis. Labeling of the SNAP-tag is irreversible and quantitative, and thus well suited for the detection and quantitation of labeled proteins via in-gel fluorescence scanning of SDS-PAGE gels. Depending on the permeability of the BG substrate, SNAP-tag fusions can be labeled at the cell surface, and within cells and subcellular organelles when cell-permeable substrates are used. For example, SNAP-tag labeling has been described not only for proteins in the cytosol, but also in the nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus (Hinner and Johnsson, 2010; Srikun et al., 2010; Tomat et al., 2008). Non-permeable substrates can be microinjected or bead loaded and do not normally lead to significant background staining, as excess dye usually diffuses out of the cell (Keppler et al., 2006). In addition to the SNAP-tag, a companion labeling system, known as the CLIP-tag, is available that covalently binds O²-benzylcytosine (BC) substrates. SNAP and CLIP fusion proteins can be labeled simultaneously and specifically with different substrates in living cells, and have been used in simultaneous pulse-chase experiments to visualize different generations of two different proteins (Gautier et al., 2008). A number of applications have featured SNAP-tag labeling, including FRET (Comps-Agrar et al., 2011; Maurel et al., 2008; Zhang et al., 2011), chromophore-assisted light inactivation (Keppler and Ellenberg, 2009), sensors for ions (Kamiya and Johnsson, 2010; Tomat et al., 2008) and hydrogen peroxide (Srikun et al., 2010), and super resolution microscopy (Banala et al., 2012; Klein et al., 2011).

This unit will describe basic strategies for labeling SNAP-tag fusion proteins, both for live cell imaging and for *in vitro* analysis. Due to the expanding repertoire of uses for the SNAP-tag system, a complete inventory is not practical. This unit will present a set of procedures that we have found useful for studying endocytosis and turnover of cell surface proteins. This includes a description of a releasable SNAP-tag probe that allows the user to chemically cleave the fluorophore from the labeled SNAP-tag fusion. *In vitro* labeling of purified SNAP-tag fusions is briefly described.

Familiarity with molecular cloning protocols to generate N- or C-terminal SNAP-tag fusions, as well as DNA transfection techniques and cell culturing is assumed for this unit, as is a general understanding of fluorescence microscopy and live-cell imaging. A variety of SNAP-tag building blocks and labels as well as protocols for SNAP-tag labeling can be found at the New England Biolabs website: www.neb.com. In addition, a video link sponsored by New England Biolabs that demonstrates SNAP-tag labeling can be found at: <http://www.jove.com/video/1876/fluorescent-labeling-cos-7-expressing-snap-tag-fusion-proteins-for?ID=1876>.

Basic Protocol 1. Site-specific labeling of cell surface SNAP-tag fusion proteins

Proteins of interest can be expressed with the SNAP-tag as either an N- or a C-terminal fusion, but the tag needs to be exposed to the extracellular surface of the plasma membrane for labeling with cell impermeable SNAP-Surface substrates. This includes, for example, transmembrane proteins that are initially synthesized in the endoplasmic reticulum and then trafficked through the Golgi complex en route to the plasma membrane. A notable advantage of the SNAP-tag system over fluorescent proteins is that SNAP-tag fusions can be specifically labeled on the cell surface without interference from internal fluorescent pools. The protocol will describe labeling with cell-impermeable fluorescent BG derivatives (e.g.

SNAP Surface-Alexa Fluor 488) that require several washes to reduce background, but it should be noted that a number of “no washout” probes have been developed that are essentially non-fluorescent until a quencher compound is released upon SNAP-tag binding (Komatsu et al., 2011; Sun et al., 2011).

Materials

Target cells

Culture medium (the presence of serum does not inhibit the SNAP-tag reaction)

Plasmid encoding SNAP-tag fusion protein

The original SNAP-tag, SNAP26m, has been replaced by a faster labeling variant, SNAP_f, (available from NEB) that allows for reduction of substrate concentration and incubation times.

Glass coverslips (Daigger, Fisher, etc.) or chambered coverslips (Lab-Tek II Chambered Coverglass, Nunc)

Transfection reagent (Fugene, Roche; Lipofectamine, Invitrogen; electroporation, Amaxa)

Cell impermeable BG-labeled probe (“Cell Surface” probes from NEB or custom synthesized – see Support Protocol 1)

Non-fluorescent SNAP-cell blocking reagents: SNAP Cell Block (NEB, S9106, cell permeable), SNAP-Surface Block (NEB, S9143, cell impermeable), BG-NH₂ (Toronto Research Chemicals, A614425, cell permeable)

Hanks buffered salt solution (HBSS) (with CaCl₂ and MgCl₂) or phosphate-buffered saline (PBS) (with CaCl₂ and MgCl₂).

Fixative (2–3.7% formaldehyde/PBS or 100% methanol)

Mounting medium (Fluoromount-G, Southern Biotechnologies, etc.)

37°C humidified incubator with 5 % CO₂

1. Plate target cells on glass coverslips or Lab-Tek chambers so that they will be less than 50% confluent the next day.

For microscopy on fixed cells, use glass coverslips; for live cell imaging, use Lab-Tek chambers (1–8 well formats). Alternatively, cells can be cultured and transfected on plastic dishes and subsequently detached and replated on glass coverslips or Lab-Tek chambers.

2. The next day, transfect target cells with plasmid encoding SNAP-tag fusion protein according to transfection reagent manufacturer’s directions.

Incubation for 8–24 h at 37°C is usually sufficient to observe protein expression.

3. Add BG-labeled probe to a final concentration of 1–5 μM.

Commercially available probes from NEB are usually dissolved to 0.6–1 mM in DMSO. The concentrations of custom-made probes are usually higher and should be diluted accordingly. For cells on coverslips, probe can be diluted in media and spotted onto parafilm. Coverslips are then placed upside down for the labeling period.

4. Cells are placed in the incubator for 5–30 min.

Labeling at 37°C for as little as 1 min is often sufficient. Alternatively, cells can be labeled at 4°C for 30 min. In this case, add HEPES buffer to 20 mM to the medium. Labeling is slightly reduced at low temperatures, but uptake of the SNAP-tag fusion via endocytosis is blocked.

5. Rinse cells 3X with media, HBSS or PBS.

This is usually sufficient to remove unreacted probe. For staining with cell-permeable probes, more extensive washing is usually necessary (see Alternate protocol 1).

6. (Optional) For doing “pulse-chase” experiments, any remaining unlabeled SNAP-tag fusion can be blocked using nonfluorescent cell permeable or impermeable BG substrates.

Using in-gel detection experiments, we have found that preincubation with a 20 fold molar excess of unlabeled blocking reagent is sufficient to prevent subsequent labeling of SNAP-tag fusions. Cell impermeable blocking agents react with unlabeled SNAP-tag fusions on the plasma membrane, whereas cell permeable agents react with fusion proteins throughout the cell, including the secretory pathway.

7. (Optional) Return the cells to the incubator to “chase” the labeled SNAP-tag fusion as it is internalized from the plasma membrane into the cell by endocytosis.
8. (Optional) Fix cells with either 2–3.7% formaldehyde/PBS for 10–15 min at room temperature or with 100% methanol for 5 min at –20°C. Rinse cells with PBS, and then mount onto glass slides.

SNAP-tag probes are compatible with a number of fixatives, including formaldehyde and methanol. Labeled cells can subsequently be permeabilized and the SNAP-tag protein colocalized with markers of interest.

9. Image the cells using an appropriate filter set. SNAP-tag fusion proteins labeled with SNAP-Surface Alexa Fluor 488 should have an excitation maximum at 496 nm and an emission maximum at 520 nm, and can be imaged with standard fluorescein filter sets.

Alternate Protocol 1. Site-specific labeling of intracellular SNAP-tag fusion proteins

In this protocol, labeling of SNAP-tag fusions with the SNAP-tag localized to intracellular compartments (nucleus, mitochondria, cytosol, etc.) is described. The essential features of the Basic Protocol are retained, although, in this case, cell permeable BG probes are required, or alternatively, cell impermeable probes can be microinjected (Keppler et al., 2006) or bead loaded (Maurel et al., 2010).

Materials

Cell permeable BG probe (e.g. SNAP-Cell TMR Star, NEB #S9105S). Other probes from NEB include SNAP-Cell 505, SNAP-Cell Oregon Green, and SNAP-Cell Fluorescein.

While the majority of SNAP-tag substrates are BG derivatives, the SNAP-tag is also able to recognize chloropyrimidine (CP) derivatives. SNAP-Cell TMR-Star is one such example.

1. Plate and transfect cells as in the Basic Protocol 1.
2. Add BG-labeled probe to a final concentration of 1–5 μ M. Label for 5–30 min at 37°C.
3. Wash the cells three times with tissue culture medium with serum or HBSS and incubate in fresh medium for 30 minutes. This allows any unconjugated probe to diffuse out of the cell. Replace the medium one more time to remove unreacted SNAP-tag substrate that has diffused into the medium.

Background staining using SNAP-Cell TMR Star can be a problem in some cell types (e.g. HeLa), whereas little background is observed in others (e.g. COS). This background often consists of vacuolar accumulation of unconjugated BG dye. For fixed cells, this can be eliminated by methanol fixation. For live cells, reducing the labeling time, concentration of probe, or increasing the number of washes during the incubation may reduce background.

4. Image the cells using an appropriate filter set. SNAP-tag fusion proteins labeled with SNAP-Cell TMR-Star should have an excitation maximum at 554 nm and an emission maximum at 580 nm, and can be imaged with standard rhodamine filter sets.

Support Protocol 1. Releasable SNAP-tag probe for studying endocytosis and recycling

In this protocol, a SNAP-tag probe has been designed that incorporates a reducible disulfide bond between the BG moiety and fluorescent label (Fig. 2) (Cole and Donaldson, 2012). Following labeling of SNAP-tag fusions at the cell surface, endocytosis is allowed to occur for varying lengths of time. A cell impermeable reducing agent, tris (2-carboxyethyl) phosphine (TCEP), is then added that rapidly and effectively releases the fluorophore from the labeled SNAP-tag into the surrounding medium. This reduces background surface staining, and allows SNAP-tag fusion proteins within endosomes to be clearly visualized (see Video).

Materials

Cell impermeable BG-S-S-Alexa Fluor 488 (For probe synthesis, see Cole and Donaldson, 2012).

TCEP (Sigma or Thermo Scientific) (A 500 mM stock is prepared in water, then adjusted to pH ~7 with NaOH)

1. Plate and transfect cells as in the Basic Protocol 1.
2. Add BG-S-S-Alexa Fluor 488 to a final concentration of 1–5 μ M. Label for 5 min to several hours at 37°C.

Continuous labeling for extended periods at 37°C allows SNAP-tag labeled fusions to be internalized via endocytosis. Control experiments on cells not expressing the SNAP-tag fusion should be tested to rule out uptake of the unconjugated BG probe by fluid phase endocytosis. If this is detected, reduce the concentration of probe. For cell surface only labeling or for “pulse-chase” studies, cells can be labeled at 4°C for 30 min.

3. Add TCEP to 10 mM final concentration for 1–3 min at 37°C.

TCEP works well in complete medium with serum. Alternatively, cells can be washed with HBSS or PBS prior to TCEP treatment. Cells can also be fixed in formaldehyde before TCEP treatment, although it is generally less effective.

4. (Optional) For live cell imaging by confocal microscopy, a continuous time series is collected prior to and after TCEP treatment. For quantitation of endocytosis, images of the same cells before and after TCEP are collected and fluorescence image intensity measured (using imaging analysis software, e.g. Metamorph). The ratio of total cell fluorescence after TCEP versus before TCEP reflects the fractional uptake. Microscope settings should not change during imaging.

Reduction of the releasable probe with TCEP is usually complete by 30 sec-1 min at 37°C. This can be determined experimentally by measuring total cell surface fluorescence in cells in which endocytosis is blocked or is minimal (e.g. with a number of G protein coupled receptors in the absence of agonist). The time for which fluorescence is no longer reduced reflects the maximal amount of probe reduction. Remaining fluorescence may represent uncleaved probe and/or background staining and should be subtracted from all measurements.

5. (Optional) To image recycling of internalized labeled SNAP-tag fusions in live cells, continue imaging in TCEP-containing media for up to 20 min., acquiring images every 1 min.

Loss of signal over time represents delivery of internalized protein back to the cell surface where the probe is cleaved by TCEP. For quantitation of recycling, images of the same cells during the time course are collected and fluorescence image intensities measured (Metamorph or other software). The loss of total cell fluorescence is a measure of protein recycling. Microscope settings should remain unchanged during image acquisition. Measuring total fluorescence in cells not treated with TCEP can test signal loss due to photobleaching. Cells treated with TCEP often show signs of rounding after ~20 min. Washing out the TCEP and/or adding an excess of a TCEP substrate, such as oxidized glutathione, can offset this. The resulting reduced glutathione product is not effective in reducing the BG-S-S-Alexa Fluor 488 probe.

6. (Optional) Fix cells with either 2–3.7% formaldehyde/PBS for 10–15 min at room temperature or with 100% methanol for 5 min at –20°C. Rinse cells with PBS, and then mount onto glass slides.
7. Image the cells using an appropriate filter set. SNAP-tag fusion proteins labeled with BG-S-S-Alexa Fluor 488 should have an excitation maximum at 496 nm and an emission maximum at 520 nm, and can be imaged with standard fluorescein filter sets.

Basic Protocol 2. Pulse-Chase labeling of SNAP-Tag fusions detected by In-gel fluorescence

The covalent nature of the bond between SNAP-tag fusions and fluorescent substrates allows for in-gel detection of bound fluorophores under denaturing conditions by SDS-PAGE. This has been an invaluable benefit for the evolution of SNAP-tag mutants and development of new BG-based probes (Kobayashi et al., 2012; Mollwitz et al., 2012; Sun et al., 2011; Zhang et al., 2011). In this protocol, analysis by SDS-PAGE of the turnover of

plasma membrane SNAP-tag fusions labeled with BG conjugated to infrared probes is described (see Fig. 3).

Materials

BG-800 (synthesized from BG-NH₂ and IRDye® 800CW NHS (LI-COR), see Support Protocol 2)

12 or 24-well tissue culture treated plates (e.g. Falcon; Costar)

Cell lysis buffer (50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 1 % Triton X-100), containing protease inhibitors (Complete tablets, Roche) and 20 µM unlabeled BG-NH₂.

Coomassie blue R250 (Sigma)

Polyacrylamide gels (e.g. Novex® Tris-Glycine, Invitrogen) and SDS-PAGE apparatus

Infrared fluorescence scanner (Odyssey, LI-COR Biosciences)

In this protocol, detection of IRDye® 800CW is with an Odyssey infrared scanner. Depending on the fluorophore used, gels can be scanned using a number of commercially available imagers (e.g. Typhoon, GE Healthcare; Pharos FX, Biorad) using appropriate lasers and filter sets.

1. Plate and transfect cells as in the Basic Protocol 1.

For turnover measurements, we have used 12 or 24-well dishes, with each well expressing equivalent amounts of labeled SNAP-tag fusion protein. To ensure this, rather than transfect wells separately, we plate cells and perform a single transfection in a 10 cm dish. After 16 h, cells are released from the dish with 10 mM EDTA/PBS. The cells are then spun down and replated in equal numbers in the multi-well dishes. They are then incubated for an additional 16–24 h before labeling with the BG-800 probe. Alternatively, stable cell lines can be generated and directly plated into multi-well dishes.

2. Add BG-800 to a final concentration of 1–5 µM. Label for 30 min at 4°C.

Alternately, cells can be labeled for shorter times (5–15 min) at 37°C during which internalization by endocytosis can occur.

3. Rinse the cells 3X with HBSS.

4. Add media containing unlabeled BG-NH₂ (20 µM final) to bind to any unreacted SNAP-tag fusion.

BG-NH₂ is cell permeable and will react with SNAP-tag fusions throughout the secretory pathway. For experiments in which a second population of fusion molecules delivered to the surface is to be labeled (i.e. via exocytosis), a cell impermeable blocking agent (SNAP-Surface Block, NEB #S9143S) should be used.

5. Place dish at 4°C for 15 min.

6. For the “pulse” rinse the wells for the 0 time point (we usually do this in triplicate wells) 2X with PBS.

7. Add 0.1–0.25 ml lysis buffer to each well of the 0 time point. Scrape cells into microcentrifuge tubes and keep at 4°C.

8. Return the dishes to the incubator for the “chase”.

9. At selected time points (e.g. 2, 4, 8, 16 h), repeat steps 6–8.
10. For each time point, after lysis for 5 min at 4°C, spin the microcentrifuge tubes at 13,000 × g for 5 min to pellet nuclei and insoluble material. Place the supernatant into a fresh tube, then place at –20°C until all of the samples have been collected.
11. For SDS-PAGE, add denaturing sample buffer containing reducing agent (e.g. DTT), heat to 95°C for 3 min, then load onto gel (see Chapter 10, Unit 10.1 for electrophoresis details).
12. After electrophoresis, scan gel directly on infrared scanner at the appropriate wavelength (e.g. 800 nm for the IRDye® 800CW dye).

The only band that should be detected is that from the SNAP-tag fusion. The signal should decrease during the “chase”, reflecting protein turnover. Interestingly, we have observed that a proportion of several labeled SNAP-tag fusions are shed into the medium during the chase. This likely reflects the activity of “shedases”, and is a property of various cell surface proteins and not due to addition of the SNAP-tag. For measuring protein turnover, this should be a consideration.

13. Signal intensities from each gel are quantified by using the LI-COR/Odyssey infrared image system, or images can be exported for analysis using other software packages.
14. (Optional) Fix and stain the gel with Coomassie R250 (see Chapter 10, unit 10.5 for fixation and staining protocols)

One reason for using an 800 nm dye for in-gel SNAP-tag labeling experiments is that Coomassie-stained proteins exhibit strong infrared fluorescence when excited in the 700 nm channel, with little to no spectral bleed through into the 800 nm channel (Luo et al., 2006) (LI-COR website: www.licor.com). By selecting a single or multiple Coomassie-stained bands from the 700 nm channel, the amount of protein loaded within each well of the gel can be normalized. Thus, total protein assays (e.g. BCA) or western blotting with antibodies to cellular proteins (e.g. actin) are not necessary.

Alternate Protocol 2. Labeling cell surface proteins with cell-impermeable BG-PEG-Biotin probes

A traditional method for labeling plasma membrane proteins, for example to study their turnover, is to non-specifically biotinylate proteins on the cell surface with amine-reactive biotin-NHS esters. Labeled proteins can then be purified by immunoprecipitation with specific antibodies followed by detection with streptavidin-conjugated probes. In the protocol described here, cell surface SNAP-tag fusions are specifically labeled with a cell impermeable BG-PEG₁₂-biotin probe, and then isolated by pull-down with streptavidin agarose. Not only does this result in quantitative recovery of biotinylated SNAP-tag proteins from cell lysates, but also it has allowed us to identify interacting partners for several cell surface membrane proteins. Scale-up of this protocol can potentially be effective for proteomic analysis.

Materials

BG-NH₂ (Toronto Research Chemicals)

EZ-Link NHS-PEG₁₂-Biotin (Thermo Scientific)

Streptavidin agarose resin (Thermo Scientific)

Blocking Buffer (LI-COR) or other western blocking buffer

PBS-T (PBS containing 0.1% Tween-20)

NeutrAvidin, DyLight 800 conjugate (Thermo Scientific)

700 nm conjugated species-specific secondary antibodies (Invitrogen; Li-COR)

10 cm dishes or 6-well plates (Falcon, Costar, etc.)

- 1 Conjugate BG-NH₂ to EZ-Link NHS-PEG₁₂-Biotin to synthesize the BG-PEG₁₂-biotin SNAP-tag substrate.

See the Support Protocol 2 below for reactions between BG-NH₂ and NHS esters. Also see the Thermo Scientific website:
www.thermoscientific.com

- 2 Plate cells onto 10 cm or 6-well dishes.

- 3 Transfect with SNAP-tag fusion constructs (See Step 2, Basic protocol 1)

- 3 Add BG-PEG₁₂-biotin to a final concentration of 1–5 μM.

- 4 Incubate cells at 4°C to biotinylate the SNAP-tag fusion at the cell surface.

Alternatively, add the probe for extended periods of time at 37°C to allow the labeled protein to undergo endocytosis. We have observed little to no labeling of cytosolic SNAP-tag fusions with the BG-PEG₁₂-biotin probe, even after 2 hr of incubation. Thus, the BG-PEG₁₂-biotin probe is effectively membrane impermeable.

- 5 Incubate cells with an excess of unlabeled BG blocking reagent (e.g. 20 μM BG-NH₂) for 15 min at 4°C.

- 6 Wash the cells 2X with PBS.

- 7 Lyse cells in 1 ml lysis buffer, containing protease inhibitors and 20 μM unlabeled BG-NH₂. Scrape cells into microcentrifuge tube and keep at 4°C.

- 8 After lysis for 5 min at 4°C, spin the microcentrifuge tube at 13,000 × g for 5 min to pellet nuclei and insoluble material. Place the supernatant into a fresh tube.

- 9 Add 40 μl 1:1 slurry of streptavidin agarose beads.

- 10 Rotate end-over-end at 4°C for 1 h.

- 11 Pellet beads by centrifuging at 5,000 rpm for 30 sec in a microfuge.

Save the supernatant to check for the effectiveness of the pull-down.

- 12 Wash the beads 3-4X in lysis buffer or PBS.

- 13 For SDS-PAGE, remove the last traces of wash buffer and add denaturing sample buffer containing reducing agent (e.g. DTT), heat to 100°C for 3 min, then load precipitated sample and an aliquot of the remaining cell lysate onto gel (see Chapter 10, Unit 10.1 for electrophoresis details).

- 14 After electrophoresis, transfer proteins to nitrocellulose (or PVDF) membranes (See Chapter 10, Unit 10.7).

- 15 Block membrane with blocking buffer for 30 min at room temperature.

- 16 Rinse membrane 3X in PBS-T.
At this point, primary antibodies to suspected interacting proteins can be incubated with the membrane for 1 h at room temperature in PBS-T. Following this incubation, blots should be washed 3X over 15 min with PBS-T.
- 17 Incubate with NeutrAvidin, DyLight 800 conjugate (1:20,000 dilution) for 15 min-1 h at room temperature with rocking.
Species-specific 700 nm-conjugated secondary antibodies (1:10-20,000 dilution) can be included to detect any interacting proteins. In addition, there are 4 endogenously biotinylated carboxylases in mammalian cells, one (acetyl-CoA carboxylase, ~260 kDa) in the cytosol, and 3 (propionyl-CoA carboxylase, ~73 kDa; methyl-crotonyl-CoA carboxylase, ~75 kDa; and pyruvate carboxylase, ~125) in the mitochondrial matrix. These will be detected with NeutrAvidin or Streptavidin conjugates. Thus, for distinguishing endogenous versus SNAP-tag biotinylation, include one sample on the SDS-PAGE gel from cells not incubated with BG-biotin ligands.
- 18 Rinse 3X with PBS-T over 15 min. Rinse 1X with water.
- 19 Image by infrared scanning.

Support Protocol 2. Synthesis of BG-800 substrate

The commercial availability of a number of SNAP-tag building blocks and fluorophores that contain reactive groups makes synthesis of BG-labeled probes straightforward. Depending on the nature of the chemistry, probe synthesis and analysis can be accomplished in a number of hours, often without further purification. In this protocol, small-scale synthesis of BG-800 is described.

Materials

BG-NH₂, (O6-[4-(Aminomethyl)benzyl]guanine (Toronto Research Chemicals # A614425; NEB #S9148S)

IRDye® 800CW N-hydroxysuccinimide (NHS) ester (#929-70020, Licor)

Anhydrous N, N-dimethyl formamide (DMF, Sigma-ALdrich)

Triethylamine (TEA, Sigma)

High-resolution mass spectrometer and UV-Vis absorption

1. Dissolve BG-NH₂ to 20 mM in DMF.
BG-NH₂ does not easily dissolve, so heating to 80–90°C may be necessary.
2. Add BG-NH₂ (1 equivalent) to solid IRDye® 800CW NHS ester (1 equivalent).
3. Add TEA (2 equivalents).
4. Incubate with shaking for 2–16 h at 30°C.
5. Monitor reaction completion by LC-MS.

Free BG-NH₂ and IRDye® 800CW NHS ester should be consumed in the reaction. If further purification is necessary, the solvent can be evaporated

under vacuum. The reaction should then be diluted in 1 ml water/ acetonitrile (9:1) and the product purified by reverse phase HPLC.

6. Label cells expressing SNAP-tag fusions as in Basic Protocol 2 and analyze cell lysate after SDS-PAGE. Alternatively, purified SNAP-tag or SNAP-tag fusion proteins can be labeled *in vitro* and analyzed after SDS-PAGE (see Basic Protocol 3).

Basic Protocol 3. *In Vitro* labeling of SNAP-Tag fusions

This protocol describes labeling of purified SNAP-tag or SNAP-tag fusion proteins, or those from cell lysates, with BG-fluorophores. Analysis of binding is *via* SDS-PAGE and in-gel detection. Other types of detection methods, such as binding-induced changes in probe fluorescence, have been important in the development of quenched or caged BG-probes (Kobayashi et al., 2012; Komatsu et al., 2011; Maurel et al., 2010; Zhang et al., 2011).

Materials

Purified SNAP-tag or SNAP-tag fusion proteins

Purified SNAP-tag protein, as well as a plasmid for generating and expressing SNAP-tag fusions in bacteria, is available from NEB. Alternatively, plasmids can be custom generated and expressed in inducible bacterial systems. Expression and purification of proteins from bacteria is often empirically determined and beyond the scope of this unit (see Chapter 5, Units 5.1 and 5.2). See the NEB or other commercial websites for protocols and troubleshooting tips. SNAP-tag fusion proteins are generally purified before labeling, but the labeling reaction also works in non-purified protein solutions, such as bacterial cell lysates. The SNAP-tag protein or expression vector, as provided by NEB, does not contain an affinity tag, so it should be purified by standard separation methods of protein chemistry (e.g. ion exchange chromatography, gel filtration). If an affinity tag has been introduced into the fusion protein, use protocols adapted for the tag.

Reaction buffer (PBS) or (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20)

Fluorophore-labeled BG-substrate (e.g. SNAP Vista Green (NEB #S9147))

1. Combine purified SNAP protein (5 μ M) with BG conjugate (10 μ M) in reaction buffer. Add DTT or other reducing agent to 1–5 mM.

SNAP-tag stability and reactivity *in vitro* is improved by the presence of a reducing reagent. The presence of chelating reagents such as EDTA should be avoided in the expression, purification, and reaction of the SNAP-tag, as the protein contains a structural Zn^{2+} ion.

2. Incubate at 37°C for 30 min in the dark.

SNAP-tag fusions can also be labeled at lower temperatures (4–37°C). An optimization of various parameters (e.g. labeling time, substrate and protein concentrations) should be carried out.

3. Quench reactions by addition of 6X SDS-PAGE sample buffer and heating for 3 min at 95°C.
4. Analyze by SDS-PAGE followed by fluorescence in-gel scanning.

Image with the appropriate laser and filter sets. For SNAP-tag fusion proteins a band of molecular weight ~20 kDa bigger than the protein of

interest should be seen. Unreacted BG-substrates will run in front of the protein bands in the gel. If the fluorescence from the unreacted substrate interferes with imaging of the protein, the labeled protein can be separated from unreacted substrate by gel filtration prior to SDS-PAGE.

Support Protocol 3. Labeling of SNAP-tag proteins from cell lysates

It is not always necessary to use purified SNAP-tag proteins to assess BG-substrate binding. This protocol describes labeling of mammalian cell lysates that express SNAP-tag fusions.

Materials

Cells expressing SNAP-tag fusion protein (see Basic Protocol 2, step 1)

Tissue culture-treated dishes (35 or 60 mm) or multi-well plates (6 or 12 well) (Falcon; Costar, etc.)

Trypsin (0.25%, Lonza)

Cell lifter (Corning)

Lysis buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20, 1 mM DTT and an EDTA-free protease inhibitor cocktail (e.g., Complete™, Roche) or CelLytic M (Sigma))

1. Rinse cells 2X with PBS.
2. Harvest cells by trypsinization.

Do not use trypsin that contains versene (EDTA), since this may affect SNAP activity (See Basic Protocol 3, step 1). For plasma membrane SNAP-fusions, gently scrape or lift the cells off the dish in PBS. SNAP-tag cell surface fusions may be damaged by trypsin treatment.

3. Gently pellet cells in microfuge (5,000 rpm for 1 min). 3. Wash cells 2X with PBS.
4. Lyse cell pellet in lysis buffer.

Add lysis buffer so that a 20 µl reaction contains between 10⁴–10⁵ cells.

5. Add BG-conjugate to 1–10 µM final concentration.
6. Incubate in the dark for 30 min at 37°C (see Basic Protocol 3, step 2).
7. Add denaturing sample buffer and process for SDS-PAGE and image analysis, as in Basic Protocol 3, steps 3 and 4.

Commentary

Background Information

Fluorescence microscopy is an invaluable method for the visualization of protein function and biochemical activity in living cells. The identification and modification of a broad number of fluorescent proteins (FPs) to genetically tag individual proteins has revolutionized our ability to study protein function in the cell. As a complement to FPs, various technologies have been developed for specific protein labeling with synthetic probes (Jing and Cornish, 2011; Lin and Wang, 2008; O'Hare et al., 2007; Wombacher and Cornish, 2011). These methods rely on the fusion of the protein of interest to a tag that can be covalently labeled with a small molecule probe, thereby combining the simplicity of fusion protein expression with the diversity of molecular probes provided by synthetic chemistries (Gautier et al., 2008; Hinner and Johnsson, 2010; Jing and Cornish, 2011). Some of these peptide or protein tags include: non-enzymatic peptide tags [e.g. FIAsh, (Griffin et

al., 1998)]; enzyme mediated peptide tags [e.g. biotin acceptor peptide (Chen et al., 2005)], and lipoic acid acceptor peptide (Uttamapinant et al., 2010); proximity-induced protein tags [e.g. eDHFR (Cai et al., 2010)]; enzyme mediated protein tags [e.g. acyl carrier protein (George et al., 2004)], and self-labeling protein tags [e.g. SNAP-tag and CLIP-tag (Gautier et al., 2008; Keppler et al., 2003) and HaloTags (Los et al., 2008)].

There are several applications in which the SNAP-tag (and/or CLIP tag) may be advantageous over fluorescent proteins (FPs) and other chemical labeling approaches. These include time-resolved fluorescence, that is the ability to initiate fluorescence at a given time; pulse-chase analysis when used with non-fluorescent BG substrates; the ability to change colors and functionalities on the BG-probe; the ability to specifically label proteins on the cell surface; and the capacity to perform pull down studies without the need for specific antibodies.

The one drawback to all of these systems, including FPs, is a requirement for exogenously expressed fusion proteins. The fusion-tag may not necessarily disrupt the function of the protein to which it is attached, i.e. a cell surface SNAP-tagged receptor may still signal upon adding an agonist. However, detection by all of these methods dictates that the protein needs to be overexpressed, and this may affect pathways that are saturable. For example, we have observed that overexpression of some SNAP-tagged membrane proteins that enter cells *via* a clathrin-independent pathway and normally recycle in long tubules, are also found in numerous vesicular structures that are targeted for degradation. This is not due to the SNAP-tag itself, since overexpression of an untagged version of the membrane protein shows a similar phenotype. Until robust methods for specifically labeling endogenous molecules with small molecule probes are developed, this remains an issue.

Critical Parameters

One of the most critical parameters in SNAP-tag labeling is to achieve a high labeling efficiency with minimal background. For fusion proteins exposed to the cell exterior this is straightforward, since minimal washing steps are all that are required. In published work, “no wash” probes have been developed that contain quencher compounds attached to the probe that are displaced upon SNAP-tag binding, thereby eliminating the need for washing steps, even for live cell imaging (Komatsu et al., 2011). For labeling intracellular SNAP-tag fusions this has been a bit more problematic, since background can be an issue. Reducing substrate concentration and/or incubation time or allowing the final wash step to proceed for longer periods of time may alleviate some of these problems. We have found that cell type has a significant influence on background staining (COS-7, very low; HeLa, significant), and should be a consideration when choosing an experimental system.

For binding using purified proteins or from cell lysates, the presence of chelating reagents such as EDTA should be avoided as the SNAP-tag protein contains a structural Zn^{2+} ion.

Troubleshooting

Low labeling efficiencies can often be overcome simply by adjusting reaction conditions outlined in the various protocols. Incubation times, substrate concentrations, labeling temperatures, can all be varied to obtain an optimal signal.

For plasma membrane proteins, low surface labeling may indicate a failure of the SNAP-tag fusion to reach the plasma membrane. Mis- or low-efficiency folding can result in accumulation of proteins in the endoplasmic reticulum. Staining fixed and permeabilized cells with antibodies to the target protein or to the SNAP-tag (available from Sigma or NEB)

can check this. These antibodies can also be used to check fusion protein expression by SDS-PAGE and immunoblotting.

It also may be useful to test other types of tagging systems when problems are encountered. For example, we have observed that a releasable BC-S-S-Alexa 488 probe (analogous to the BG-S-S-Alexa 488 used in the SNAP-tag system, Support Protocol 1) is not cleaved by TCEP when it is bound to the CLIP-tag. This probe also shows cross-reactivity with SNAP-tag fusions. For simultaneous double labeling experiments, switching to a releasable HaloTag fusion probe worked well in this case.

Anticipated Results

When cells are imaged by fluorescence microscopy, the localization of the SNAP-tag fusion should be as predicted from the untagged target protein. The specific signal should be high and background low. For purified proteins or proteins from cell lysates, in-gel scanning should show one specific protein band ~20 kDa larger than the untagged protein.

Time Considerations

With proper expression of the SNAP-tag fusion, and depending on the nature of the experiment, labeling can be achieved and cells imaged within 20 min. With labeling of intracellular proteins with cell permeable probes, this will take longer. Once labeled cells are fixed, the fluorescent signal is stable. For purified proteins or proteins from cell lysates, the labeling time can be as short as 15 min, especially using the newer SNAP_F-tag. SDS-PAGE and in-gel scanning should take around 2 hours total. If the gels are to be stained with Coomassie dye, staining and destaining may take 30 min to overnight, depending on the staining method. Labeling conditions vary with the target protein and must be optimized for the particular application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A protein of interest (POI) is fused to the SNAP-tag for expression in cells or *in vitro*. The reaction of the SNAP-tag with O⁶-benzylguanine (BG) derivatives results in the covalent attachment of the label to the active site cysteine.

Figure 2.

Diagram of the releasable SNAP-tag probe, BG-S-S-Alexa 488 (see Cole and Donaldson, 2012). After binding to SNAP-tag fusion proteins on the surface of cells, the fluorophore (e.g. Alexa Fluor 488) can be released from the protein with the cell-impermeable reducing agent tris (2-carboxyethyl) phosphine (TCEP).

Figure 3.

An example of a pulse-chase turnover experiment using a SNAP-CD147 fusion construct. CD147 is a transmembrane cargo molecule that is internalized into cells via clathrin-independent endocytosis (Eyster et al., 2009). Cells are labeled with the cell impermeable probe BG-800 then chased for the indicated times. Cells are lysed and aliquots of the lysate run on SDS-PAGE and imaged by in-gel fluorescence in the 800 nm channel (Odyssey, LI-COR). The turnover of SNAP-CD147 over time can be seen in (A). In (B), the gel was fixed and stained by Coomassie blue R250. It was then imaged by infrared scanning in the 700 nm channel. Quantitation of image intensity from both channels can be used to normalize for protein loading to obtain an accurate reflection of protein turnover. Each time point was done in triplicate. This figure was kindly provided by Darya Karabasheva (Laboratory of Cell Biology/NHLBI/NIH).