CHAPTER

SNAP-Tagging the Retrograde Route

Ludger Johannes* and Massiullah Shafaq-Zadah†

*Institut Curie—Centre de Recherche, Trafic, Signaling and Delivery group, Paris Cedex 05, France
†UMR144 CNRS, Paris, France

CHAPTER OUTLINE

Introduction ............................................................................................................ 140
9.1 Materials ........................................................................................................ 141
9.2 Methods ......................................................................................................... 142
  9.2.1 Cell Surface Modification Reagent ................................................ 142
  9.2.2 Capture Reagent ................................................................................ 144
  9.2.3 Validation of the BG/SNAP-tag Strategy ......................................... 144
     9.2.3.1 SNAP-tag Localization to Golgi Membranes by Immunofluorescence ....................................................................................... 144
     9.2.3.2 SNAP-tag Validation Using STxB ........................................... 145
  9.2.4 BG/SNAP-tag Strategy in Proteomics Format ................................. 147
     9.2.4.1 BG-PEG9-NHS is a Cell Impermeable Amine Reactive Linker ... 147
     9.2.4.2 Proteomics Format ............................................................... 148
  9.2.5 Validation of Retrograde Cargo Candidates—Antibody Uptake ...... 150
     9.2.5.1 BG Tagging of Anti-GFP Antibody ........................................ 150
     9.2.5.2 BG-tagged Antibody Uptake Assay ....................................... 150
9.3 Conclusion/Discussion .................................................................................... 152
Summary ................................................................................................................ 153
Acknowledgments ................................................................................................... 153
References ............................................................................................................. 153

Abstract

We have developed a chemical biology strategy to identify proteins that follow the retrograde transport route from the plasma membrane to the Golgi apparatus, via endosomes. The general principle is the following: plasma membrane proteins are covalently tagged with a first probe. Only the ones that are then transported to
trans-Golgi/TGN membranes are covalently bound to a capture reagent that has been engineered into this compartment. Specifically, the first probe is benzylguanine (BG) that is conjugated onto primary amino groups of plasma-membrane proteins. The capture reagent includes an $O^6$-alkylguanine-DNA alkyltransferase-derived fragment, the SNAP-tag, which forms a covalent linkage with BG. The SNAP-tag is fused to the GFP-tagged Golgi membrane anchor from galactosyl transferase for proper targeting to trans-Golgi/TGN membranes. Cell-surface BG-tagged proteins that are transported to trans-Golgi/TGN membranes (i.e., that are retrograde cargoes) are thereby covalently captured by the SNAP-tag fusion protein. For identification, the latter is immunopurified using GFP-Trap, and associated retrograde cargo proteins are identified by mass spectrometry. We here provide a step-by-step protocol of this method.

INTRODUCTION

The retrograde transport route ensures the trafficking of proteins and lipids from the plasma membrane to the Golgi apparatus, via endosomes, and in some cases on to the endoplasmic reticulum (Bonifacino & Rojas, 2006; Johannes & Popoff, 2008). Retrograde transport was described as an entry gate for protein toxins such as the bacterial Shiga (Johannes & Romer, 2010) and cholera toxins (Lencer & Tsai, 2003), and the plant toxin ricin (Sandvig et al., 2004). Some viral proteins including the HIV-1 envelope protein and the herpes virus glycoprotein M also traffic via this pathway, which thereby contributes to viral infection (Blot, Janvier, Le Panse, Benarous, & Berlioz-Torrent, 2003; Crump et al., 2004; Duncan & Kornfeld, 1988; Snider & Rogers, 1985).

Several endogenous proteins use the retrograde transport route. Historically, the first to be identified were the transferrin and mannose 6-phosphate receptors (Duncan & Kornfeld, 1988; Snider & Rogers, 1985). More recently, the list of retrograde cargoes has greatly increased (for a review, see Bonifacino & Rojas, 2006; Johannes & Popoff, 2008). Striking examples are glucose and copper transporters (Shewan et al., 2003; Voskoboinik & Camakaris, 2002), Wnt morphogen and glutamate receptors (Belenkaya et al., 2008; Coudreuse, Roel, Betist, Destree, & Korswagen, 2006; Yang et al., 2008; Zhang et al., 2012). The discovery of these proteins as retrograde cargoes highlights the importance of retrograde transport for essential cellular functions such as morphogen gradient formation, compartmentalization of ion transporter activity, and postsynaptic activity in neurons. Yet, no systematic study has ever been performed to identify retrograde cargoes.

We have taken advantage of the SNAP-tag system to develop a vectorial proteomics approach that should enable such systematic analysis with the ultimate goal of determining the retrograde proteome in different cell types and physiological or pathological situations (Shi et al., 2012) (Fig. 9.1). The SNAP-tag is derived from the human DNA repair protein $O^6$-alkylguanine-DNA alkyltransferase. It mediates the covalent linkage of $O^6$-benzylguanine (BG) to a specific residue of the protein, even when $O^6$-BG is itself conjugated to other chemical entities (Keppler et al., 2003).

Our vectorial proteomics approach involves the following steps (Fig. 9.1): (i) Cell surface proteins are modified with BG. (ii) BG surface-modified cells are incubated at
37 °C to allow for retrograde transport to occur. (iii) At the TGN, BG-labeled plasma membrane proteins that have undergone retrograde transport react with a SNAP-GFP construct that has been positioned in this compartment by fusion with a cytosolic and transmembrane domain fragment of galactosyl transferase (GalT). (iv) The conjugates between BG-tagged retrograde cargoes and GalT-GFP-SNAP fusion protein are collected by immunopurification and identified by mass spectrometry.

Here, we expose the experimental details of this approach. Furthermore, we discuss its limitations, and describe how hits (i.e., retrograde cargo candidates) can be validated.

### 9.1 MATERIALS

#### PLASMIDS
- pGalT-EGFP vector (Frank Perez, UMR144 CNRS, Curie Institute, Paris, France)
- pSNAPm (New England Biolabs)

#### ANTIBODIES
- Rabbit antigiantin from Curie Institute Antibody Platform, reference A-R-R#05
- Secondary Cy3-labeled goat antirabbit (Reference 111-166-045, Jackson ImmunoResearch)
- Mouse anti-GFP antibody (Reference 11814460001, Roche) supplied in 50 mM sodium phosphate buffer, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA)
- Rabbit anti-SNAP-tag antibody (Reference P9310S, NEB), supplied in 10 mM HEPES, pH 7.5, 150 mM NaCl, 100 μg/ml bovine serum albumine (BSA), and 50% glycerol
- Mouse anti-STxB antibody (clone 13C4, purified from hybridoma, ATCC cat. no. CRL 1794)
- Rabbit anti-STxB antibody (homemade polyclonal)

**BUFFERS**
- PBS (phosphate-buffered saline): in 1 l distilled water dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, adjust pH to 7.4 with HCl
- PBS⁺⁺: PBS containing 130 mM Ca²⁺ and 200 mM Mg²⁺
- TNE buffer: 5 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.0
- TNE lysis buffer: TNE buffer containing 1% NP-40 (Sigma-Aldrich) and protease inhibitor cocktail (1 mg/ml of each aprotinin, leupeptin, antipain, pepstatin, 1 M benzamidine, and 40 mg/ml phenylmethanesulfonyl fluoride in DMSO)

**IMMUNOPRECIPITATION**
- Protein G-Sepharose® Fast Flow beads supplied in 20% ethanol (Reference P3296, Sigma-Aldrich)
- GFP-Trap®_A beads supplied in 20% ethanol (Reference GTA-20, Chromotek)

**SNAP-TAG REAGENTS**
- SNAP-Cell® TMR-Star (Reference S9105S, NEB), stock solution at 0.6 mM in DMSO (Reference D2650, Sigma-Aldrich)
- SNAP-Cell™ Block stock solution at 2 mM in DMSO (Reference S9106S, NEB)
- BG-NHS stock solution at 4 mM in DMSO (Reference S9151S, NEB)
- BG-PEG₉-NHS obtained as described in Shi et al. (2012), stock solution at 100 mM in DMSO

**IMMUNOFLUORESCENCE**
- Fixation: 4% paraformaldehyde (PFA), in PBS
- Quenching: 50 mM NH₄Cl in PBS
- Permeabilization: 0.2% saponin (Reference 102855, MP Biomedicals), 2% BSA (Reference 04-100-811-C, Euromedex) in PBS
- Mounting: 0.13 g/ml mowiol, 0.33 g/ml glycerol, 0.2 M Tris/HCl, pH 8.5

**9.2 METHODS**

**9.2.1 Cell surface modification reagent**
The cell surface modification reagent is composed of the following elements (Fig. 9.2A): BG moiety for TGN capture (blue), polyethylene glycol (PEG) linker arm to render the reagent membrane impermeable (red), and primary amino-reactive N-hydroxysuccinimidyl (NHS) moiety for modification of cell surface proteins
This reagent is termed BG-PEG9-NHS. The chemical synthesis of this reagent is achieved as follows: BG-NH2 (78.2 mg, 0.29 mmol; Fig. 9.2A) is obtained according to published procedure (Keppler et al., 2004), dissolved under argon in anhydrous dimethylformamide (10 ml), and successively added to commercially available bis-succinimide ester-activated PEG compound BS(PEG)9 (266.9 mg, 0.38 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (78.3 mg, 0.41 mmol), and hydroxybenzotriazole (55.4 mg, 0.41 mmol) solution at $-5^\circ C$. The solution is

...
stirred at room temperature for 6 h. The solvent is removed under reduced pressure and the product is purified by preparative high-performance liquid chromatography (HPLC) (Shi et al., 2012).

NHS is an amino-specific functionality that covalently reacts with cell surface proteins, preferentially via primary amines on the side chains of lysine residues (Fig. 9.2B). This reaction can be achieved on living cells on ice, conditions under which endocytosis is inhibited. The NHS moiety transfers other chemical labels onto amino acceptors (here: plasma membrane proteins) if these are part of the same molecule. In the context of the current vectorial proteomics system, NHS is linked to BG for the reaction with the trans-Golgi/TGN-localized capture reagent (see below). Both NHS and BG can diffuse across membranes, and would thereby spontaneously (i.e., independent of retrograde transport) react with the capture reagent. We have therefore introduced nine PEG repeats into the molecule to render it hydrophilic, yielding BG-PEG₉-NHS (Fig. 9.2A). The lack of membrane permeability of this reagent is described in Section 9.3.4.

9.2.2 Capture reagent

The capture reagent is molecularly designed to be localized on trans-Golgi/TGN membranes. This is achieved by fusing the SNAP-tag to a fragment from GalT. GalT is a single pass membrane protein that is localized at the trans-cisternae of the Golgi apparatus and on TGN membranes (Roth & Berger, 1982). We use an existing pEGFP-GalT-GFP vector to obtain the GalT-GFP fragment, which is then inserted into the pSNAPm vector (New England Biolabs, NEB), yielding pGalT-GFP-SNAP. The GFP-tag allows for localization of the fusion protein by immunofluorescence microscopy, and its enrichment using GFP-Trap for proteomics.

As mentioned above, BG-tagged plasma membrane proteins that are shuttled to trans-Golgi/TGN membranes (putative retrograde cargoes, termed here ProtX) covalently react with the GalT-GFP-SNAP fusion protein, through the transfer of the benzyl group onto an internal cysteine residue within the SNAP-tag, yielding after the release of the guanine base the conjugate GalT-GFP-SNAP-ProtX (Fig. 9.1).

9.2.3 Validation of the BG/SNAP-tag strategy

In this section, we describe the step-by-step validation of the BG/SNAP-tag strategy on a stable HeLa cell line expressing the GalT-GFP-SNAP fusion protein. This cell line is termed HeLa-GGS in the following sections.

9.2.3.1 SNAP-tag localization to Golgi membranes by immunofluorescence

$4 \times 10^5$ HeLa-GGS cells are seeded on glass coverslips in four-well plates 1 day prior to the experiment. The cells are cultured at 37 °C in complete DMEM medium (containing 10% fetal calf serum) in a 5% CO₂ incubator, washed three times with PBS⁺⁺ (PBS containing 130 mM Ca²⁺ and 200 mM Mg²⁺), and then fixed for 10 min at room temperature with PFA (4% in PBS). PFA is quenched by incubation of cells
for 10 min at room temperature with 50 mM NH₄Cl prepared in PBS. Cells are permeabilized for 30 min at room temperature with 0.2% saponin in a solution of 2% BSA in PBS. Primary (here: rabbit antigiantin antibody from Curie antibodies platform, Reference A-R-R#05) and secondary antibodies (here: Cy3-labeled goat antirabbit from Jackson ImmunoResearch) are appropriately diluted in saponin–BSA–PBS solution and incubated with cells for 30–45 min. After three washes with saponin–BSA–PBS (5 min each), coverslips are dipped into water and mounted on Mowiol.

Confocal microscopy analysis reveals a consistent codistribution of GaLT-GFP-SNAP (green)– and giantin (red)–specific labeling, strongly indicating that the GaLT-GFP-SNAP fusion protein is indeed correctly localized in Golgi membranes (Fig. 9.3A).

To validate the enzymatic activity of the SNAP-tag in the context of the GaLT-GFP-SNAP fusion protein, 4 × 10⁵ HeLa-GGS cells are incubated for 15 min at 37 °C in complete DMEM medium with 3 μM TMR-Star (0.6 mM stock solution in DMSO, NEB), a rhodamine-labeled membrane permeable BG derivative. TMR-Star labeling (red) colocalizes with green GFP fluorescence in a perinuclear location (Fig. 9.3B), indicating that BG-SNAP reaction occurs on living cells within the membranes of the Golgi apparatus.

### 9.2.3.2 SNAP-tag validation using STxB

A model cargo of the retrograde route, the B-subunit of Shiga toxin (STxB) (Johannes & Romer, 2010), is chosen to demonstrate that the BG-SNAP reaction can also occur when BG is chemically linked onto the surface of a protein. STxB binds to its cellular receptor, the glycosphingolipid Gb3, is internalized by clathrin-dependent and independent endocytosis, transported from early endosomes to the TGN, Golgi cisternae, and the endoplasmic reticulum (Johannes & Romer, 2010; Sandvig, Torgersen, Engedal, Skotland, & Iversen, 2010).

STxB is purified according to established procedures (Mallard & Johannes, 2003). Purified STxB (1 mg/ml final concentration in PBS) is incubated for 2 h at room temperature with BG-NHS (4 mM stock solution in DMSO, NEB) at a molar ratio of 1:3. Excess BG-NHS is removed by overnight dialysis at 4 °C against PBS, using 10 kDa molecular weight cut-off point dialysis cassettes (Thermo Scientific). HeLa-GGS cells are then incubated with 1 μM STxB-BG (or STxB as a negative control) for 30 min on ice in complete DMEM medium (membrane-binding step), washed, and shifted for 4 h to 37 °C (intracellular transport).

For immunofluorescence analysis, cells are washed with PBS³⁺⁺, fixed with PFA, permeabilized with saponin, and labeled with anti-STxB antibody (clone 13C4), as described above. Confocal microscopy is used to analyze the localization pattern of wild-type STxB or STxB-BG in comparison to the GaLT-GFP-SNAP fusion protein.

STxB is in part localized after 4 h in a perinuclear site and overlaps with the GaLT-GFP-SNAP fusion protein (likely in Golgi membranes), and in part found in a peripheral network, likely the endoplasmic reticulum (Fig. 9.3C). Such distribution pattern between Golgi and endoplasmic reticulum has already been described in earlier studies.
In contrast, STxB-BG exclusively localizes in a perinuclear site with GalT-GFP-SNAP fusion protein (Fig. 9.3D). This differential behavior clearly demonstrates that BG-tagged STxB is retained in the perinuclear Golgi area, likely because of a reaction with the GalT-GFP-SNAP fusion protein.

(Johannes, Tenza, Antony, & Goud, 1997).
The formation of such a GalT-GFP-SNAP-STXB reaction product is tested by immunoprecipitation. HeLa-GGS cells are continuously incubated for 4 h at 37 °C in complete DMEM medium with 1 μM of either unmodified STxB, or BG-STxB. The cells are then washed three times with DMEM to eliminate free ligands. Prior to cell lysis, unreacted GalT-GFP-SNAP fusion protein is quenched to prevent its reaction with STxB-BG in the cell lysate. The membrane permeable quenching reagent is termed SNAP-Cell-Block (2 mM stock solution in DMSO, NEB). Quenching is performed by incubation of cells for 20 min at 37 °C with 10 μM SNAP-Cell-Block in complete DMEM medium.

Cells are then lysed at 4 °C by a 30-min incubation under agitation on a rotating mixer with TNE buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA) containing 1% nonyl phenoxypolyethoxylethanol (NP-40). Cells are scrapped off the culture dish using a 1-ml end-cut tip, and the cell lysate is cleared of debris and DNA by a 13,200-rpm centrifugation for 10 min at 4 °C in an Eppendorf table top centrifuge. For immunoprecipitation, GFP-Trap beads (Chromotek), which recognize GFP with high affinity, are washed three times at 4 °C with TNE buffer, including bead collection by centrifugation for 3 min at 4600 rpm at the same temperature. 30 μl of beads are used for immunoprecipitation from 4 × 10^5 HeLa-GGS cells. The washed beads are suspended in cell lysate from above, and incubated overnight at 4 °C by end-over-end rotation. After three rapid washes at 4 °C in TNE buffer, excess washing buffer is removed using a Hamilton syringe, and beads are suspended in 3 × sample buffer (2 M Tris/HCl, pH 6.8, 20% SDS, 30% glycerol, 0.03% phenol red), and heated for 5 min at 95 °C. The protein eluate from the beads is separated by electrophoresis on 10% SDS-PAGE gels, and immunoblotted using rabbit anti-STxB antibodies (homemade rabbit polyclonal).

A protein species of the expected size of the GalT-GFP-SNAP-STxB reaction product can be detected only when cells are incubated with BG-tagged STxB, and not with untagged STxB (Fig. 9.3E) The inhibition of retrograde transport using specific small molecule tools (i.e., Retro-2 from Stechmann et al., 2010) or the disassembly of the Golgi apparatus using brefeldin-A prevent the formation of this reaction product (Shi et al., 2012), strongly indicating that the encounter of STxB-BG and GalT-GFP-SNAP indeed occurs in trans-Golgi/TGN membranes.

9.2.4 BG/SNAP-tag strategy in proteomics format

In this section, we describe the application of the SNAP-tag/BG reaction scheme to the proteomics analysis of the retrograde route.

9.2.4.1 BG-PEG₉-NHS is a cell impermeable amine reactive linker

To covalently label cell surface proteins with BG, we use a modified version of BG-NHS, termed NHS-PEG₉-BG (Fig. 9.2A). The PEG₉ unit is intended to increase the hydrophilicity of the compound, and thereby prevents its spontaneous diffusion across membranes, which would lead to the quenching of the TGN-localized SNAP-tag of the GalT-GFP-SNAP fusion protein. The membrane impermeability of NHS-PEG₉-BG is documented as follows.
HeLa-GGS cells are incubated for 1 h at 4 °C with 4 mM BG-NHS, 4 mM NHS-PEG9-BG, or 10 μM SNAP-Cell-Block, all in PBS++ buffer, washed three times with the same buffer, and shifted for 15 min to 37 °C in complete DMEM medium. After this primary incubation, cells are again incubated in DMEM for 30 min at 37 °C with TMR-Star (NEB), a membrane permeable compound in which BG is linked to a red fluorophore, 6-carboxytetramethylrhodamine. This molecule reacts with the trans-Golgi/TGN-localized GalT-GFP-SNAP only under conditions under which the BG derivatives of the prior incubation have not reached this compartment (i.e., compounds that have been rendered sufficiently hydrophilic such that they cannot diffuse passively across membranes). After washing (three times, as described above), cells are lysed in TNE buffer with 1% NP-40, and postnuclear supernatents from cell lysates are loaded onto 10% SDS-PAGE gels, followed by direct visualization using a fluorescence scanner (Typhoon 9400, GE).

In control cells (i.e., cells that are not treated with BG-modified compounds during the primary incubation), a fluorescent band is detected at the expected size of GalT-GFP-SNAP (Fig. 9.4, lane 2; see arrow). For cells that are incubated during the primary phase with the SNAP-Cell-Block reagent, this band cannot be detected (lane 3), confirming that this compound indeed diffuses passively across membranes and quenches GalT-GFP-SNAP, such as to prevent the secondary modification by TMR-star. This condition thereby defines the maximal level of inhibition. A similar level of inhibition is observed with BG-NHS (lane 4), showing that this compound is also fully membrane permeable. In contrast, NHS-PEG9-BG does not quench SNAP-tag reactivity on GalT-GFP-SNAP during the primary incubation (lane 5), demonstrating that this compound is not membrane permeable.

### 9.2.4.2 Proteomics format

4 × 10⁶ HeLa-GGS cells are incubated for 30 min at 4 °C with 1 μM STxB in complete DMEM medium, which leads to STxB binding to cells via its glycolipid receptor Gb3 with high apparent affinity in the nanomolar range (Johannes et al., 1997). Cells are then washed in the same medium, and incubated for 1 h at 4 °C with 1 mM NHS-PEG9-BG in PBS++ buffer, to BG-modify plasma membrane proteins. Cells are washed and incubated in complete DMEM medium for 10 h at 37 °C to allow for internalization of BG-tagged cell surface proteins.

Further treatments are as described above in Section 9.3.3.2: Quenching using SNAP-Cell Block reagent, cell lysis in TNE/NP-40 buffer, GFP-Trap, and SDS-PAGE. Proteins are then submitted to in-gel digestion.

For this, the SDS-PAGE gel is stained using colloidal blue; migration lanes are excised and subjected to 10 mM DTT in 100 mM ammonium bicarbonate (1 h at 60 °C) for reduction prior to alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (45 min at room temperature). Gel pieces are washed with 100 mM ammonium bicarbonate and shrinked by incubation with 100% acetonitrile.
(10 min at room temperature), before being subjected to an overnight trypsin digestion at 30 °C in 25 mM ammonium bicarbonate. Peptide concentration and separation are achieved using an actively split capillary HPLC system (Ultimate 3000 system) connected to the LTQ Orbitrap XL™ mass spectrometer (Thermo Scientific). The resulting spectra are analyzed via the Mascot™ Software created with Proteome Discoverer (version: 1.2.0.92, Thermo Scientific) using the NCBIInr human Protein Database.

STxB is clearly identified among the retrograde cargo candidate proteins (Table 9.1). Other proteins of this list (data not shown) may represent plasma membrane proteins for which trafficking via the retrograde route has previously not been shown. We describe below a method by which such retrograde cargo candidates can be further validated.

From Shi et al. (2012).
9.2.5 Validation of retrograde cargo candidates—Antibody uptake

To confirm the trafficking via the retrograde route of putative retrograde cargo candidates from the proteomics list, we have developed an antibody uptake assay (Fig. 9.5). Here, this protocol is illustrated for a well-characterized cargo of the retrograde route, the cation-independent mannose 6-phosphate receptor (CI-MPR) (Duncan & Kornfeld, 1988). CI-MPR shuttles newly synthesized mannose 6-phosphate-modified lysosomal enzymes from the TGN to endosomes (Brown, Goodhouse, & Farquhar, 1986). After cargo delivery, the empty receptor is retrieved from endosomes to the TGN. A small fraction of CI-MPR is also found at the plasma membrane where it binds to extracellular ligands for subsequent internalization (Lin, Mallet, Huang, & Maxfield, 2004). For our study, we use a stable cell line expressing a GFP-tagged CI-MPR fusion protein (Waguri et al., 2003). An anti-GFP antibody (Roche) specifically recognizes this fusion protein whose GFP part is exposed to the extracellular medium (Fig. 9.5).

The experimental principle is the following: Upon transport of the GFP-CI-MPR fusion protein from the cell surface to the Golgi compartment, the anti-GFP-BG antibody (that will bind to GFP-CI-MPR at the cell surface upon addition to the cell culture medium) is dragged along and reacts with the trans-Golgi/TGN-localized GalT-GFP-SNAP capture reagent once it has reached this compartment (Fig. 9.5).

### 9.2.5.1 BG tagging of anti-GFP antibody

Anti-GFP antibody (Roche) is prepared at a final concentration of 5 mg/ml in 50 mM sodium phosphate buffer pH 7.5, containing 1 mM EDTA. BG-NHS reagent is added in 10-fold molar excess over antibody. This mixture is incubated 6 h at 4 °C prior to dialysis against 50 mM sodium phosphate pH 7.5, 1 mM EDTA, using 10 kDa molecular weight cut-off (MWCO, Thermo Scientific) dialysis cassettes. Additional quenching of the BG-NHS linker can be performed by adding 20 mM Tris (containing primary amine) for 20 min at room temperature prior to dialysis.

### 9.2.5.2 BG-tagged antibody uptake assay

4 × 10^5 MPR-GFP-expressing HeLa cells transiently transfected with pGalT-GFP-SNAP in 6-well culture plates, are incubated for 4 h at 37 °C with 15 μg/ml of unmodified or BG-tagged anti-GFP antibodies in complete DMEM medium. Cells

---

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Size (aa)</th>
<th>MW (Da)</th>
<th>Matching peptides</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalTGFP</td>
<td>366</td>
<td>39608</td>
<td>11</td>
<td>28.1</td>
</tr>
<tr>
<td>SNAP-tag</td>
<td>193</td>
<td>20239.3</td>
<td>4</td>
<td>38.3</td>
</tr>
<tr>
<td>STxB</td>
<td>70</td>
<td>7793.8</td>
<td>1</td>
<td>14.3</td>
</tr>
</tbody>
</table>

STxB-bound HeLa-GGS cells are incubated for 1 h at 4 °C with NHS-PEG9-BG cell surface modification reagent, and then after washing for 10 h at 37 °C. Immunoprecipitated GFP-tag material is analyzed by mass spectrometry, as described in the proteomic format section. Note that STxB peptides are clearly identified among other retrograde cargo candidates. From Shi et al. (2012).
are washed, trans-Golgi/TGN-localized GalT-GFP-SNAP that has not reacted with BG-tagged cargo proteins is quenched with SNAP-Cell-Block reagent, and cells are lysed as described in Section 9.3.3.2. Cell lysate is incubated overnight at 4 °C by end-over-end mixing with 50 μl of protein G-Sepharose beads (Sigma-Aldrich). Immunoprecipitated proteins are separated on 8% SDS-PAGE gels in nonreducing condition to avoid the dissociation of antibody chains, and finally blotted with anti-GFP (lanes 1–4) or anti-SNAP (lanes 5–8) antibodies (Fig. 9.6)

Positive bands just below 250 kDa are observed only in conditions in which the anti-GFP antibody is BG-tagged and cells are expressing the GalT-GFP-SNAP capture reagent (Fig. 9.6, lanes 1 and 5). This size corresponds to the GalT-GFP-SNAP fusion protein (70 kDa) linked to the nonreduced antibody (150 kDa). Under conditions in which either non BG-tagged anti-GFP antibody (lanes 2 and 6) or the GalT-GFP-SNAP capture reagent (lanes 3 and 7) are missing, or cells are used that do not express GFP-tagged CI-MPR (lanes 4 and 8), no band is detected at the expected size.

This approach can in principle be transposed to any candidate protein from the retrograde proteomics hit list, provided that either antibody is available against the extracellular domain of the protein, or the protein can be GFP tagged.
9.3 CONCLUSION/DISCUSSION

At the basis of the approach that is detailed in this methods review, the study by Shi et al. (2012) was the first to demonstrate that the BG-SNAP-tag ligation can occur on living cells between full size proteins within the lumen of an intracellular compartment. We apply this finding to the proteomics analysis of the retrograde transport route, for which plasma membrane proteins are BG-tagged, and the SNAP-tag capture reagent is localized to trans-Golgi/TGN membranes. In principle, this experimental design can be transposed more generally to other endocytic pathways, by placing the SNAP-tag into other intracellular compartments (early or late endosomes, endoplasmic reticulum, cytosol, etc.). SNAP-tag constructs for the endoplasmic reticulum and the cytosol are indeed already available (Geiger, Luisoni, Johnsson, Greber, & Helenius, 2013).

The current approach relies on the labeling of primary amino groups of plasma membrane proteins with BG-NHS. Only cargo proteins can thereby be identified whose intracellular trafficking characteristics are not abolished by this chemical modification. Weak hits in the proteomics format might also represent proteins that are partially affected by BG tagging. In such cases, the antibody uptake protocol (which does not require BG tagging of the cargo candidate itself) is a valid means to obtain complementary confirmation on the authenticity of apparently weak hits.

Another reason for obtaining apparently weak hits (i.e., proteins on the retrograde proteomics hit list that are present in low peptide numbers) that might in reality be efficient cargoes of the retrograde route resides in the experimental design of the current approach. Our method relies on the modification of the plasma membrane proteome on cells that are kept on ice, thereby introducing a bias toward proteins that are
abundant at the cell surface at steady state. Some retrograde cargo proteins may only transiently cycle through the plasma membrane, and only small numbers of these molecules would be BG-tagged under our experimental conditions.

**SUMMARY**

Retrograde transport from the plasma membrane to the TGN is increasingly attracting attention of scientists in diverse fields ranging from cellular neurobiology, Alzheimer’s disease research and infection biology to development. The vectorial proteomics approach that is described in this method’s review enables an integrated analysis of the retrograde proteome. We describe step-by-step how to move from tagging plasma membrane proteins with an amino-reactive BG probe to the TGN-localized and SNAP-tag-based capture, enrichment, and identification of retrograde cargo candidates, including their validation using an antibody uptake protocol. In its current format, the approach is limited to cell lines that can be stably transfected with the capture reagent. The possible development of virus-based expression systems should in the future also allow working on primary nondividing cells.

**Acknowledgments**

The work in the laboratory of the authors was supported by Agence Nationale de la Recherche (ANR-09-BLAN-283, ANR-11 BSV2 018 03, ANR-11 BSV2 014 03) and Institut National du Cancer (PLBIO11-022-IDF-JOHANNES).

**References**


