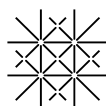


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Analysis of endocytic uptake and retrograde transport to the trans-Golgi network using functionalized nanobodies in cultured cells --Manuscript Draft--

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To the Editors of *JoVE*

Dear Editors,

Here we submit our article "**Analysis of endocytic uptake and retrograde transport to the trans-Golgi network using functionalized nanobodies in cultured cells**" for your consideration for *JoVE*.

Nanobodies are an emerging class of protein binders with many advantages over conventional antibodies (small size, non-crosslinking, no disulfide bonds and thus allowing bacterial expression). They have recently been implemented as intracellular or cell surface traps, and for high-resolution protein localization. In our previous study (Buser et al., 2018, PMID: 29915061), we developed bacterially expressed and derivatized anti-GFP nanobodies as tools to follow GFP-tagged cell surface proteins on their retrograde route to endosomes and the Golgi. They allow the quantitative analysis of protein uptake and transport to intracellular compartments either biochemically, by fixed- and live-cell imaging, or by electron microscopy. Our tool thus allows to dissect the retrograde fate and identify the molecular players involved in retrograde transport of any GFP fusion of interest. Our here submitted protocol allows a detailed methodological description of bacterial nanobody expression and their use to study endocytic uptake and retrograde transport to the Golgi in mammalian cells.

Since our study is of exceptional importance in the investigation of endocytic and retrograde traffic, and moreover is intelligible to a broad scientific audience, we consider submission of our protocol to *JoVE* as suited.

We hope that you will also consider our article to be of interest and are looking forward to your response.

Yours sincerely,

Dominik Buser
Postdoctoral Researcher (Spiess Group)

Martin Spiess
Professor of Biochemistry

TITLE:

Analysis of Endocytic Uptake and Retrograde Transport to the Trans-Golgi Network Using Functionalized Nanobodies in Cultured Cells

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KEYWORDS:

nanobodies, retrograde transport, Golgi complex, tyrosine sulfation, bacterial expression, radiolabeling, EGFP, HeLa cells

SUMMARY:

Retrograde transport of proteins from the cell surface to the Golgi is essential to maintain membrane homeostasis. Here, we describe a method to biochemically analyze cell surface-to-Golgi transport of recombinant proteins using functionalized nanobodies in HeLa cells.

ABSTRACT:

Transport of proteins and membranes from the cell surface to the Golgi and beyond is essential for homeostasis, organelle identity and physiology. To study retrograde protein traffic, we have recently developed a versatile nanobody-based toolkit to analyze transport from the cell surface to the Golgi complex, either by fixed and live cell imaging, by electron microscopy, or biochemically. We engineered functionalized anti-green fluorescent protein (GFP) nanobodies – small, monomeric, high-affinity protein binders – that can be applied to cell lines expressing membrane proteins of interest with an extracellular GFP moiety. Derivatized nanobodies bound to the GFP reporters are specifically internalized and transported piggyback along the reporters' sorting routes. Nanobodies were functionalized with fluorophores to follow retrograde transport by fluorescence microscopy and live imaging, with ascorbate peroxidase 2 (APEX2) to investigate the ultrastructural localization of reporter-nanobody complexes by electron microscopy, and with tyrosine sulfation (TS) motifs to assess kinetics of trans-Golgi network (TGN) arrival. In this methodological article, we outline the general procedure to bacterially express and purify functionalized nanobodies. We illustrate the powerful use of our tool using the mCherry- and TS-modified nanobodies to analyze endocytic uptake and TGN arrival of cargo proteins.

INTRODUCTION:

Retrograde traffic of proteins and lipids from the cell surface to various intracellular compartments is crucial for maintenance of membrane homeostasis to counterbalance secretion and to recycle components of anterograde transport machineries^{1,2}. Following internalization via clathrin-dependent or -independent endocytosis, protein and lipid cargo first

populate early endosomes from where they are further redirected either along the endo-lysosomal system, recycled to the plasma membrane, or targeted to the trans-Golgi network (TGN). Recycling from endosomes and/or the cell surface to the TGN is part of the functional cycle of a number of anterograde transmembrane cargo receptors, such as the cation-dependent and cation-independent mannose-6-phosphate receptors (CDMPR and CIMPR) delivering newly synthesized lysosomal hydrolases from the TGN to late endosomes and lysosomes³⁻⁵, sortilin and SorLA^{6,7}, and Wntless (WLS) transporting Wnt ligands to the cell surface⁸⁻¹¹. Other proteins retrieved back to the TGN are TGN46 and its related isoforms¹²⁻¹⁴, SNAREs (soluble *N*-ethylmaleimide-sensitive fusion factor attachment receptors)¹⁵⁻¹⁷, amyloid precursor protein (APP)^{18,19}, progressive ankylosis (ANK) protein²⁰, metal transporters such as ATP7A/B or DMT1^{21,22}, and transmembrane processing enzymes including carboxypeptidase D, furin or BACE1²³⁻²⁵. Apart from these endogenous proteins, bacterial and plant toxins (e.g., Shiga and cholera toxin, ricin and abrin) hijack retrograde transport machineries to reach the ER for retrotranslocation into the cytosol²⁶⁻²⁹.

In order to directly analyze retrograde traffic, we have previously developed a nanobody-based toolkit to label and follow cargo proteins from the cell surface to intracellular compartments³⁰. Nanobodies represent a new family of protein binders derived from homodimeric heavy-chain-only antibodies (hcAbs) that naturally occur in camelids and cartilaginous fishes^{31,32}. They constitute the variable heavy-chain domain (VHH) of hcAbs and have many advantages over conventional antibodies (e.g., IgGs): They are monomeric, small (~15 kDa), highly soluble, devoid of disulfide bonds, can be bacterially expressed, and selected for high-affinity binding³³⁻³⁶. To make our nanobody tool versatile and broadly applicable, we employed functionalized anti-GFP nanobodies to surface-label and track proteins tagged with GFP at their extracellular/luminal domain. By functionalization of nanobodies with mCherry, ascorbate peroxidase 2 (APEX2)³⁷, or tyrosine sulfation (TS) sequences, retrograde transport of bona fide transmembrane cargo proteins can be analyzed by either fixed and live cell imaging, by electron microscopy, or biochemically. Since tyrosine sulfation mediated by tyrosylprotein sulfotransferases (TPST1 and TPST2) is a posttranslational modification restricted to the trans-Golgi/TGN, we can directly study transport and kinetics of proteins of interest from the cell surface to this intracellular Golgi compartment³⁸⁻⁴⁰.

In this methods article, we describe the ease of production of functionalized nanobodies (VHH-2xTS, -APEX2, -mCherry and derivatives) suited for a number of applications to analyze retrograde transport in mammalian cells³⁰. We mainly focus on the use of TS site-modified nanobody for analysis of intracellular traffic from the cell surface to the compartment of sulfation.

PROTOCOL:

1. Bacterial Transformation with Functionalized Nanobodies

NOTE: This protocol has been optimized for the expression, purification, and analysis of functionalized anti-GFP nanobodies as previously described³⁰. Derivatization with other protein

moieties might require modification of this standard protocol.

1.1 Thaw chemocompetent bacteria (~100 µL) suited for protein expression (e.g., *Escherichia coli* Rosetta BL21 (DE3) cells) by placing them on ice.

NOTE: Prepare chemocompetent bacterial cells according to standard lab procedures. Alternatively, chemically competent bacterial cells can be purchased commercially.

1.2 Add 50 ng of a plasmid encoding functionalized nanobodies. To allow sufficient site-specific biotinylation of the nanobody reporter during bacterial expression, also add a threefold excess (150 ng) of a bacterial expression plasmid encoding biotin ligase BirA (see also **Table 1** for plasmid information). Gently pipet up and down.

NOTE: If nanobody biotinylation is not necessary or the nanobody reporters are devoid of a biotin acceptor peptide (BAP), co-transformation with a plasmid encoding BirA is not required.

1.3 Incubate the bacterial cells for 30 min on ice.

1.4 Heat shock the bacterial cells by placing them for 1 min at 42 °C in a water bath or a heating block.

1.5 Add 1 mL of room-temperature (RT) Luria broth (LB) medium and incubate the transformed bacteria in a thermoshaker for 1 h at 37 °C to allow phenotypic expression of resistance genes. To prepare 1 L LB medium, balance 5 g of yeast extract, 10 g of tryptone and 10 g of NaCl, fill up with water and sterilize by autoclaving. If the functionalized nanobody has been subcloned in an expression vector containing resistance to ampicillin/carbenicillin, step 1.5 can be omitted.

1.6 Pellet the bacteria at 11,000 x g for 1 min and resuspend in 100 µL of fresh LB medium.

1.7 Plate the suspended bacteria on LB plates containing the respective antibiotics (e.g., with 50 µg/mL kanamycin and 50 µg/mL carbenicillin when the bacteria have been co-transformed with nanobody reporter and BirA as stated above (step 1.2)).

1.8 Place the LB plates in an incubator at 37 °C and let grow overnight (O/N).

NOTE: The protocol can be paused here by storing the LB plate with grown colonies at 4 °C. Use parafilm to seal LB plates.

2. Bacterial Liquid Culture and Induction of Functionalized Nanobody Expression

2.1 Pick a bacterial colony containing the vector of interest from the plate and let it grow in a flask containing 20 mL of LB medium supplemented with antibiotics O/N in a shaking incubator at 37 °C (see also step 1.7 regarding selection antibiotics).

2.2 Next day, dilute the grown 20 mL bacterial culture into a flask containing 1 L of LB medium with selection antibiotics.

2.3 Continue to incubate the bacterial culture at 37 °C until it reaches an OD₆₀₀ of 0.6-0.7. Allow the culture to cool down to RT before inducing protein expression.

2.4 Induce protein expression of functionalized nanobodies and BirA by the addition of 1 mL of 1 M isopropyl-β-D-thiogalactopyranosid (IPTG) to a final concentration of 1 mM of the inducer (1:1000 dilution). Also add 10 mL of a 20 mM D-biotin stock solution resulting in 200 μM D-biotin in the growth medium to allow biotinylation of the BAP epitope present in functionalized nanobodies (see also **Table 1** for plasmid information)

NOTE: The D-biotin stock solution is prepared in ddH₂O and brought into solution by drop-wise addition of 500 mM NaH₂PO₄. Alternatively, D-biotin can also be dissolved in DMSO. To produce nanobodies fused to APEX2 (VHH-APEX2), the LB medium must be complemented in addition with 1 mM 5-aminolevulinic acid (dALA) hydrochloride to promote heme incorporation. dALA is prepared as a 100 mM stock solution in ddH₂O.

2.5 Incubate the IPTG-induced 1 L bacterial culture at 30 °C for 4 h for VHH-2xTS, at 20 °C or RT O/N for VHH-APEX2, or at 16 °C O/N for VHH-mCherry (see also **Table 1** for plasmid information).

NOTE: Expression conditions for a new nanobody construct must be optimized by the experimenter.

2.6 Transfer the bacterial culture from the flask into a 1 L centrifugation bottle and pellet the cells at 5,000 x g at 4 °C for 45 min. Decant the supernatant and continue with the purification.

NOTE: The protocol can be paused here by storing the bacterial pellet at –80 °C indefinitely. The pellet for VHH-APEX2 or VHH-mCherry is typically brownish (because of the incorporated heme) or pink, respectively.

3. Purification of Functionalized Nanobodies

3.1. If necessary, thaw the frozen bacterial pellet on ice (see also step 2.6).

3.2. Add 30 mL of ice-cold binding buffer (20 mM imidazole in 1x PBS) to the bacterial cell pellet and resuspend by pipetting up and down. Transfer resuspended bacteria into a labeled 50 mL tube.

3.3. Supplement the 30 mL binding buffer with 200 μg/mL lysozyme, 20 μg/mL DNase I, 1 mM MgCl₂ and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubate first for 10 min at RT,

and then for 1 h at 4 °C on an end-over-end shaker.

3.4. Mechanically lyse bacterial cells in a 50 mL tube by placing a tip sonicator into the suspension. Apply constant 3x 1-min pulses with 1-min cooling periods in-between.

3.5. Centrifuge the bacterial lysate at 15,000 x *g* at 4 °C for 45 min to pellet the debris and intact cells. Transfer the sonicated bacterial cell lysate either into an appropriate centrifugation bottle for centrifugation or divide the lysate into several 5 mL tubes for tabletop centrifugation.

3.6. Transfer the supernatant into a new 50 mL tube and discard the pelleted debris.

3.7. Store the cleared lysate on ice while preparing purification by immobilized metal affinity chromatography (IMAC). To isolate histidine-tagged functionalized nanobodies, employ single-use His columns (see **Table of Materials**) allowing fast and simple gravity-flow purification.

NOTE: Alternatively, batch purification instead of commercial columns can be also used.

3.8. Mounting His Columns at a Metal Stand.

3.8.1. Taper off the columns' storage solution, and equilibrate the His column with 10 mL of binding buffer (20 mM imidazole in 1x PBS).

3.8.2. Empty by gravity flow (the flow-through can be discarded).

3.9. Gradually load the cleared bacterial lysate (~30 mL) onto the column. Empty by gravity flow (the flow-through can be discarded).

3.10. Wash the His column 2x with 10 mL of binding buffer (20 mM imidazole in 1x PBS).

3.11. Elute the nanobodies with 2 mL of elution buffer (500 mM imidazole in 1x PBS) into a 2 mL tube and apply a buffer exchange.

3.12. Buffer Exchange.

3.12.1. Equilibrate a desalting column (see **Table of Materials**) placed in a 50 mL tube column adapter 5 times with 5 mL of 1x PBS.

3.12.2. Allow the buffer to enter the packed bed. Discard the flow-through.

3.12.3. After the fifth PBS equilibration, spin the column at 1,000 x *g* for 2 min.

3.12.4. Discard the flow-through.

3.12.5. Place the column with the adapter onto a new 50 mL tube. Load the 2 mL of eluted

functionalized nanobody (from step 3.11) onto the PBS-equilibrated desalting column and spin at 1,000 x g for 2 min and collect the eluate.

NOTE: Instead of commercial desalting columns, dialysis can be applied to exchange buffer composition.

3.13. Determine the protein (nanobody) concentration of the eluate using the bicinchoninic (BCA) or Bradford assay according to the manufacturer's instructions.

NOTE: For nanobody uptake assays by GFP reporter cell lines, a stock concentration of 2-10 mg/mL is optimal.

4. Validation of Functionalized Nanobody Expression (Coomassie Staining)

4.1. Prepare 10-12.5% gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard protocols.

NOTE: Alternatively, use commercially available precast gradient gels.

4.2. Pipet 20 µg of purified functionalized nanobody into a 1.5 mL tube and boil in sample buffer at 95 °C for 5 min.

NOTE: As an example, add 10 µL of a 2 mg/mL nanobody stock solution into a 1.5 mL tube with 10 µL of 2x sample buffer. If the volume is too small, further dilute nanobody in PBS before adding sample buffer.

4.3. Load the purified nanobody boiled in sample buffer onto an SDS-polyacrylamide gel, and run according to the standard PAGE protocol until the reference dye (e.g., bromophenol blue) has reached the end of the separating gel, followed by Coomassie staining and destaining.

4.4. Coomassie Gel Staining and Destaining.

4.4.1. Uncast the gel and stain it with Coomassie staining solution (5% of a 10 g/L Coomassie stock solution in 10% acetic acid and 45% methanol in ddH₂O) for 20-30 min at RT on a moving shaking platform. The gel should be completely covered in the staining solution.

4.4.2. Destain the gel 2-3 times with destain solution (7.5% acetic acid and 15% methanol in ddH₂O) for 1 h each at RT, before leaving the gel O/N for further destaining.

NOTE: Excess Coomassie can be efficiently soaked up by placing kitchen paper towels around the gel.

4.5. Image the gel with an imager or camera of choice.

NOTE: Nanobody expression can be further validated by immunoblot analysis using antibodies targeting its epitopes (see also **Figure 1A**).

5. Uptake of Functionalized Nanobodies by Cultured Cells for Fluorescence Staining

5.1 In a cell culture hood, seed ~400,000 to 500,000 HeLa cells stably expressing a GFP-tagged reporter protein on 18-mm glass coverslips (No. 1.5H) in 35-mm dishes or 6-well clusters with complete medium containing antibiotics (Dulbecco's Modified Eagle medium, DMEM, supplemented with 10% fetal calf serum (FCS), 100 units/mL streptomycin, 2 mM L-glutamine, and 1.5 µg/mL puromycin).

NOTE: Here, we use HeLa cells stably expressing EGFP-Calnexin, EGFP-CDMPR and TfR-EGFP for illustration purposes. Apart from stable cell lines, also transiently transfected HeLa cells can be used. Stable cell lines can be co-cultivated at this step with parental non-transduced HeLa cells, for direct comparison.

5.2 Incubate the cells O/N at 37 °C in a 7.5% CO₂ incubator to proliferate.

NOTE: Cells should have a confluency of ~80% the next day.

5.3 Pipet 2 µL of a 2 mg/mL nanobody stock solution into a 15 or 50 mL tube containing 2 mL of complete medium (this volume is sufficient to cover a 35-mm dish or one well of a 6-well cluster, adjust the volume of medium and nanobody proportionally to include more cell culture dishes or clusters).

NOTE: For the purpose of illustration, we here use VHH-mCherry, since no further antibody staining is required to see nanobody internalized by the EGFP reporters (see **Figure 2C**).

5.4 Remove growth medium from the cells and add 2 mL of prewarmed complete medium containing 2 µg/mL functionalized nanobody per 35-mm dish or 6-well unit.

5.5 Incubate the cells for 1 h at 37 °C in a 7.5% CO₂ incubator to allow reporter-mediated nanobody uptake.

NOTE: Depending on the planned experiment, the time of nanobody uptake needs to be adjusted. For the experiment shown here, 1 h is sufficient to reach steady-state of nanobody uptake by EGFP-CDMPR and TfR-EGFP.

5.6 Remove the medium and wash the cells 2-3 times with 1 mL of 1x PBS at RT.

5.7 Fix the cells with 1 mL of 3% paraformaldehyde (PFA) for 10 min at RT.

5.8 Remove PFA solution and quench remaining fixative with 1 mL of 50 mM NH₄Cl in 1x PBS for 5 min at RT.

NOTE: 3% PFA should be disposed of separately as fixative waste.

5.9 Wash the cells 3 times with 1 mL of 1x PBS at RT.

5.10 Permeabilize the cells with 1 mL of 0.1% Triton X-100 in 1x PBS for 10 min at RT.

NOTE: Although no permeabilization is required, EGFP and mCherry fluorescence is better when afterwards the cells are embedded in mounting medium.

5.11 Wash the cells 3 times with 1 mL of 1x PBS at RT.

5.12 Place the coverslips with forceps on a drop (~100 μ L) of 1% BSA in 1x PBS containing 5 μ g/mL DAPI (4',6-diamidino-2-phenylindole) for 5 min at RT.

5.13 Place the coverslips back into the dish/well and wash 3 times with 1 mL of 1x PBS at RT.

5.14 Label the glass slides and mount the cells with Fluoromount-G. Let the mounting medium harden in the dark for 3-4 h and store the glass slides at 4 °C under light protection.

5.15 Image staining patterns using a microscope of choice (e.g., Point Scanning Confocal upright microscope).

6. Uptake of Functionalized Nanobodies by Cultured Cells (for Sulfation Analysis)

6.1 In a cell culture hood, seed ~400,000 to 500,000 HeLa cells stably expressing a GFP-tagged reporter protein in 35-mm dishes or on 6-well clusters with complete medium containing antibiotics (Dulbecco's Modified Eagle medium, DMEM, supplemented with 10% fetal calf serum (FCS), 100 units/mL streptomycin, 2 mM L-glutamine, and 1.5 μ g/mL puromycin).

NOTE: As mentioned above, we here use HeLa cells stably expressing EGFP-Calnexin, EGFP-CDMPR and TfR-EGFP for illustration purposes. Apart from stable cell lines, also transiently transfected HeLa can be used.

6.2 Incubate the cells O/N at 37 °C in a 7.5% CO₂ incubator to proliferate.

NOTE: Cells should have a confluency of ~80% next day.

6.3 Remove the complete medium, wash 2 times with 1x PBS at RT, and starve the cells with sulfate-free medium (SFM) for 1 h at 37 °C in a 7.5% CO₂ incubator.

NOTE: SFM is prepared by adding 10 mL of MEM amino acids (50x) solution, 5 mL of L-

glutamine (200 mM), 5 mL of vitamin solution (100x), and 900 μ L of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to 500 mL of Eagle's balanced salts. Pass through a 0.22 μ m filter and aliquot.

6.4 In a ventilated hood or bench designated for radioactivity work, prepare sulfate labeling medium containing 0.5 mCi/mL [^{35}S]sulfate as sodium salt in SFM.

CAUTION: Carefully handle all solutions containing radioactivity. Only work in designated hoods or benches. All material (tips, tubes, plates, etc.) or wash buffers in contact with radioactivity have to be collected and disposed of separately. Do this for all steps below (step 6.5-6.20) as well.

6.5 Add VHH-2xTS in 1x PBS into sulfate labeling medium to a final concentration of 2 μ g/mL.

NOTE: As control, also include VHH-std or another nanobody devoid of TS sites to demonstrate specific labeling.

6.6 Replace SFM by 0.7 mL of sulfate labeling medium containing 0.5 mCi/mL [^{35}S]sulfate and 2 μ g/mL VHH-2xTS and incubate the cells for 1 h at 37 $^{\circ}\text{C}$ in a 7.5% CO_2 incubator designated for work with radioactivity.

NOTE: Depending on the planned experiment, the time of nanobody sulfation needs to be adjusted. In addition, to determine TGN arrival kinetics, labeling is performed for different times (e.g., for 10 min, 20 min, 30 min, etc.).

6.7 Remove the labeling medium and wash the cells 2-3 times with ice-cold 1x PBS on a cooling plate or ice.

6.8 Add 1 mL of lysis buffer (PBS containing 1% Triton X-100 and 0.5 % deoxycholate) supplemented with 2 mM PMSF and 1x protease inhibitor cocktail and incubate the cells for 10-15 min on a rocking platform at 4 $^{\circ}\text{C}$.

6.9 Scrape and transfer the lysate into a 1.5 mL tube, vortex the lysate, and place for 10-15 min on an end-over-end rotator at 4 $^{\circ}\text{C}$.

6.10 Prepare a postnuclear lysate by centrifugation at 10,000 x g for 10 min at 4 $^{\circ}\text{C}$.

6.11 Using a new 1.5 mL tube, prepare 20-30 μ L of a slurry of Nickel beads in a 1.5 mL tube and wash once with 1x PBS by gently pelleting them at 1,000 x g for 1 min.

NOTE: Instead of Nickel beads, streptavidin beads (if nanobody is biotinylated) or protein A beads with an IgG against an epitope of the nanobody (T7- or HA-tag) can be used.

6.12 Transfer the postnuclear lysate into a 1.5 mL tube containing Nickel beads and incubate for 1 h at 4 °C on an end-over-end rotator to isolate the nanobodies. Remove an aliquot (50-100 µL) of the postnuclear lysate and boil in SDS sample buffer for subsequent immunoblot analysis of total cell-associated nanobody, GFP reporter and loading control.

6.13 Wash the beads 3 times with 1x PBS or lysis buffer by gently pelleting at 1,000 x *g* for 1 min.

NOTE: For more stringent washing of beads, 20 mM imidazole can be included in PBS or lysis buffer.

6.14 Carefully remove all washing buffer from the beads, add 50 µL of 2x sample buffer, and boil for 5 min at 95 °C.

6.15 Load both boiled beads on a midi 12.5% SDS-PAGE gel and run according to the standard PAGE protocol until the reference dye (e.g., bromophenol blue) has reached the end of the separating gel.

NOTE: Immunoblot analysis of total cell-associated nanobody, GFP reporter and loading control can also be performed using a mini 12.5% SDS-PAGE or precast gradient gel.

6.16 Fix the separating gel in ~30 mL of fixation buffer (10% acetic acid and 45% methanol in ddH₂O) for 1 h at RT, wash the gel three times with ~50 mL of deionized water and prepare for gel drying.

6.17 Drying SDS-PAGE Gels.

6.17.1 Carefully place the fixed gel on stretched cling film and cover it with precut filter paper.

6.17.2 Place the gel with the filter paper down on top of the drying platform, place the vacuum cover, and switch on the vacuum pump for gel drying. Dry the gel for 3-5 h.

6.17.3 Remove the cling film and place the dried gel into an autoradiography cassette equipped with a Phosphor Screen.

6.18 Image autoradiograph using a Phosphor Screen Imager. Follow the instructions of the manufacturer.

NOTE: Depending on the strength of the signal, dried gels need to be exposed longer with Phosphor screens.

REPRESENTATIVE RESULTS:

To investigate retrograde protein transport to various intracellular destinations, we have recently established an anti-GFP nanobody-based tool to label and follow recombinant fusion

proteins from the cell surface³⁰. Here, we demonstrate the bacterial production of such derivatized nanobodies and demonstrate their application to study endocytic uptake by fluorescence microscopy and immunoblotting, as well as their use to investigate TGN arrival by sulfation analysis. The latter application is the methodological focus of this protocol.

We have generated a toolbox of different functionalized anti-GFP nanobodies with common features by standard molecular cloning³⁰. Our simplest (standard) nanobody, VHH-std, contains the VHH domain, a T7 and an HA epitope for subsequent detection by specific antibodies, a carboxyterminal hexahistidine (His6)-tag for purification, and a biotin acceptor peptide (BAP) sequence for biotinylation and high-affinity streptavidin pull-down experiments (**Figure 1A**). Derivatization of VHH-std yields different nanobody variants to examine endocytic uptake and retrograde transport biochemically, by fixed and live cell imaging, and by electron microscopy.

To assess protein traffic from the plasma membrane to the trans-Golgi/TGN, we took advantage of the exclusive localization of tyrosylprotein sulfotransferases in these compartments and thus modified VHH-std with a tandem tyrosine sulfation site (2xTS) derived from rat procholecystokinin⁴¹. While modification of VHH-std with mCherry allows direct visualization of retrograde transport by fixed and live cell imaging, functionalization with a peroxidase, such as APEX2³⁷, enables electron microscopy studies, cytochemical compartment ablation, or proximity-dependent biotinylation reactions. In addition, insertion of a cleavage site for the tobacco etch virus (TEV) protease allows to biochemically distinguish between intracellular and surface-bound nanobodies (**Figure 1A**). We have previously used VHH-tev to monitor endocytic recycling kinetics of nanobody-bound EGFP-CDMPR and TfR-EGFP³⁰. Nanobody reappearance at the cell surface after uptake could be simply assessed by chasing with extracellularly applied recombinant TEV protease causing an inverse loss of the nanobody's carboxyterminal epitope cassette. Including a cleavage site in the mCherry or APEX2 nanobody fusion is useful to study recycling kinetics of EGFP reporters by live cell imaging in a similar way to VHH-tev, or to limit cytochemical reactions to intracellular compartments, respectively. Functionalization with other protein domains (e.g., other fluorophores or enzymes) or target sequences for other posttranslational modifications can be easily achieved by subcloning a respective insert into the plasmid vector encoding VHH-std (see also **Table 1** for plasmid information) using available SpeI and EcoRI restriction sites.

Using the protocol as described above, all nanobodies illustrated here can be isolated to high purity and yield (**Figure 1B**). Only mCherry fusions showed minor clipping of protein domains post purification (**Figure 1B**, see lanes 7-8). In the absence of BirA expression, we typically obtained 25-35 mg for VHH-std, VHH-2xTS and their tev-modified counterparts, or ~20 mg for VHH-APEX2 and VHH-mCherry and their tev-containing derivatives. Our experience shows that BirA coexpression lowers nanobody yield by a third to a half.

To illustrate reporter-mediated nanobody uptake, we have used HeLa cell lines stably expressing EGFP-Calnexin (CNX), EGFP-CDMPR, and TfR-EGFP (**Figure 2A and B**). EGFP was fused to the extracellular/luminal end of each protein (i.e., between the signal and reporter sequence of CNX or CDMPR), and to the carboxyterminus of the TfR, leaving the cytosolic

sorting signals unobstructed. All of these EGFP-tagged cargo molecules have different intracellular trafficking itineraries after ER targeting. Since EGFP-Calnexin is resident in the ER and normally does not reach post-Golgi compartments, it serves as a negative control for potential and nonspecific nanobody uptake. In contrast, both EGFP-CDMPR and TfR-EGFP have transport functions beyond the Golgi and thus steadily appear at the plasma membrane. As expected, when VHH-mCherry was added to a mixed population of unmodified HeLa and cells stably expressing EGFP-CNX, no nanobody internalization could be observed in either case. However, both EGFP-CDMPR and TfR-EGFP captured VHH-mCherry at the cell surface and transported them piggyback to the reporter's homing compartments (**Figure 2C**).

VHH-mCherry colocalization with a resident TGN marker could be used in principle to assess TGN arrival. Yet, we established a biochemical approach based on tyrosine sulfation. In order to take advantage of this modification occurring in the trans-Golgi/TGN, nanobodies were functionalized with TS sites (**Figure 1A**). To demonstrate the specificity and functionality of these protein binders, we incubated unmodified HeLa cells and cells stably expressing EGFP-CNX, EGFP-CDMPR and TfR-EGFP with VHH-std or VHH-2xTS for 1 h in the presence of radioactive sulfate. Radiolabeling of VHH-2xTS only occurs when a reporter transports the nanobody retrogradely to the TGN where it is exposed to the tyrosine sulfation machinery. HeLa expressing EGFP-CNX and their parental cells display no nanobody uptake and thus no sulfation. Both EGFP-CDMPR and TfR-EGFP internalized nanobodies, the latter considerably more than the former. Nevertheless, only EGFP-CDMPR caused VHH-2xTS to be sulfated (**Figure 3A**). This confirms efficient retrograde transport of CDMPR from the cell surface to the TGN and provides evidence against TfR returning to the TGN (as has been suggested in some studies^{42,43}).

Kinetics of internalization and TGN arrival of reporter proteins can also be determined by analyzing cell lysates after different times of nanobody uptake and sulfation. Using EGFP-CDMPR as reporter, we found sulfation to start only after a lag period of ~15 min and to reach saturation only after >75 min (**Figure 3B**, lanes 1-6, and C, square symbols). Uptake and sulfation kinetics appeared shifted by almost 30 min, reflecting the transport to the TGN. In addition, the method allows to investigate the sorting machinery involved in MPR transport by pharmacological interference or protein silencing approaches such as knockdown, knockout, or knocksideways. Here, we used brefeldin A (BFA), an inhibitor of guanine nucleotide exchange factors (GEFs) of several Arf GTPases, to generally interfere with retrograde transport to the TGN. When treated with BFA, sulfation was completely abolished, while uptake remained unaffected both in kinetics and in extent (**Figure 3B**, lanes 7-12, and C, round symbols).

FIGURE AND TABLE LEGENDS:

Figure 1. Design and production of functionalized nanobodies to track EGFP-modified cell surface proteins. (A) Schematic representation of the derivatized nanobodies. The standard nanobody consists of the GFP-specific VHH domain, T7 and HA epitope tags, a BAP, and a hexahistidine (His6) purification tag. Other nanobodies in addition comprise two TSs, APEX2, or mCherry, each with or without a cleavage site for TEV protease (tev). Scale bar is in amino acids (aa). (B) Bacterially expressed and purified nanobodies (50 µg) were analyzed by gradient SDS-

gel electrophoresis and stained with Coomassie. Marker proteins with molecular mass in kDa are shown on the left. Only VHH-mCherry and VHH-tev-mCherry showed minimal clipping between the VHH and the mCherry domains.

Figure 2. Uptake and intracellular localization of VHH-mCherry by different EGFP reporter proteins. (A) Schematic representation of EGFP fusion proteins. Sequences derived from secretory membrane proteins are shown in black with N-terminal signal peptides and internal transmembrane segments in yellow, and EGFP is illustrated in green. EGFP was fused to full-length CDMPR, TfR, and Calnexin (CNX). Scale bar in amino acids (aa). EGFP-CDMPR and TfR-EGFP have been described³⁰. (B) HeLa cells stably expressing EGFP reporter proteins were harvested, lysed, and analyzed by SDS-gel electrophoresis and immunoblotting using anti-GFP and anti-actin antibodies. Molecular mass in kDa is indicated on the right. (C) HeLa cells stably expressing the EGFP-tagged reporter proteins were mixed with parental HeLa cells, incubated with 5 µg/mL VHH-mCherry (~100 nM) for 1 h at 37 °C and processed for fluorescence microscopy. Scale bar is 10 µm. Uptake only occurs by cells expressing a reporter with EGFP exposed at the cell surface.

Figure 3. Sulfation analysis and kinetics of retrograde transport to the TGN using TS-tagged nanobodies. (A) Parental and HeLa cells stably expressing EGFP-modified reporter proteins were labeled using 0.5 mCi/mL [³⁵S]sulfate for 60 min with 2 µg/mL VHH-std or VHH-2xTS. Nanobodies were isolated using Nickel beads, separated on a 12.5 % SDS-PAGE followed by autoradiography. Aliquots of cell lysates were collected before the Nickel pull down and immunoblotted for cell-associated nanobody (His6), EGFP, and actin. (B and C) HeLa cells stably expressing EGFP-CDMPR were labeled with 0.5 mCi/mL [³⁵S]sulfate for up to 75 min in the presence of 2 µg/mL VHH-2xTS with and without 2 µg/mL BFA before analysis as in (A). Quantitation of VHH-2xTS uptake and sulfation from three independent experiments is shown in percent of the value without BFA after 75 min (mean and SD). Black squares, without BFA; grey circles, with BFA; open symbols, uptake; filled symbols, sulfation. This figure has been modified and extended from Buser et al., 2018, PNAS³⁰.

Table 1. Protein sequences of the functionalized nanobodies shown in this study. T7 epitope in grey, VHH in black, HA epitope in blue, BAP sequence in orange, hexahistidine (His6) in red, TEV protease cleavage site in purple, myc epitope in magenta, the tyrosine sulfation sequence in yellow, APEX2 in green, and mCherry in pink. The expression vectors encoding these functionalized anti-GFP nanobodies have been deposited (see also **Table 1** for plasmid information).

DISCUSSION:

Nanobodies represent an emerging class of protein binder scaffolds with many advantages over conventional antibodies: they are small, stable, monomeric, can be selected for high affinity and lack disulfide bonds^{33,35,44,45}. They are used in a number of applications, such as in cell culture systems and organisms in developmental biology⁴⁶⁻⁴⁹, as crystallization chaperones to stabilize conformational states in structural biology⁵⁰⁻⁵³, as inhibitors or activity modulators of enzymes⁵⁴⁻⁵⁶, as immunohistochemical reagents for biochemical analyses⁵⁷, or as "secondary

nanobodies" to detect anti-mouse and anti-rabbit immunoglobulins on immunoblots and immunofluorescence⁵⁸. In our previous study, we have developed and applied functionalized nanobodies produced by bacterial expression to surface-label proteins and track their intracellular route to various compartments, in particular to the TGN³⁰. We used an anti-GFP nanobody to make the tool versatile, able to target fusion proteins with extracellular GFP, YFP, or mCerulean that might already be available and characterized. Modification of anti-GFP nanobodies with mCherry, APEX2, or TS sites allowed us to monitor reporter uptake by fixed and live cell imaging, to ultrastructurally visualize retrograde transport compartments, to ablate these compartments, or to study transport kinetics to the TGN. One drawback of our tool is that recombinant modification of target cell lines (stable expression or endogenous tagging) is required before functionalized anti-GFP nanobodies can be applied. Functionalization can of course be also applied to the rapidly increasing number of nanobodies directed against untagged endogenous target proteins established by animal immunization or by ribosome-display and phage-display selection of synthetic VHH libraries⁵⁹. Using nanobodies modified with TS sites (e.g., VHH-2xTS), we can directly measure transport and kinetics of TGN arrival of diverse EGFP-modified target proteins. We have applied VHH-2xTS to study the contribution of the adaptor protein complex 1 (AP-1) in retrograde transport of MPRs by direct kinetic analysis in knocksideways cells allowing rapid inactivation of the given transport machinery³⁰. Nanobody sulfation and hence retrograde transport of EGFP-labeled MPRs was partially, but not completely blocked. Our nanobody-based approach using sulfation as TGN arrival sensor confirmed previous results from other laboratories indicating a functional contribution of AP-1 in retrograde endosome-to-TGN transport^{17,60,61}. Sulfatable nanobodies can thus provide a useful biochemical tool to dissect the contribution of other retrograde transport machineries, such as epsinR, Rab9/TIP47 or SNX-BAR/retromer complexes, on cargo proteins by genomic, genetic or chemical manipulations. The protocol presented here offers a basis of how one can make use of functionalized nanobodies in general to determine target compartments, pathways, and transport kinetics in cultured cells.

Other groups have already made use of TS sites to follow transport from the cell surface to the TGN. Ricin, Shiga or pertussis toxin subunits have previously been modified with sulfation sites to demonstrate a transport route through the TGN⁶²⁻⁶⁶. Moreover, TS peptides had also been chemically coupled to IgGs to assay retrograde transport of GFP-CIMPR and endogenous TGN46 to the TGN^{67,68}. Our sulfatable nanobodies have the advantage of simple and reproducible bacterial production and of a 1:1 stoichiometry with the target protein. On the contrary, it is for instance well known that divalent protein binders, such as IgGs, can crosslink cell surface proteins and alter their intracellular trafficking to lysosomes after endocytosis^{69,70}, highlighting the significance of monomeric protein binders with respect to existing methods. A critical step of our technique is that handling with radioactivity is required to perform sulfate labeling of nanobodies. However, our tool of functionalized nanobodies with TS sites to study TGN arrival may be potentially applied without radioactivity using anti-sulfotyrosine antibodies.

Our previous study suggests that nanobody sulfation are not very efficient, since sulfation of MPRs had not yet reached a maximum after 75 min, even though nanobody uptake was saturated after 45 min³⁰. Screening for other natural TS sites might improve sulfation efficiency.

Alternatively, to potentially enhance sulfation efficiency, components of the sulfation machinery itself, such as TPST1 and TPST2, might be overexpressed. Indeed, it has been previously shown that overexpression of one of the transporters delivering 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the substrate for TPSTs, from the cytosol to the lumen of the Golgi could alter the sulfation status of proteoglycans⁷¹.

Apart from sulfatable nanobodies, other derivatizations also allow tracing transport through endocytic compartments and to the TGN. APEX2 can be applied as a promiscuous labeling enzyme for proximity-dependent biotinylation for proteomic analysis of retrograde transport. APEX2 nanobody that is internalized by a cargo reporter of interest will label proteins in close proximity within the target compartments. Comparative proteomics should allow to identify other endogenous proteins in the different types of endosomes and the TGN that the nanobody accesses. Many variations of nanobodies in combination with its target protein are conceivable. A recent report, for instance, applied an inverted approach to the one described here: derivatized GFP was used to study and trap cellularly expressed anti-GFP nanobody-tagged vacuolar sorting receptors in anterograde and retrograde compartments of the plant endomembrane system⁷². Similarly, functionalized nanobody-traps may be designed in mammalian cell culture systems to capture and accumulate EGFP-modified reporters in different compartments during retrograde transport.

Our here presented method and protocol with the focus on TS site-tagged nanobodies allows quantitative and qualitative tracking of cargo proteins from the cell surface to endocytic compartments and the TGN in cultured mammalian cells.

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DISCLOSURES:

The authors have nothing to disclose.

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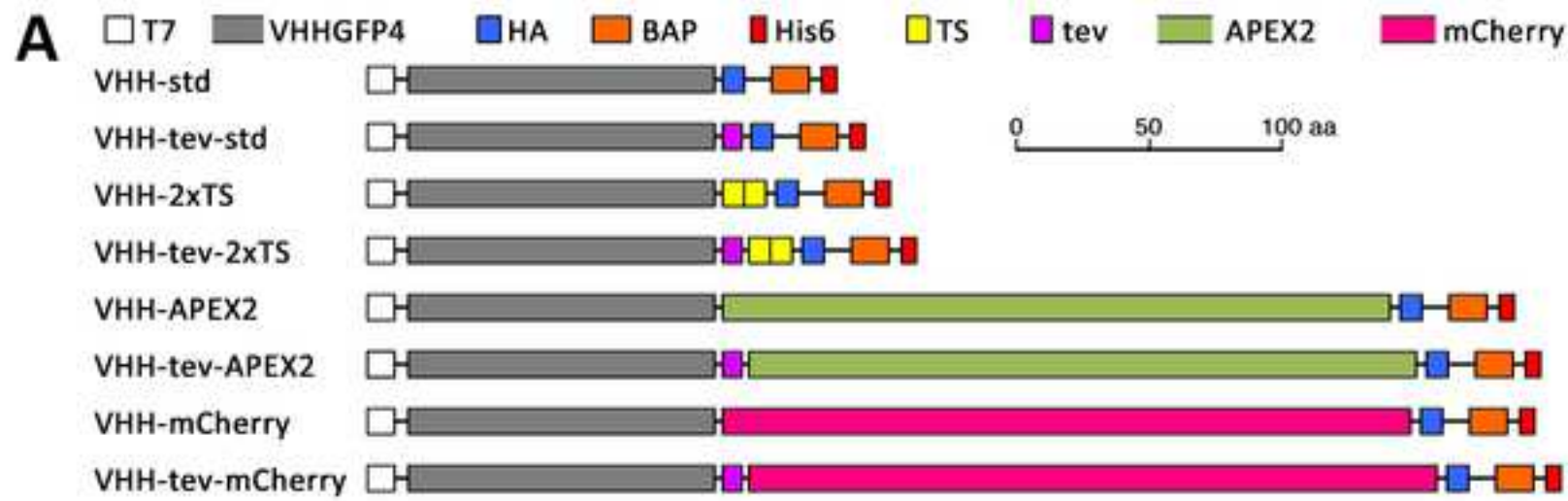
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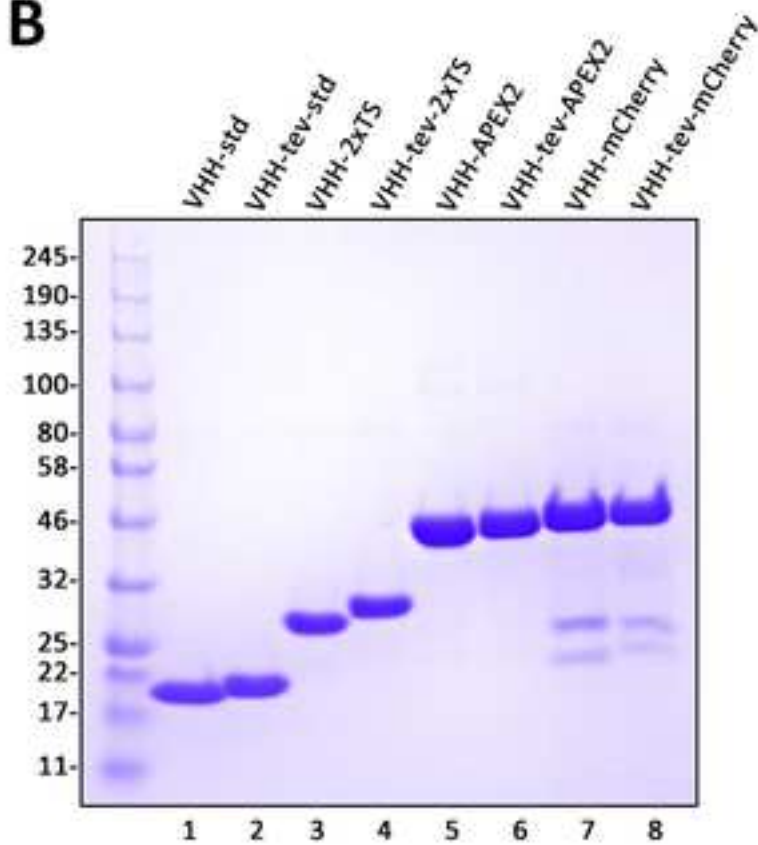
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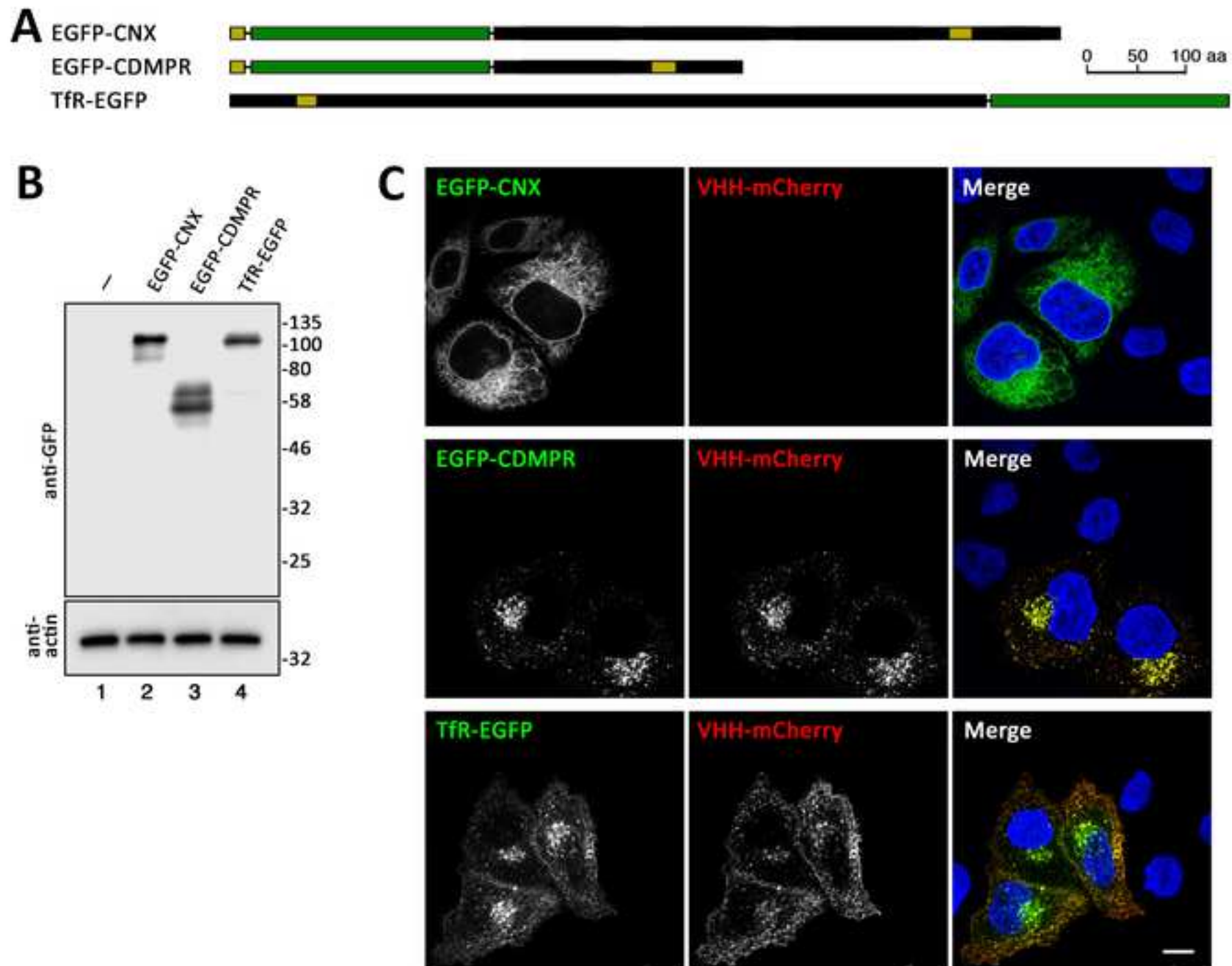
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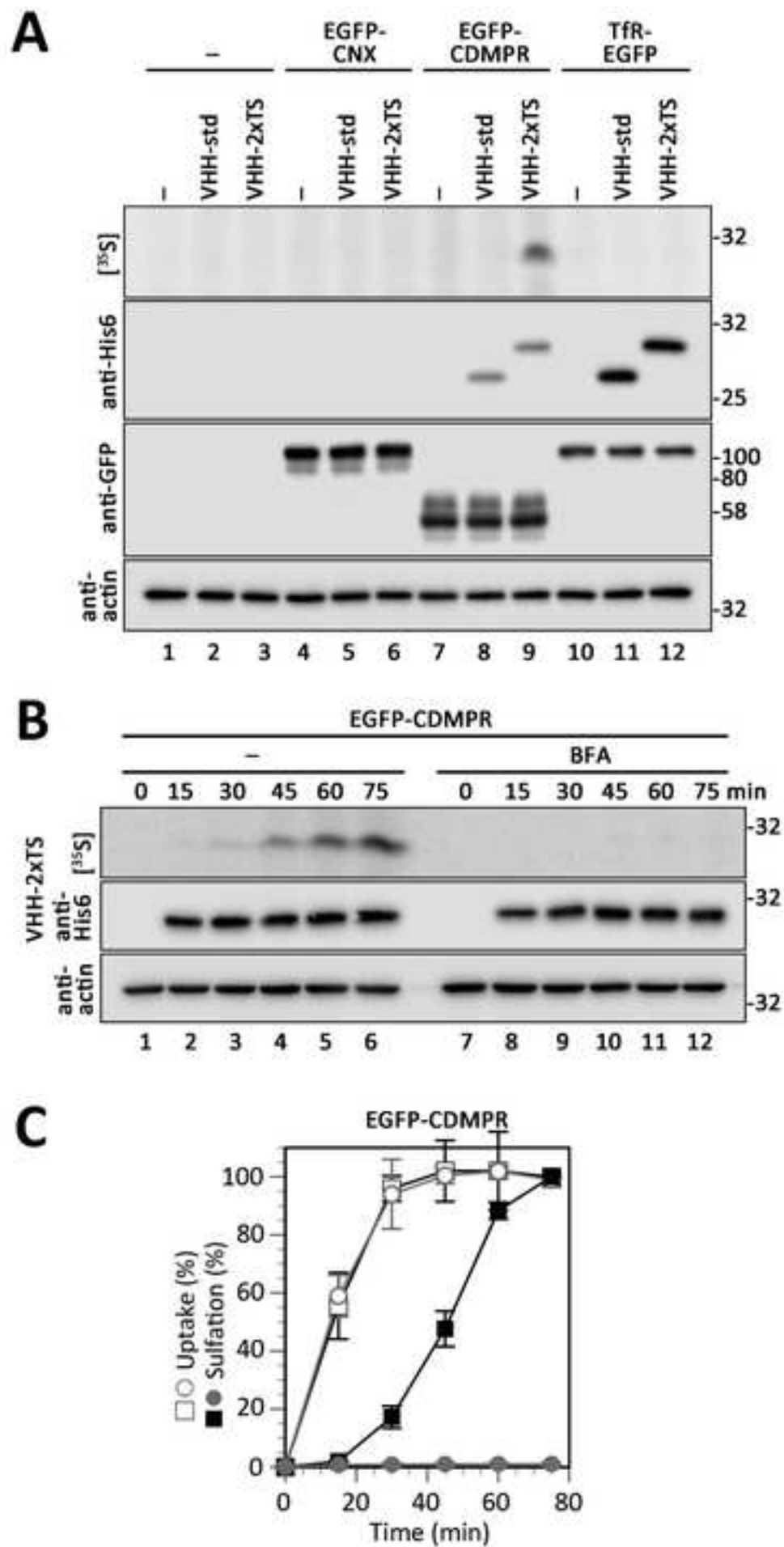


Table 1

Nanobody
VHH-std (Addgene 109417)
VHH-tev (Addgene 109423)
VHH-2xTS (Addgene 109419)
VHH-tev-2xTS (Addgene 117750)
VHH-APEX2 (Addgene 109420)
VHH-tev-APEX2 (Addgene 117751)
VHH-mCherry (Addgene 109421)
VHH-tev-mChery (Addgene 117752)

Protein sequence

[illegible]

'EDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFYWGQGTQVTVSSTSEFDYPYDVPD'
'EDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFYWGQGTQVTVSSTSENLYFQSEFDY
'EDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFYWGQGTQVTVSSTSAEDYEYPSAE
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'DYEYPS EFDYPYDVDPDYAGAQPARGGGGLNDIFEAQKIEWHEGALEHHHHHH
'YEYPSAEDYEYPS EFDYPYDVDPDYAGAQPARGGGGLNDIFEAQKIEWHEGALEHHHHHH
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'PTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAGTFDKGKTGGPFGTIKHPAELAHSANGLDIAVF
'AAIIEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTGGPLPFAWDILSPQFMYGSKAYVKHPA
KGEEDNMAIIEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTGGPLPFAWDILSPQFMYGSK/

EFPILSYADFYQLAGVVAVEVTGGPKVPFHPPGREDKPEPPPEGRLPDPTKGS DHLRDVFGKAMGLTDQDIVALS
RLEPLKAEFPILSYADFYQLAGVVAVEVTGGPKVPFHPPGREDKPEPPPEGRLPDPTKGS DHLRDVFGKAMGLT
DIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSEF
AYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMG

SGGHTIGAAHKERSGFEGPWTSNPLIFDNSYFTELLSGEKEGLQLPSDKALLSDPVFRPLVDKYAADEDAFFADY
DQDIVALSGGHTIGAAHKERSGFEGPWTSNPLIFDNSYFTELLSGEKEGLQLPSDKALLSDPVFRPLVDKYAADE
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WEASSERMYPEDGALKGEIKQRLKLDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYER

/AEAHQKLSELGFADAEFDYPYDVPDYAGAQPARGGGLNDIFEAQKIEWHEGALEHHHHHH
:DAFFADYAEAHQKLSELGFADAEFDYPYDVPDYAGAQPARGGGLNDIFEAQKIEWHEGALEHHHHHH
TGGMDELYKEFDYPYDVPDYAGAQPARGGGLNDIFEAQKIEWHEGALEHHHHHH
AEGRHSTGGMDELYKEFDYPYDVPDYAGAQPARGGGLNDIFEAQKIEWHEGALEHHHHHH

Name of Material/ Equipment	Company	Catalog Number
Anti-GFP antibody	Sigma-Aldrich	118144600001
Anti-His6 antibody	Bethyl Laboratories	A190-114A
Anti-actin antibody	EMD Millipore	MAB1501
Goat anti-rabbit HRP	Sigma-Aldrich	A-0545
Goat anti-mouse HRP	Sigma-Aldrich	A-0168
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542
Dimethyl sulfoxide (DMSO)	Applichem	A3672
D-biotin	Sigma-Aldrich	B4501
5-aminolevulinic acid (dALA) hydrochloride	Sigma-Aldrich	A3785
DNase I	Applichem	A3778
Lysozyme	Sigma-Aldrich	18037059001
Brefeldin A (BFA)	Sigma-Aldrich	B5936
Puromycin	Invivogen	ant-pr-1
Penicillin/Streptomycin	Bioconcept	4-01F00-H
L-glutamine	Applichem	A3704
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich	D5796
Fetal calf serum (FCS)	Biowest	S181B-500
Sulfur-35 as sodium sulfate	Hartmann Analytics	ARS0105
Earle's balanced salts	Sigma-Aldrich	E6267
MEM amino acids (50 x) solution	Sigma-Aldrich	M5550
MEM vitamin solution (100 x)	Sigma-Aldrich	M6895
cOmplete, Mini Protease inhibitor cocktail	Sigma-Aldrich	11836153001
Isopropyl- β -D-thiogalactopyranosid (IPTG)	Applichem	A1008
Carbenicillin disodium salt	Applichem	A1491
Kanamycin sulfate	Applichem	A1493
Coomassie-R (Brilliant Blue)	Sigma-Aldrich	B-0149
Paraformaldehyde (PFA)	Applichem	A3813
Bovine serum albumin (BSA)	Sigma-Aldrich	A2153

Fluoromount-G	Southern Biotech	0100-01
Ni Sepharose High Performance	GE Healthcare	17-5268-01
His GraviTrap columns	GE Healthcare	GE11-0033-99
His buffer kit	GE Healthcare	GE11-0034-00
Disposable PD10 desalting columns	GE Healthcare	GE17-0851-01
Mini-Protean TGX gels, 4-20%, 15-well	Bio-Rad	456-1096
Dulbecco's phosphate buffered saline (DPBS) w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$	Sigma-Aldrich	D8537
35-mm dishes	Falcon	353001
6-well plates	TPP	92406
Glass coverslips (No. 1.5H)	VWR	631-0153
Phenylmethanesulfonyl fluoride (PMSF)	Applchem	A0999.0025
Tryptone	Applchem	A1553
Yeast extract	Applchem	A1552
Magnesium chloride hexahydrate	Merck Millipore	105833
Calcium chloride dihydrate	Merck Millipore	102382
Sodium chloride	Merck Millipore	106404

Comments/Description

Product is distributed by Sigma-Aldrich, but manufactured by Roche

dissolved in 1 x PBS/1%BSA

dissolved in sterile 500 mM NaH_2PO_4 or DMSO

dissolved in sterile water

dissolved in sterile water

Product is distributed by Sigma-Aldrich, but manufactured by Roche

Product contains 5 mCi

Product is distributed by Sigma-Aldrich, but manufactured by Roche

dissolved in sterile water, stock is 1 M

dissolved in sterile water, stock is 100 mg/mL

dissolved in sterile water, stock is 100 mg/mL

dissolved in 40% DMSO 60% isopropanol, stock in 500 mM

dissolved in sterile water, stock is 1 M

dissolved in sterile water, stock is 1 M

dissolved in sterile water, stock is 5 M



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Author(s):

Dominik P. Buser, and Martin Spiess

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Institution:

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Article Title:

Analysis of endocytic uptake and retrograde transport to the trans-Golgi network using fluorescently labeled nanobodies in cultured cells

Signature:

[Handwritten Signature]

Date:

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Dear Dr. Wu,

Many thanks for editing our paper “Analysis of endocytic uptake and retrograde transport to the trans-Golgi network using functionalized nanobodies in cultured cells” that has been reviewed and sent to us by October 17, 2018. Please find below our point-to-point response to the reviewers’ and editor’s comments (we mentioned the corrections here and not the manuscript file).

Answers to comments of reviewer #1

1. Line 89: it is probably better to indicate "Rosetta" as the trade marker from Novagen. Can the authors comment on if Rosetta strain of BL21 cells necessary here?

Since we use Rosetta cells for expression of other proteins as well, we have no experimental experience how nanobody expression in other strains look like. We corrected this by mentioning Rosetta as an example strain for our purifications as follows:

1. Thaw chemocompetent bacteria (~100 µL) suited for protein expression (e.g., *Escherichia coli* Rosetta BL21 (DE3) cells) by placing them on ice.

NOTE: Prepare chemocompetent bacterial cells according to standard lab procedures. Alternatively, chemically competent bacterial cells can be purchased commercially.

2. In part 3 of the protocol—purification of functionalized nanobodies, can the authors comment on the more commonly used and economical method: batch purification and dialysis?

We did not perform other purifications than outlined in this protocol. But, we think that any other isolation and desalting procedure (batch purification and dialysis) will work out with high confidence as well. We used His GraviTrap and PD-10 desalting columns for the ease of handling and for shortening the purification process. While desalting with columns takes minutes, dialyzing is often performed overnight and thus prolongs the purification process. Nevertheless, we added notes to step 3.7 and 3.13 that other purification and desalting procedures can be employed as well.

3. Figure 1: to facilitate the potential modification of the toolkit by other users, it would be very helpful to label key restriction enzyme sites flanking each functional elements.

We did not include restriction sites in Figure 1 A, but mentioned in the part “Representative Results” that our standard nanobody (VHH-std) can be easily derivatized by using SpeI and EcoRI restriction sites. To allow the researcher to do any subcloning, full plasmid sequences are available on Addgene. Our new plasmids encoding additional nanobodies (VHH-tev-2xTS, VHH-tev-APEX2, VHH-tev-mCherry) that we describe in this protocol for the first time have been in the meantime catalogued by Addgene. The plasmids will be released immediately after publication.

4. Figure 2 A: signal peptide and transmembrane domain should be labeled with different colors to distinguish the two elements.

To have overlaps to our previous publication Buser et al., 2018¹, we kept the color codes in the reporter cartoons. In case this would pose any conflict for the editorial decision, we would revise this and implement new color coding.

5. Figure 3: Molecular weights are not labeled in A and B. In C, the time of BFA treatment and corresponding gel lanes are misaligned from lane 1 to lane 12.

We added molecular weights to Figure 3 A and B, and also properly aligned BFA time indications to Figure 3 B.

6. The following comments from the authors would be helpful for the usage of the toolkit:
 - a. What types of commonly used GFP variants can GFP nanobody bind?
The GFP nanobody binds to other GFP variants (e.g., YFP, etc.). We mentioned this in the discussion already (lane 560-562 from the old manuscript).
 - b. Does the binding of GFP nanobody increase or decrease the GFP fluorescence intensity?
The GFP nanobody has been reported to slightly alter GFP fluorescence properties by a 1.47-fold increase ^{2,3}. In our previous article ¹, we normalized to any potential changes in GFP fluorescence properties due to nanobody binding.
 - c. What is the epitope (amino acid sequence of GFP) of GFP nanobody?
The anti-GFP nanobody binds to a three-dimensional epitope present on GFP, and thus no primary amino acid sequence can be given. Different anti-GFP nanobodies with different properties (e.g., affinity, etc.) exist and they all have distinct epitopes they recognize on GFP. Binding sites of some nanobodies have been systematically analyzed previously ⁴. The nanobody used in our study (VHHGFP4) potentially binds to the epitope as described in reference ⁵.
 - d. Can the authors compare GFP nanobody and commercial GFP binding products such as GFP-Trap from ChromoTek?
We do not have any comparison between our nanobody and the GFP protein binder immobilized on GFP-Trap. We do not know which GFP protein binder ChromoTek might be using.
7. There are very few grammar errors.
 - 1 Line 77: "the ease production of" should be "the ease of production".
 - 2 Line 342: The full stop is missing.
 - 3 Line 520: "have been described in 30." ; "in" can be removed.

We corrected the mentioned grammar mistakes in the respective parts.

Answers to comments of reviewer #2

1. One typo noticed: line 442, EGFR-CDMPR should be EGFP-CDMPR.
We corrected this mistake in the manuscript.

Answers to comments of reviewer #3

1. Figure 1A: The schematic structure of nanobody derivatives should reflect precisely the number of the amino acids shown in table 1 (e.g. VHH-APEX2 and -mCherry derivatives).
The cartoons in Figure 1 A are to scale and reflect the number of amino acids presented in Table 1.
2. Figure 3B: In Line 491, lane 1-7 must be 1-6. In line 498, lane 8-14 must be 7-12.
We corrected these mistakes in the manuscript.
3. Figure 3C: In line 539, Black and grey must be removed.
The indication of black and grey is required to follow the meaning of the symbols.
4. In Table of Materials, cOmpete must be Complete.
The manufacturer advertises the product with the name "cOmpete". However, since this apparent spelling mistake was also mentioned in the editorial comments, we substituted "cOmpete" by "Complete".

Answers to editorial comments

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[We proofread the manuscript several times and tried to find and correct as many spelling and/or grammar mistakes as possible.](#)
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[We removed any commercial names from our manuscript \(e.g., we wrote 1.5-mL tube instead of 1.5-mL Eppendorf tube\)](#)
4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
[We reformatted the numbering.](#)
5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
[We removed any personal pronouns from the protocol text. However, they are still present in other subparts \(e.g., introduction, discussion, etc.\).](#)
6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
 - a. Lines 185-187: Please specify centrifugation parameters.
[Parameters were already specified.](#)
 - b. Line 197: Please provide the composition of binding buffer. If it is purchased, please cite the Table of Materials.
[We added the binding buffer's composition \(20 mM imidazole in 1 x PBS\). Now, the composition is outlined at several positions in the manuscript.](#)
 - c. Lines 199-200: Is the flow-through discarded?
[We added that the flow-through can be discarded.](#)

- d. Lines 216-217: Please add more details about how this is done.
Protein concentration determination procedures using a BCA or Bradford assay are familiar to most readers. We added the comment “according to the manufacturer’s instructions” to refer to how to perform such an assay.
- e. Lines 251-255: Because these steps have been highlighted for filming, please provide more details about imaging and immunoblot analysis. We need specific details for filming.
There is no special requirement for providing more details for this step. Theoretically, the gel can also be imaged using a scanner of a commercial multi-purpose printer. The filming (the responsible person) should just record how the researcher (Dominik Buser) is taking a gel image (can be discussed locally).
- f. Line 344: Please describe how to prepare sulfate labeling medium.
The preparation of this medium has been already outlined (lines 340-342). However, we think that this does not need to be filmed. Therefore, we did not highlight this part in yellow anymore.
- g. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
We combined some actions in the protocol steps 1-6.
- h. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We only highlighted the most necessary parts of the outlined protocol. We think that the highlighted passages will tell the most cohesive story of our application.
- i. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
If necessary, we corrected some of the sentences so that at least one action is written in imperative tense. Also we colored full sentences, however we did not color number of Addgene plasmids that also appear in some of the sentences.
- j. Please include all relevant details that are required to perform the step in the highlighting.
For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
We added and specified some sub-steps of the protocol.
- k. Please upload Table 1 to your Editorial Manager account as an .xls or .xlsx file.
A new table as an .xlsx file is provided.
- l. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
We think that we covered most of the points a)-e). But, we mention here again the specific points you are addressing. We think that our discussion provides a cohesive summary of the applied method, its potential and limitation. We hope that you consider our revised discussion for publication.

a) Critical steps within the protocol.

The following discussion point informs about critical steps of our method: *“A critical step of our technique is that handling with radioactivity is required to perform sulfate labeling of nanobodies. However, our tool of functionalized nanobodies with TS sites to study TGN arrival may be potentially applied without radioactivity using anti-sulfotyrosine antibodies.”*

b) Any modifications and troubleshooting of the technique

In the first paragraph we wrote: *„Functionalization can of course be also applied to the rapidly increasing number of nanobodies directed against untagged endogenous target proteins established by animal immunization or by ribosome-display and phage-display selection of synthetic VHH libraries.“*

c) Any limitations of the technique

The following discussion point informs about the limitation of our method: *“One drawback of our tool is that recombinant modification of target cell lines (stable expression or endogenous tagging) is required before functionalized anti-GFP nanobodies can be applied.”*

d) The significance with respect to existing methods

The following paragraph in the discussion informs about the significance when compared to other methods: *“Other groups have already made use of TS sites to follow transport from the cell surface to the TGN. Ricin, Shiga or pertussis toxin subunits have previously been modified with sulfation sites to demonstrate a transport route through the TGN. Moreover, TS peptides had also been chemically coupled to IgGs to assay retrograde transport of GFP-CIMPR and endogenous TGN46 to the TGN. Our sulfatable nanobodies have the advantage of simple and reproducible bacterial production and of a 1:1 stoichiometry with the target protein. On the contrary, it is for instance well known that divalent protein binders, such as IgGs, can crosslink cell surface proteins and alter their intracellular trafficking to lysosomes after endocytosis, highlighting the significance of monomeric protein binders with respect to existing methods.”*

e) Any future applications of the technique

The following paragraphs (subparagraphs) in the discussion informs about future applications: *“Apart from sulfatable nanobodies, other derivatizations also allow tracing transport through endocytic compartments and to the TGN. APEX2 can be applied as a promiscuous labeling enzyme for proximity-dependent biotinylation for proteomic analysis of retrograde transport. APEX2 nanobody that is internalized by a cargo reporter of interest will label proteins in close proximity within the target compartments. Comparative proteomics should allow to identify other endogenous proteins in the different types of endosomes and the TGN that the nanobody accesses. Many variations of nanobodies in combination with its target protein are conceivable. A recent report, for instance, applied an inverted approach to the one described here: derivatized GFP was used to study and trap cellularly expressed anti-GFP nanobody-tagged vacuolar sorting receptors in anterograde and retrograde compartments of the plant endomembrane system. Similarly functionalized nanobody-traps may be designed in mammalian cell culture systems to capture and accumulate EGFP-modified reporters in different compartments during retrograde transport.”*

„Sulfatable nanobodies can thus provide a useful biochemical tool to dissect the contribution of other retrograde transport machineries, such as epsinR, Rab9/TIP47 or SNX-BAR/retromer complexes, on cargo proteins by genomic, genetic or chemical manipulations. The protocol presented here offers a basis of how one can make use of functionalized nanobodies in general to determine target compartments, pathways, and transport kinetics in cultured cells.“

- m. References: Please do not abbreviate journal titles.
[We corrected this in the manuscript.](#)

Answers to editorial comments (“after Revision 1”)

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
[We corrected the manuscript for potential spelling or grammar mistakes.](#)
2. Please do not highlight note/caution for filming.
[We did not highlight “note/caution” anymore.](#)
3. The highlighted protocol steps are over the 2.75-page limit (including spacing and headings). Please highlight fewer protocol steps for filming.
[We highlighted less parts for filming. According to us, we are now in the 2.75-page range.](#)
4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
[We only have commercial names in the material and methods. We already removed them during our first corrections.](#)

References:

- 1 Buser, D. P., Schleicher, K. D., Prescianotto-Baschong, C. & Spiess, M. A versatile nanobody-based toolkit to analyze retrograde transport from the cell surface. *Proceedings of the National Academy of Sciences of the United States*. 115 (27), E6227-E6236, (2018).
- 2 Harmansa, S., Alborelli, I., Bieli, D., Caussin, E. & Affolter, M. A nanobody-based toolset to investigate the role of protein localization and dispersal in *Drosophila*. *eLife*. 6, (2017).
- 3 Kirchhofer, A. et al. Modulation of protein properties in living cells using nanobodies. *Nature Structural & Molecular biology*. 17 (1), 133-138, (2010).
- 4 Fridy, P. C. et al. A robust pipeline for rapid production of versatile nanobody repertoires. *Nature Methods*. 11 (12), 1253-1260, (2014).
- 5 Kubala, M. H., Kovtun, O., Alexandrov, K. & Collins, B. M. Structural and thermodynamic analysis of the GFP:GFP-nanobody complex. *Protein Science: a Publication of the Protein Society*. 19 (12), 2389-2401, (2010).

Nanobody	Protein sequence
VHH-std (Addgene 109417)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSEFDYPYDVPDYAGAQPARGSGGLNDIFEAQKIEWHEGALEHHHHHH
VHH-tev (Addgene 109423)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSENLYFQSEFDYPYDVPDYAGAQPARGSGGLNDIFEAQKIEWHEGALEHHHHHH
VHH-2xTS (Addgene 109419)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSAAEDYEYPSAEDYEYPSFEFDYPYDVPDYAGAQPARGSGGLNDIFEAQKIEWHEGALEHHHHH H
VHH-tev-2xTS (Addgene 117750)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSENLYFQSSAEDYEYPSAEDYEYPSFEFDYPYDVPDYAGAQPARGSGGLNDIFEAQKIEWHEGAL EHHHHHH
VHH-APEX2 (Addgene 109420)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSGKSYPTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAGTFDKGKTGGPFGTIKHPAE LAHSANNGLDIAVRLLEPLKAEFPILSYADFYQLAGVVAVEVTGGPKVPFHPGREDKPEPPPEGRLPDP TKGSDHLRDVFGKAMGLTDQDIVALSGGHTIGA AHKERSGFEGPWTSNPLIFDNSYFTELLSKEGL LQLPSDKALLSDPVFRPLVDKYAADEDAFFADYAEAHQKLSLGFADA EFDYPYDVPDYAGAQPARG GGGLNDIFEAQKIEWHEGALEHHHHHH
VHH-tev-APEX2 (Addgene 117751)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSENLYFQSGKSYPTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAGTFDKGKTGGPF GTIKHPAE LAHSANNGLDIAVRLLEPLKAEFPILSYADFYQLAGVVAVEVTGGPKVPFHPGREDKPEPP PEGRLPDPTKGS DHLRDVFGKAMGLTDQDIVALSGGHTIGA AHKERSGFEGPWTSNPLIFDNSYFTEL LSKEGELLQLPSDKALLSDPVFRPLVDKYAADEDAFFADYAEAHQKLSLGFADA EFDYPYDVPDYA GAQPARGSGGLNDIFEAQKIEWHEGALEHHHHHH
VHH-mCherry (Addgene 109421)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSMVSKGEEDNMAIIKEFMRFKVHMEG SVNGHEFEIEGEGEGRPYEGTQTAKLKVT KGGPLPFA WDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDG EFIYKVKL RGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLDGGHYDAEVKTTYKAKKPVQL PGAYNVNIKLDITSHNEDYTIVEQYERA EGRHSTGGMDELYKEFDYPYDVPDYAGAQPARGSGGLND IFEAQKIEWHEGALEHHHHHH
VHH-tev-mCherry (Addgene 117752)	MASMTGGQQMGRGSDQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREW VAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGGGTQVT VSSTSENLYFQSMVSKGEEDNMAIIKEFMRFKVHMEG SVNGHEFEIEGEGEGRPYEGTQTAKLKVT K GGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDG EFYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLDGGHYDAEVKTTYK AKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERA EGRHSTGGMDELYKEFDYPYDVPDYAGAQPARG SGGGLNDIFEAQKIEWHEGALEHHHHHH