Dear Dr. Wu,

Many thanks for editoring 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.

1. 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.

1. 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.

1. 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., 2018 1, 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.

1. 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.

1. The following comments from the authors would be helpful for the usage of the toolkit:
2. 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).

1. 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.

1. 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.

1. 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.

1. 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.

1. 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.

1. 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.

1. In Table of Materials, cOmplete must be Complete.

The manufacturer advertises the product with the name “cOmplete”. However, since this apparent spelling mistake was also mentioned in the editorial comments, we substituted “cOmplete” by “Complete”.

**Answers to editorial comments**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proofread the manuscript several times and tried to find and correct as many spelling and/or grammar mistakes as possible.

1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

We asked for copyright permission by PNAS.

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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)

1. 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.

1. 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.).

1. 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.
   1. Lines 185-187: Please specify centrifugation parameters.

Parameters were already specified.

* 1. 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.

* 1. Lines 199-200: Is the flow-through discarded?

We added that the flow-through can be discarded.

* 1. 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.

* 1. 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).

* 1. Line 344: Please describe how to prepare sulfate labeling medium.

The preparation of this medium has been already outlined (lanes 340-342). However, we think that this does not need to be filmed. Therefore, we did not highlight this part in yellow anymore.

* 1. 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.

* 1. 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.

* 1. 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.

* 1. 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.

* 1. Please upload Table 1 to your Editorial Manager account as an .xls or .xlsx file.

A new table as an .xlsx file is provided.

* 1. 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.“*

* 1. 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.

1. Please do not highlight note/caution for filming.

We did not highlight “note/caution” anymore.

1. 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.

1. 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., Caussinus, 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).