**Editorial comments:**

Changes to be made by the author(s) regarding the manuscript:

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

Thank you for the suggestion. The manuscript has now been checked using proofreading services, so we believe that there are no spelling/grammar issues.

2. Please revise lines 209-226 to avoid previously published text.

To address this issue, we have revised the discussion section, so as not to include the same sentences as in the previous publication.

3. 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 have uploaded the copyright permission obtained via the Copyright Clearance Center. We have also revised the figure legends to include the phrase “This figure has been modified from”, followed by the citation information.

4. Please provide an email address for each author.

The email address for each author is:

* leclerc@uthscsa.edu (Simon Leclerc)
* youri.arntz@unistra.fr (Youri Arntz)
* taniguchi@riken.jp (Yuichi Taniguchi)

5. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have added a Summary section:

“Here, we present a protocol to assess the labeling homogeneity for each protein species in a complex protein sample at the single molecule level.”

6. Please define all abbreviations (DTT, CHAPS, EDC, etc.) before use.

We have added definitions for the abbreviations in the main text, including FBS, PBS, EDTA, SDS, DTT, CHAPS, HEPES, PEG and EDC.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Thank you for the suggestion. We have revised the protocol to include only the imperative tense. Text that cannot be written in the imperative tense was moved to a “Note” or the discussion section. We have also described all safety procedures in the text, including laser safety.

8. 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.

We revised descriptions for cell collection (step 1.2) by providing a reference and details with multiple steps. We also provided further details for image analysis (step 6).

9. 1.2: Please specify centrifugation parameters (force in x g and time) as well as reaction conditions of trypsinization.

We added centrifugation parameters in step 1.2.6. We also described the reaction conditions of trypsinization in step 1.2.2.

10. 2.4: Please describe how to homogenize the solution.

We added the description “by slowly pipetting to avoid bubbles” to step 2.4. We also added the same description to steps 2.5, 2.6 and 2.7.

11. 3.1: Please provide the composition of SDS sample buffer.

We have added the composition of SDS sample buffer (200 mM Tris-HCl pH 6.8, 4% SDS, 4% glycerol and 0.4% bromophenol blue) to step 3.1.

12. 3.4: Please specify specific protein bands selected in this protocol.

We have noted the band information in step 3.4.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have added single-line spaces in the revised manuscript.

14. 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 have highlighted essential steps of the protocol in yellow.

15. 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.

16. 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 have adhered to the above points regarding the highlighting.

17. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We have discussed critical steps in the protocol in the Discussion section. Particularly, we consider the avidin and biotinylated protein glass coating step to be critical, so we created a new paragraph to discuss this (the paragraph starting from “To precisely measure”).

18. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We have sorted the items in alphabetical order in the Table of Equipment and Materials.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

The manuscript is basically a short version of a recently published paper by the authors in bioconjugation chemistry, with all figures taken from this publication (with reference and copyright statement).

The authors describe a method that allows for the analyses of the efficiency and homogeneity of fluorescent labelling of proteins samples and apply the technique to HELA lysates. It describes an initial step of Cy3 labelling of the protein sample (the labeling whose efficiency will be analyzed). Next they label the protein sample with Biotin. This will allow the protein sample to bind to a avidin labeled microscope slide later in the protocol. Once labelled, the proteins sample is separated using SDS-PAGE, electro-eluted, and spotted on a microscope slide that is coated with avidin. Next, sample can be analyses by fluorescent microscope. Since this procedure produces individual spots, that according to the authors represent single molecules, for each spot the intensity can be determines, which should be step-wise increase depending on how many fluorophores have been conjugated onto that individual molecule. Besides the ability to determine the average number of fluorophores attached, the authors can also determine how many of the molecules have been labelled by comparing the number of fluorescent spots with a positive control that is 100% labeled. Here the assumption is that the number of avidin spots on each slide is identical, so no fluorescent spots are occupied by Biotin-labeled protein that lacks fluorophore label.

Overall, the technique is interesting and valuable, as I think it is important for researchers to know what fraction of their proteins is labeled. That said, some sections were thin on their description, which might be addressed in the video production and maybe a good reason to warrant a video protocol as I cannot readily envision from my experimental background what or how it takes place ( e.g.line 130 up air plasma and microscope slide treatment).

Major Concerns:

-The third lane in figure 2 is identical to others, but shifted. One band visible is all three lanes is labeled 55 kDa in lane 2 and in lane three it is at the top of a molecular range section that spans from 54-69, suggesting it is 69 kDa. This is impossible and something is wrongly assigned.

We thank the reviewer for this important point. We agree that the 55 kDa band was wrongly assigned. We have corrected the indication of the 55 kDa band in the figure.

-Some discussion of limitations or some key potential pitfalls would be valuable.

-Technically I was not completely sure on what limits the spots on the slide, is there limited avidin, or is the protein concentration so low that there is only limited binding? I saw the supplement of the original paper has some titration curve, where depending on the protein concentration the binding plateau's. Also, doesn't avidin bind 4 biotin molecules, leaving the potential for four molecules at a spot instead of one? As the protocol is published and the current version has limited technical details, I did not dive into a full critical analyses here, but more general I think the manuscript would benefit from discussion of some potential pitfalls or points of caution. Comparing a variety of very different conditions with a very different independent positive control can be tricky and I was wondering how crucial are accurate protein concentration measurements etc. There seems to be potential for misleading result sin certain steps are not carefully executed at the right concentration. Some discussion or clarification here would be valuable.

Thank you for the useful suggestions. Although some of these questions were discussed in the original paper, we agree that it is important to cover them in this manuscript. As the referee may expect, the spot number is limited by the number of avidin molecules attached to the slide, because subsequent binding of proteins to avidin is saturated. Therefore, in our assay, it is important to use a sufficient quantity of biotinylated protein sample, and too little sample results in underestimation of *LO*. The avidin density on the slide has been optimized to provide sufficient spot numbers for statistical analysis with no overlaps between spots in images.

As for the avidin binding site issue, as the referee argued, this assay assumes that one tetravalent avidin molecule on the coverslip can bind one protein. Indeed, the positive control data in our previous study (Leclerc et al., 2018) indicated that one avidin molecule can bind to up to four fluorescently labeled biotin molecules, but the majority (55%) of avidin molecules bind only one or two biotin molecules. Because proteins are larger than biotin, their steric effects should result in fewer proteins bound to one avidin molecule. Our assumption is further supported by the observation in the previous study that dye remains constant when measuring mixed samples of labeled and unlabeled proteins at different ratios. We added these arguments to the Discussion section (the paragraphs starting with “To precisely measure” and “This assay assumes”).

Minor Concerns:

-How do authors know that positive control is truly 100% labelled?

-Is the biotin labelling 100%? Probably does that not matter as long as there is no bias in the labelling?

We referred to this as “100% labeled” because the product was created through chemical purifications following biotin conjugation reactions. In fact, the company assures at least 95% purity (http://www.nanocs.com/PEG/BPEG.htm). To avoid confusion, we have changed “100%” to “nearly 100%”.

-The authors in the original manuscript show a nice overview cartoon in figure 2 that would be a helpful overall picture of the protocol.

We agree with the reviewer. We have included the overview cartoon as a new Figure 2.

-Especially for a methods story care should be taken to clear and accurate description. Eg. Line 81. It is ambiguous if samples should be stored as pellet or as PBS resuspended sample. I assume pellet, but better to be clearly stated. Line 114 incubate at RT?

We agree that some descriptions were ambiguous. In Line 81, PBS resuspended sample was aliquoted. In Line 114, the sample was kept at room temperature. We have revised these descriptions.

-There seems to be only limited discussion on alternative ways people have addressed this, what other approaches have people used?

Thank you for the suggestion. In our protocol, the protein separation step with SDS-PAGE can be substituted with other methods such as liquid chromatography or capillary electrophoresis. In addition, the labeling method using NHS-ester can be replaced with other methods using maleimide-ester or antibodies. We have added these alternatives to the discussion section (the paragraph starting with “This paper describes”).

-Many awkward or unusual English sentence constructions are used, need to be editing, e.g. line 111 The protocol can terminate here.

This version of the manuscript has been proofread by a commercial English editing service (Life Science Editors, <https://lifescienceeditors.com/>).

-Title is hard to read.

We changed the title to ”Proteome-wide quantification of labeling homogeneity at the single molecule level”.

Original: Proteome-wide labeling homogeneity of a complex protein sample at the single molecule level

-Line 47 is CB a fluorescent dye?

Coomassie blue (CB) is indeed more often used as a visible dye, but it can also be used as a fluorescent dye. We added an additional reference demonstrating this (Butt and Coorssen, 2013).

-section 6 is important for the protocol as it is the key analyses of the data, but rather limited in its description.

We have added descriptions for the image analysis in section 6. In particular, we have added descriptions for laser pattern correction (step 6.1) and derivation of dye (step 6.5), and image analysis parameters for background subtraction (step 6.2) and spot filtering (step 6.3).

**Reviewer #2:**

Manuscript Summary:

The authors describe a method to determine labeling homogeneity of nonspecific labels across proteins in biological samples. Such labels are used for detection in SD gel electrophoresis based assays, but labeling efficiency has typically been determined at the bulk level. This method enables labeling heterogeneity to be determined. This work should be of interest to those performing labeling studies of proteins.

Major Concerns:

None

Minor Concerns:

-The authors state on Line 38 that "Proteome analysis, which quantifies the entire set of protein molecules expressed in the cell, is a demanded approach in current biological and medicinal studies." In fact, proteomic analyses never quantify the entire set of proteins despite efforts to do so. We only ever quantify a fraction of the ~20000 expressed proteins. This should be corrected.

We agree with the reviewer. We have changed the expression “which quantifies the entire set” to “which aims to quantify the entire set”.

-Mass spectroscopy on Line 40 should be changed to mass spectrometry.

Thank you for the suggestion. The word has been corrected.

-The rest of the manuscript is in need of some copy editing.

This version of the manuscript was copy-edited using a professional proofreading service.