**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript (58922):

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

The manuscript was proofread by a native speaker.

2. 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 editorial policy statement of Frontiers in Neuroanatomy, granting us permission to re-use the figures. We have also changed the citation in the manuscript, now saying:

“This figure has been modified from Wallrafen and Dresbach (2018)1”

3. Please provide an email address for each author.

We have added the email addresses for TD and JSV.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have included an ethics statement before the protocol saying:

“ETHICS STATEMENT:

No experiments involving live animals were conducted for this study. Experiments involving euthanizing of animals to obtain brain samples were approved by the local animal protection authorities (Tierschutzkommission der Universitätsmedizin Göttingen) under the approval number T 10/30.”

5. 1.2, 2.7: Please number the tables, instead of referring to table Solutions, table antibodies, etc.

We have numbered the tables and added the numbers at the appropriate positions in the protocol.

6. 1.3: Please specify the age, gender and type of the animal used.

We have specified the age, gender and genetic background of the animals. The section now says:

“For this protocol, 3 adult male C57BL/6 mice were used.”

7. 1.4: Please specify all surgical instruments used.

We have added the surgical instruments used in step 1.4. in the protocol. This section now says:

“1.4. Open the skull with scissors and carefully isolate the brain using a spoon with blunt edges to avoid damaging the tissue.”

8. 1.6: What volume of PB is used to wash? How long is the washing process?

We have added the volume and duration of the washing process. This section now says:

“Remove the fixative and wash the brain in 50 mL 0.1 M PB on a shaker for 30 min.”

9. 3.1: Please remove commercial language and use generic terms: ZEN2, Zeiss, etc.

We have removed the commercial terms and refer to it now as imaging software.

10. Figures 2 and 3: Please explain what the black bars and dots are in the bar graphs.

We have explained the bars and dots. This section now says:

“Black dots represent single data points. Bars show the mean ± S.E.M.”

11. Table 1: Please include a space between numbers and their units (0.1 M PB).

We have included a space between numbers and their units.

12. Tables 1 and 2: Please remove commercial language (Triton X-100, Alexa Fluor) and use generic terms instead.

We have removed the commercial language. Instead of Triton we refer to “detergent”. We have exchanged the name of the fluorophore for the emission wavelength.

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

We have adjusted the discussion. The following paragraph now mentions the critical steps more explicitly:

“As with every immunofluorescence protocol, be it qualitative or quantitative, several factors can influence the success and thereby confound the analysis. Therefore, special attention should be paid to critical steps of the protocol. First, a proper fixation of the tissue is needed. This fixation can usually be achieved by a successful transcardial perfusion. The quality of perfusion can be verified by checking the liver shortly after washing out the blood. A first indicator for a successful perfusion is the clearing of the liver and extremities27. The presence of blood clots can indicate that the perfusion might have been too slow and should be performed faster next time. Some proteins require different fixation protocols, as chemical fixation with PFA can cause epitope blockage29. In this case, freeze fixation or fixation with a different chemical, such as methanol, should be considered. Second, after sectioning, it is critical to stain the brain slices as quickly as possible, preferably on the same or the next say. Longer storage in PB can lead to bacterial infection, and while adding NaN3 can prevent this to some extent, the tissue quality usually deteriorates with storage time. Third, during the staining procedure, it is important to perform washing steps well-by-well to avoid drying of the slices. When the slices dry out, background fluorescence can increase and thus cause a bias in the analysis. Fourth, after application of the secondary antibody, it is vital to perform all following steps in the dark. The fluorophores are light-sensitive, and light exposure can severely distort the fluorescence signal.”

The following paragraph deals with the limitations of the method:

“A caveat of the approach is that the different levels in the brain cannot directly be compared with each other. Hemispheres with many regions rich in the protein of interest will have a different average value than hemispheres with only few protein-rich regions. Values of 20% above average, for example, will therefore reflect a different absolute quantity of protein in one level relative to Bregma as compared to a second one. One has to keep in mind as well that this method does not allow the determination of absolute protein levels, only the relative abundance compared to the internal reference marker and the average across the hemisphere.”

14. References: Please do not abbreviate journal titles.

We changed the referencing style so that Journal titles are no longer abbreviated.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

This is an interesting and straightforward approach which adds to the collection of existing protocols to analyze synapses in brain.

Authors provided a good degree of details and elaborated on technical aspects of their new protocol. It appears that their approach can be tweaked and applied to other synaptic markers and even to 4-6 multiplex fluorescence IHC.

Major Concerns:

Authors need to show example images of 2-color staining of Mover and Synaptophysin in couple brain regions.

We have added an overlay of the two different channels (i.e. Mover and Synaptophysin) across the hemispheres to figure one. Mover can be seen in red, Synaptophysin in green. We have adjusted the figure legend accordingly, now saying:

“Figure 1: Representative immunofluorescence images of DAPI (first row), Mover (second row), Synaptophysin (third row) and their overlay (fourth row, Mover in red, Synaptophysin in green) at the 5 rostro-caudal levels (A-E).”

We have also added a description to the results section:

“An overlay of the two proteins (fourth row) shows the differential distribution of Mover (red) compared to the marker protein Synaptophysin (green).”

Authors are in favor of confocal microscopy but comparable image quality can be achieved using conventional fluorescence microscopy combine with deconvolution software - please mention this in the text which should expand the usability of your protocol.

We have mentioned the option to use epifluorescence microscopy combined with deconvolution software. In the protocol, there is a “Note” stating:

“Note: Epifluorescence microscopy combined with deconvolution software can achieve similar image quality.”

We have also added this aspect to the discussion. The paragraph now says:

“While the use of a confocal microscope is the authors’ method of choice, a combination of epifluorescence microscopy and deconvolution software should yield the same data quality and thus expand the usability of the protocol.”

Minor Concerns:

Check English grammar (e.g. Stocksolution should read Stock Solution) and adjust technical terminology to accepted norms. Elaborate more on calculating the relative abundance described in 5.3: give couple examples with real measured fluorescence intensities.

The manuscript was proofread by a native speaker. Additionally, we have added a new table (table 3), showing example calculations and how to perform them.

**Reviewer #2:**

Manuscript Summary:

In the protocol" Quantifying the heterogeneous distribution of a synaptic protein in the mouse brain

using immunofluorescence" by Wallrafen R et al., the authors propose a method for quantifying the relative abundance of a synaptic protein in the mouse brain, using immunofluorescence staining and confocal analysis. In detail, they describe the sample preparation, the immunostaining technique and the image analysis. As a representative result, they report the immunolocalization of Mover, a vesicle associated protein important for neurotransmitter release at synapses.

There are several quantitative immunofluorescence methods reported in literature. In the proposed article, the authors quantify the relative staining rather than the absolute staining of the protein of interest (Mover /synaptotagmin) in a particular brain region and they normalize this ratio to the "two stainings mean fluorescence ratio" measured in the corresponding hemisphere. In this way, they avoid biases due to the non homogeneous distribution of structures (synapses) in the brain or the immunostaining procedure itself. This is the "novelty" and the most important part of the method.

Major Concerns:

INTRODUCTION. Several information about the relative abundance of proteins in brain areas has been obtained by immunocytochemistry and/or immunofluorescence and several methods report how to obtain quantitative data from immunofluorescence images. The authors should discuss these methods in the introduction and explain the limitation of previous immunofluorescence /immunohistochemistry studies.

We added information on a study comparing immunofluorescence to mass spectrometry. We would be happy to include a comparison with other papers that the reviewer deems worth mentioning.

The affirmation (pag 4 ) "The protocol here described takes this into consideration and avoids such biases, as well as other caveats that arise from immunohistochemical methods" does not explain the caveats arising from immunochemical methods, please explain.

We added an explanation on the heterogeneity of the structures themselves (synapses) and a comment on the intrinsic variability of immunohistochemistry. The paragraph now says:

“Therefore, this non-homogeneous distribution of structures, in this case synapses, can lead to a false estimation of the distribution of the protein of interest itself. Furthermore, there is an intrinsic variability in staining intensities across samples in immunohistochemical stainings.”

PROTOCOL.

-The immunofluorescence staining reported in the protocol (section 2) is relatively usual; the novelty is linked to the image analysis method they adopt. This part should be emphasized and explained in details.

We have added a new table (table 3) with example calculations.

- Data analysis. As stated by the authors in the discussion, the background signal can severely affect immunofluorescence signal quantification even if the relative, rather than absolute, signal intensity is calculated. Therefore, usually, it is fundamental to subtract the background signal before data processing. The autofluorescence signal derived from brain sections (in particular those fixed with PAF and examined in the FITC channel) can be particularly high and can mask the specific antibody staining.

We have adjusted the protocol and added a step for a potential background subtraction. This is mentioned in step 2.4 and a new step 5.1. now saying:

“Note: To determine the background fluorescence, stainings should also be performed without adding the primary antibody. For that, incubate the slice in antibody solution without primary antibodies according to the protocol.”

and

“5.1. In case the background fluorescence is high (see Section “Discussion”) a background subtraction might be needed. For that, determine the mean fluorescence intensity for the slice processed without primary antibody against the reference protein (here: Synaptophysin) and subtract that value from all values obtained for the brain regions and hemispheres.”

respectively.

Why the authors in their analysis did not envisage a control sample (stained only for secondary antibodies) and exploit the average fluorescence of this sample as a background signal?

We performed the experiments suggested by the reviewer. When imaging the samples stained without primary antibody, we could detect hardly any fluorescence (intensity values lower than 200 A.U. on a 16-bit scale (i.e. on a scale of [0, 65535]). We therefore decided that a background subtraction is not necessary and refrained from doing it.

DISCUSSION. The discussion section is not well organized: for example, the importance of the internal reference is repeated twice.

We have substantially revised the discussion section, both regarding the content as well as the organization.

- The optimization of the staining procedure (selection of primary and secondary antibodies, optimal antibody concentration, and exposition time) is a prerequisite to carry out a reliable quantitative immunofluorescence analysis. In the discussion the authors must emphasize this aspect.

We have added the aspect mentioned by the reviewer to the discussion section. This part now says:

“The optimization of the staining procedure, including the selection of adequate primary and secondary antibodies, optimal antibody concentration, and exposition time, is a prerequisite to achieve the best signal-to-noise ratio and to carry out a reliable quantitative immunofluorescence analysis.”

-As above reported, the background signal has an important impact on the result. "high background fluorescence" is poorly informative and it is difficult to establish the "adequate" background fluorescence for a student approaching for the first time the method.

Is it possible to define the "adequate" background in a quantitative manner (for example the mean fluorescence intensity of the stained section must be x fold higher than that measured in the control sample)?

We have expanded the paragraph to tackle this concern. However, we think that it is not trivial to define the “adequate” background. In this section we suggest that the signal to noise ratio should at least be 2:1 (i.e., the fluorescence signal should be 2-fold higher than background), but this is a more or less arbitrary number based on our experience with Synaptophysin- and Mover stainings. For other proteins the ratio can be different. We state this in the discussion as follows:

“The amount of background fluorescence arising from autofluorescence and unspecific binding of the secondary antibody can be estimated by imaging the slices in which the primary antibody was not applied. It is not trivial to establish how much higher the intensities of the signal need to be when compared to the background to have an acceptable signal-to-noise ratio. However, based on empirical observations, the authors would suggest to aim for having a signal at least 2-fold stronger than background in order to reliably estimate the protein distribution. In case the background fluorescence is high in control conditions (without the presence of the primary antibody) the average background fluorescence should be subtracted from the experiment images.”

Minor Concerns:

- Pag 3. "The method of choice for quantification of synaptic proteins - so far - is biochemistry, specifically Western blots, rather than immunohistochemistry6-8".

Reference 6-8 only reports western blotting quantification. Several excellent papers report protein quantification by immunocytochemistry, no reference about this method has been reported in the reference section.

Protein quantification by immunocytochemistry is usually used to compare the intensity of the immunosignal for a certain protein in different areas of a tissue or with and without a certain experimental condition. The readout of these assays is quantitative. However, when applied to synaptic proteins, this quantitative readout does not reveal whether one protein has increased or decreased in expression, or whether the entire synapse with all its components has increased or decreased in size. Likewise, when applying this to - e.g. mitochondria - the quantitative readout does not reveal whether one protein or the entire organelle has changed. We describe a protocol that specifically addresses this issue, and allows the researcher to solve two questions: a) is the immunosignal for a protein changed? b) is this true for the entire compartment or specifically for this protein? We would be happy to include a comparison with other papers addressing this issue that the reviewer deems worth mentioning.

- Recommend English Grammar and Word Usage Review

The manuscript was proofread by a native speaker.