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Editorial Office

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Dear editors, dear reviewers,

Thank you for the comments and suggestions. We have uploaded the revised manuscript and the rebuttal, where we address every comment individually. The changes in the manuscript are tracked in green font color.

We hope you find the approach and the way we describe it interesting and suitable for consideration for JoVE.

Sincerely,

Rebecca Wallrafen

TITLE:

Quantifying the Heterogeneous Distribution of a Synaptic Protein in the Mouse Brain Using Immunofluorescence

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KEYWORDS:

Immunofluorescence, confocal microscopy, quantification, synaptic proteins, mouse, Mover, distribution

SUMMARY:

Here, we describe a quantitative approach to determining the distribution of a synaptic protein relative to a marker protein using immunofluorescence staining, confocal microscopy, and computer-based analysis.

ABSTRACT:

The presence, absence, or levels of specific synaptic proteins can severely influence synaptic transmission. In addition to elucidating the function of a protein, it is vital to also determine its distribution. Here, we describe a protocol employing immunofluorescence, confocal microscopy, and computer-based analysis to determine the distribution of the synaptic protein Mover (also called TPRGL or SVAP30). We compare the distribution of Mover to that of the synaptic vesicle protein synaptophysin, thereby determining the distribution of Mover in a quantitative manner relative to the abundance of synaptic vesicles. Notably, this method could potentially be implemented to allow for comparison of the distribution of proteins using different antibodies or microscopes or across different studies. Our method circumvents the inherent variability of immunofluorescent stainings by yielding a ratio rather than absolute fluorescence levels. Additionally, the method we describe enables the researcher to analyze the distribution of a protein on different levels: from whole brain slices to brain regions to different subregions in one brain area, such as the different layers of the hippocampus or sensory cortices. Mover is a vertebrate-specific protein that is associated with synaptic vesicles. With this method, we show that Mover is heterogeneously distributed across brain areas, with high levels in the ventral pallidum, the septal nuclei, and the amygdala, and also within single brain areas, such as the different layers of the hippocampus.

INTRODUCTION:

Communication between neurons happens at specialized contact sites called synapses. Synapses contain a myriad of different proteins that orchestrate synaptic transmission. Some of those proteins show a heterogeneous distribution throughout the nervous system and are not present in every synapse[1]. One example for such a protein is Munc13, which is involved in the priming process of synaptic vesicles. There are different isoforms of Munc13, which are heterogeneously distributed throughout the brain[2], and the presence or absence of specific isoforms can influence short-term synaptic plasticity and synaptic vesicle dynamics[3-5]. Therefore, it is of vital importance to be able to identify the presence of different synaptic proteins across brain areas.

The methods of choice for quantification of synaptic proteins – so far – are mass spectrometry and Western blotting, rather than immunohistochemistry[6-9]. In some cases, several methods are used to complement each other to assess both the quantity and the localization of specific proteins (*i.e.*, Wilhelm *et al.*[10]). The method we describe here allows for the localization and quantification of proteins of interest without the need of using any biochemical method, simply employing immunofluorescent stainings. Another advantage here is that the quantification can be done over areas much smaller and, therefore, more specific, than those achieved by other methods. However, one has to take into consideration that a reliable reference protein is needed to assess the distribution of the protein of interest.

Fluorescent staining by immunohistochemistry allows us to routinely identify the localization of proteins across brain areas as well as within different neuronal compartments. To identify the different compartments, specific markers are used. Typically, antibodies against synapsin and synaptophysin[11] can be used to label synaptic vesicles, while antibodies against bassoon label the active zone of a presynaptic terminal[12]. Vesicular transporters, such as the vesicular glutamate transporters (vGluT) or vesicular GABA transporter (vGAT), are used to label excitatory[13] and inhibitory[14] presynaptic terminals, respectively. On the postsynaptic side, antibodies against the Homer protein can be employed to mark postsynaptic terminals, and antibodies against postsynaptic density protein 95 (PSD95)[15-17] or gephyrin[18-20] can label excitatory or inhibitory postsynaptic terminals, respectively. By using antibodies against a protein of interest and markers such as the ones described above, one can determine the localization of such protein. Many studies to date have done this in a qualitative manner[21]. However, to reliably determine the differential distribution of a specific synaptic protein, one must not only determine its presence or absence but also its relative concentration. The heterogeneity of sizes and density of synapses makes it important to establish a ratio between the synaptic marker and the protein of interest. Otherwise, synapse-rich regions such as the non-pyramidal layers of the hippocampus and the molecular layer of the cerebellum will show a high density of synaptic proteins, only due to the higher density of synapses but not due to a strong presence of that protein in each synapse (*e.g.*, Wallrafen and Dresbach[1]). On the other hand, proteins in the neuronal soma (*e.g.*, TGN38[22]) will usually show strong presence in the hippocampal pyramidal cell layer or hippocampal or cerebellar granule cell layer due to the high concentration of neuronal cell bodies in those areas. 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. The protocol described here takes this into consideration and avoids such biases, as well as other caveats that arise from immunohistochemical methods.

In our recent study, we have used this method to describe the differential expression of Mover (also called TPRGL[23] or SVAP30[24]) across 16 different brain areas[1]. Mover is a vertebrate-specific synaptic protein that can be found in association to synaptic vesicles and influences neurotransmitter release[25-27]. We have related the Mover expression to the abundance of synaptic vesicles, by staining for synaptophysin as a synaptic vesicle reference marker. We found high levels of Mover particularly in the septal nuclei, the ventral pallidum, and the amygdala. Within the hippocampus, we found a heterogeneous distribution of Mover, with high levels in the layers associated with intra-hippocampal computation, and low levels in input- and output layers.

PROTOCOL:

This protocol does not involve experiments on live animals. 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.

NOTE: For this protocol, 3 adult male C57BL/6 mice were used.

1. Sample Preparation

1.1. Prepare fixative and 0.1 M phosphate buffer (PB; see **Table 1**).

1.2. Fix the animal by transcardial perfusion as described in Gage *et al.*[28]. First wash out the blood with 0.9% NaCl-solution, then perfuse with 30 mL of 4% paraformaldehyde (PFA).

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

1.4. Fill a 50 mL reaction tube with fixative and postfix the brain in 4% PFA at 4 °C overnight.

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

1.6. After washing, incubate the brain in a 50 mL reaction tube in 30% sucrose in 0.1 M PB for 48 h or until it sinks in the tube at 4 °C for cryoprotection.

1.7. Trim the cryoprotected brain with a sharp blade, place it in a cryomold, and embed it with optimal cutting temperature (OCT) compound. Avoid bubbles. Orient the brain and freeze the cryomold in the -80 °C freezer.

1.8. Mount the frozen tissue for sectioning. Equilibrate the tissue to the cryomicrotome temperature for at least 15 min before sectioning.

1.9. Section the brain into 25 µm thick coronal slices. Touch the OCT carefully with a glass hook without touching the brain tissue. Collect 3 adjacent slices per well in a 24 well plate and store them in 0.1 M PB at 4 °C until staining.

NOTE: The protocol can be paused here for up to two weeks. Longer storage times can interfere with the tissue quality and thus influence the outcome of the experiment.

2. Immunofluorescence

2.1. Prepare solutions including the blocking buffer, antibody buffer, washing buffer 1, and washing buffer 2 (see **Table 1**).

2.2. Rinse slices once with PB to remove excess OCT.

2.2.1. Remove the solution with a plastic pipette without sucking in the brain slices. Add 250 µL of fresh PB with a 1000 µL pipette.

CAUTION: Slices should not dry out, so remove and add fluids well by well.

2.3. Remove the PB with a plastic pipette and add 250 µL of blocking buffer per well with a 1000 µL pipette. Incubate for 3 h at room temperature (RT) on the shaker.

2.4. During the incubation time, dilute the primary antibodies in antibody buffer in a reaction tube. Use 250 µL antibody buffer per well and add the appropriate amount of antibody (see **Table 2**) by pipetting it directly into the solution using a 2 µL pipette. Mix the solution by gently pipetting up and down several times. Vortex shortly afterwards to ensure proper mixing.

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.

2.5. After the incubation time, remove the blocking buffer with a plastic pipette and add 250 µL of antibody solution containing primary antibodies per well. Incubate slices with primary antibody overnight at 4 °C on a shaker.

2.6. Next day, wash the slices with washing buffer 1 3x for 10 min at RT on a shaker.

2.6.1. Remove the medium with a plastic pipette and add 300 µL of washing buffer 1 per well. Incubate at RT for 10 min. Repeat 3 times.

2.7. During the washing steps, dilute the fluorophore-coupled secondary antibodies in antibody buffer in a reaction tube. Use 250 µL antibody buffer per well and add the appropriate amount of antibody (see **Table 2**) by pipetting it directly into the solution using a 2 µL pipette. Mix the solution by gently pipetting up and down several times. Vortex shortly afterwards to ensure proper mixing.

CAUTION: Because the antibodies are light-sensitive, all steps from this point on need to be performed in the dark.

2.8. After the washing steps, remove the washing buffer with a plastic pipette and add 250 µL of antibody solution containing secondary antibodies per well. Incubate the slices with secondary antibody for 90 min at RT in the dark.

2.9. Wash the slices with washing buffer 2 3x for 10 min at RT.

2.10. During the washing steps, dilute 4',6-diamidino-2-phenylindole (DAPI) in 0.1 M PB in a concentration of 1:2000.

2.11. Remove the washing buffer 2 with a plastic pipette and add 250 µL of DAPI solution per well. Incubate for 5 min at RT on the shaker.

2.12. Remove the DAPI solution with a plastic pipette and add 500 µL of 0.1 M PB per well with a 1000 µL pipette.

2.13. Mount slices on microscope slides.

2.13.1. Place a microscope slide under the stereoscope. With a fine brush, add three separate drops of 0.1 M PB onto the slide. Place one slice per drop onto the microscope slide.

2.13.2. Use the fine brush to flatten and orient the slices on the microscope slide.

2.13.3. When all slices are positioned correctly, remove excess PB with a tissue and dry the slide carefully.

CAUTION: Avoid drying the brain slices completely.

2.13.4. Add 80 µL of embedding medium onto the slide. Carefully lower the coverslip onto the slide, thereby embedding the brain slices.

2.13.5. Leave the slides to dry in the fume hood for 1-2 h (cover them to avoid light exposure) and store them in a microscope slide box at 4 °C.

NOTE: The protocol can be paused here.

3. Imaging

3.1. After the embedding medium is completely hardened, place the microscope slide under the confocal microscope.

NOTE: Epifluorescence microscopy combined with deconvolution software should yield similar image quality.

3.2. Adjust the laser settings by increasing or decreasing the laser intensity for every channel so that few pixels are overexposed to ensure maximum distribution of grey values.

3.3. Acquire virtual tissues of the whole brain slice for the different channels.

3.3.1. In the imaging software (see **Table of Materials**), select the **Tiles** option and manually delineate the brain slice with the **Tile Region Setup**.

3.3.2. Distribute support points throughout the tile region and adjust the focus for the different support points by pressing **Verify Tile Regions/Positions…**.

3.3.3. Adjust the settings in **Acquisition Mode** according to the desired resolution and file size of the resulting image and start the scan.

3.4. When the scan is finished, use the **Stitching** function to process the virtual tissue. Export the file as a .tif with the function **Image Export**.

4. Computer-based Analysis

4.1. Load all single channels for one image into FIJI[29] by clicking **File| Open**.

4.2. With the **Freehand selection** tool, delineate one hemisphere in the DAPI-channel. Create a mask of the selection by clicking **Edit| Selection| Create mask**.

4.3. Determine the mean fluorescence intensity for the single channels (Mover and synaptophysin) by clicking **Analyze| Measure**.

NOTE: Make sure to select the different channels to determine the mean fluorescence intensity values for each channel.

4.4. Copy the mean fluorescence intensity for the single channels into a spreadsheet.

4.5. Determine the mean fluorescence intensity for the single channels in an area of interest by delineating the area also with the **Freehand selection** tool. Use a mouse brain atlas as reference.

4.6. Repeat steps 4.1-4.5 for all hemispheres and all areas of interest.

NOTE: Determine the values for each hemisphere separately in order to later compare the values in an area of interest to that in the hemisphere (see step 5.2).

5. Data Handling

5.1. In case the background fluorescence is high (see **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.

5.2. When the mean fluorescence intensities for the single channels for every hemisphere and every area of interest have been determined (see **Table 3**), calculate the ratio of Mover to synaptophysin by dividing the value for Mover by the value for synaptophysin (yellow in **Table 3**). Perform this action for every hemisphere and every area of interest separately.

5.3. Divide the ratio obtained for one area of interest by the ratio obtained for the corresponding hemisphere (orange in **Table 3**) to determine the ratio of the area of interest to the hemisphere.

5.4. To determine the relative Mover abundance, translate the ratio obtained in 5.2 into a percentage by determining its deviation from 1 (red in **Table 3**). A ratio of 1.25 would therefore give a relative Mover abundance of 25% above average, and a ratio of 0.75 would yield a relative Mover abundance of 25% below average.

REPRESENTATIVE RESULTS:

Representative staining patterns of different markers can be seen in **Figure 1**. The pattern varies depending on the distribution of the protein. Examples of five rostro-caudal levels are shown in columns (**A**)-(**E**). A representative DAPI staining is shown in the first row: DAPI adheres to the DNA of a cell and thus nuclei are stained. This results in a punctate pattern. Regions with a high cell density are brighter than regions with low cell densities. An example for a heterogeneously distributed protein can be seen in the second row. The Mover staining reveals a differential distribution throughout the brain, with bright hotspot areas and dimmer areas. In the third row, an example for the more homogeneously distributed reference marker synaptophysin is shown. An overlay of the two proteins (fourth row) shows the differential distribution of Mover (red) compared to the marker protein synaptophysin (green).

Figure 2 shows the quantification described in step 4 of the protocol. Shown are the mean fluorescence intensity values for the different channels across the hemispheres (Mover, **Figure 2A**; synaptophysin, **Figure 2B**) and across the areas of interest (Mover, **Figure 2C**; synaptophysin, **Figure 2D**). To determine the Mover abundance relative to the number of synaptic vesicles, a ratio is taken of the Mover fluorescence values to synaptophysin fluorescence values. These ratios for the areas of interest are shown in **Figure 2E**, and already

provide an indication of the heterogeneous distribution of Mover, with areas with high and low Mover levels relative to synaptic vesicles. To additionally compensate for the inherent technical variability, the ratio in one area of interest (**Figure 2E**) is compared to that across the hemisphere (not shown) and translated into a percentage. This relative Mover abundance (**Figure 2F**) gives a measure of how much Mover is present in one area of interest relative to average.

As mentioned above, one of the major advantages of this technique is the ability to determine the abundance of the protein of interest across very small areas, even subregions and layers of areas of interest. One example of this application is shown in **Figure 3**, where the relative Mover abundance was determined for the different layers in the subfields of the hippocampus. The quantification in the different layers shown in **Figure 3D**, **Figure 3F**, and **Figure 3H** corresponds to the layers shown in **Figure 3C**, **Figure 3E**, and **Figure 3G**, with the corresponding colors. Within the hippocampus, Mover is heterogeneously distributed, with high Mover levels relative to synaptic vesicles in layers associated with intra-hippocampal computation (*i.e.*, the polymorph layer of dentate gyrus [DG], stratum radiatum, lucidum and oriens of *Cornu Ammonis* 3 [CA3], and stratum radiatum and oriens of *Cornu Ammonis* 1 [CA1]), and low levels in input- and output layers (the inner and outer molecular layer of DG, the pyramidal cell layers of CA3 and CA1, and the stratum lacunosum-moleculare of CA1).

FIGURE AND TABLE LEGENDS:

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). Areas of interest are shaded in grey in the upper row of panels. M1, primary motor cortex; IoC, islands of Calleja; ACC, anterior cingulate cortex; SNu, septal nuclei; VPa, ventral pallidum; NuA, nucleus accumbens; CP, caudate putamen; S1, primary somatosensory cortex; Hc, hippocampus; Am, amygdala; MHa, medial habenula; PAG, periaqueductal grey; SN, substantia nigra; VTA, ventral tegmental area; MLC, molecular layer of the cerebellum; GLC, granular layer of the cerebellum. Scale bar = 500 µm. This figure has been modified from Wallrafen and Dresbach[1].

Figure 2: Quantification of the Mover distribution across the 5 rostro-caudal levels. Mean fluorescence intensity of the Mover signal (**A**) and the synaptophysin signal (**B**) at the different levels. Mean fluorescence intensity of the Mover signal (**C**) and the synaptophysin signal (**D**) at the 16 manually delineated brain regions. (**E**) Ratios of Mover and synaptophysin in the 16 brain areas of interest. (**F**) Quantification of the relative Mover abundance, comparing Mover/synaptophysin ratio at the respective region to the ratio of the corresponding hemisphere. M1, primary motor cortex; IoC, islands of Calleja; ACC, anterior cingulate cortex; SNu, septal nuclei; VPa, ventral pallidum; NuA, nucleus accumbens; CP, caudate putamen; S1, primary somatosensory cortex; Hc, hippocampus; Am, amygdala; MHa, medial habenula; PAG, periaqueductal grey; SN, substantia nigra; VTA, ventral tegmental area; MLC, molecular layer of the cerebellum. Black dots represent single data points. Bars show the mean ± standard error of the mean (SEM). This figure has been modified from Wallrafen and Dresbach[1].

Figure 3: Mover distribution in the mouse hippocampus. Immunofluorescence stainings of coronal slices of the mouse hippocampus. Overview of the hippocampus showing the heterogeneous Mover expression pattern (**A**) and the corresponding Synaptophysin staining (**B**). The three regions of interest (DG, **Figure 3C**; CA3, **Figure 3E**; CA1, **Figure 3G**) are delineated with white dotted lines. (**D**,**F**,**H**) Quantification comparing the ratio in the respective layers to the ratio of the corresponding hemisphere. The colors in the bar graphs correspond to the respective shading in panels **C**, **E**, and **G**. Mover expression is high in levels associated with intra-hippocampal computation (*i.e.*, the polymorph layer of DG, stratum radiatum, lucidum and oriens of CA3, and stratum radiatum and oriens of CA1), and low in the main input- and output layers (the inner and outer molecular layer of DG, the pyramidal cell layers of CA3 and CA1, and the stratum lacunosum-moleculare of CA1). OML, outer molecular layer; IML, inner molecular layer; GrL, granular layer; PmL, polymorph layer/hilus; SO, stratum oriens; SPy, stratum pyramidale; SLu, stratum lucidum; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. Scale bar = 500 µm. Black dots represent single data points. Bars show the mean ± SEM. This figure has been modified from Wallrafen and Dresbach[1].

Table 1: Solutions used in this protocol.

Table 2: Antibodies used in this protocol.

Table 3: Example of data handling.

DISCUSSION:

The method presented here aims at quantifying the distribution of a protein of interest relative to the abundance of a marker protein with a known distribution. Immunofluorescence staining can show a high variability of staining intensities between different slices. The quantification approach described here circumvents this problem by determining the ratio of the protein of interest to the average across the hemisphere. Therefore, different staining intensities across slices are cancelled out and allow for a quantitative description.

As with every immunofluorescence protocol, 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 extremities[28]. 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 blockage[30]. 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 NaN_3 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 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. Antibodies verified in knock out tissue should be the preferred choice, albeit not always available. Always make sure to perform proper control experiments to exclude crosstalk between different antibodies. 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 aiming 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.

The major advantage of our approach is its internal reference: the immunofluorescence intensity of the target protein (*i.e.*, Mover) in a region of interest is compared to a reference marker (*i.e.*, synaptophysin) and to the overall intensity of these proteins across the entire hemisphere. Thus, from the calculation we perform, one can unequivocally conclude that the abundance of the target protein is x fold higher/lower in a certain region of interest than the abundance of the reference protein relative to the distribution of the proteins across the entire hemisphere at this level relative to Bregma[31]. This allows for the comparison of results using different antibodies, different microscopes, or even across different studies. This consistency across different samples comes from the comparative nature of this method: variability is compensated for by taking the ratio between the fluorescence in the area of interest and that of the hemisphere. Therefore, dissimilarities in absolute values arising from technical differences are nullified. Another major advantage of this technique is the fact that the areas of interest can be as small as you want them to be, only limited by the resolution of the microscope. Quantifying protein levels with biochemical methods, for example Western Blot or mass spectrometry[6-9], requires a dissection of the tissue into the area of interest. This dissection is hard for regions of the brain, such as the primary somatosensory cortex, and becomes virtually impossible when aiming for subregions, such as the different layers of the cortex or the hippocampus.

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.

This method can be easily adapted to determine the distribution of the protein of interest relative to markers for different neuronal compartments, not only presynaptic sites. It can also be easily adapted for tissues other than the brain and – with suitable antibodies – other model systems than mice[32,33]. 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. Additionally, the same stainings can be used to determine the subcellular distribution, for example with super-resolution microscopy.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;New Figure 1_Wallrafen et al-01.tif

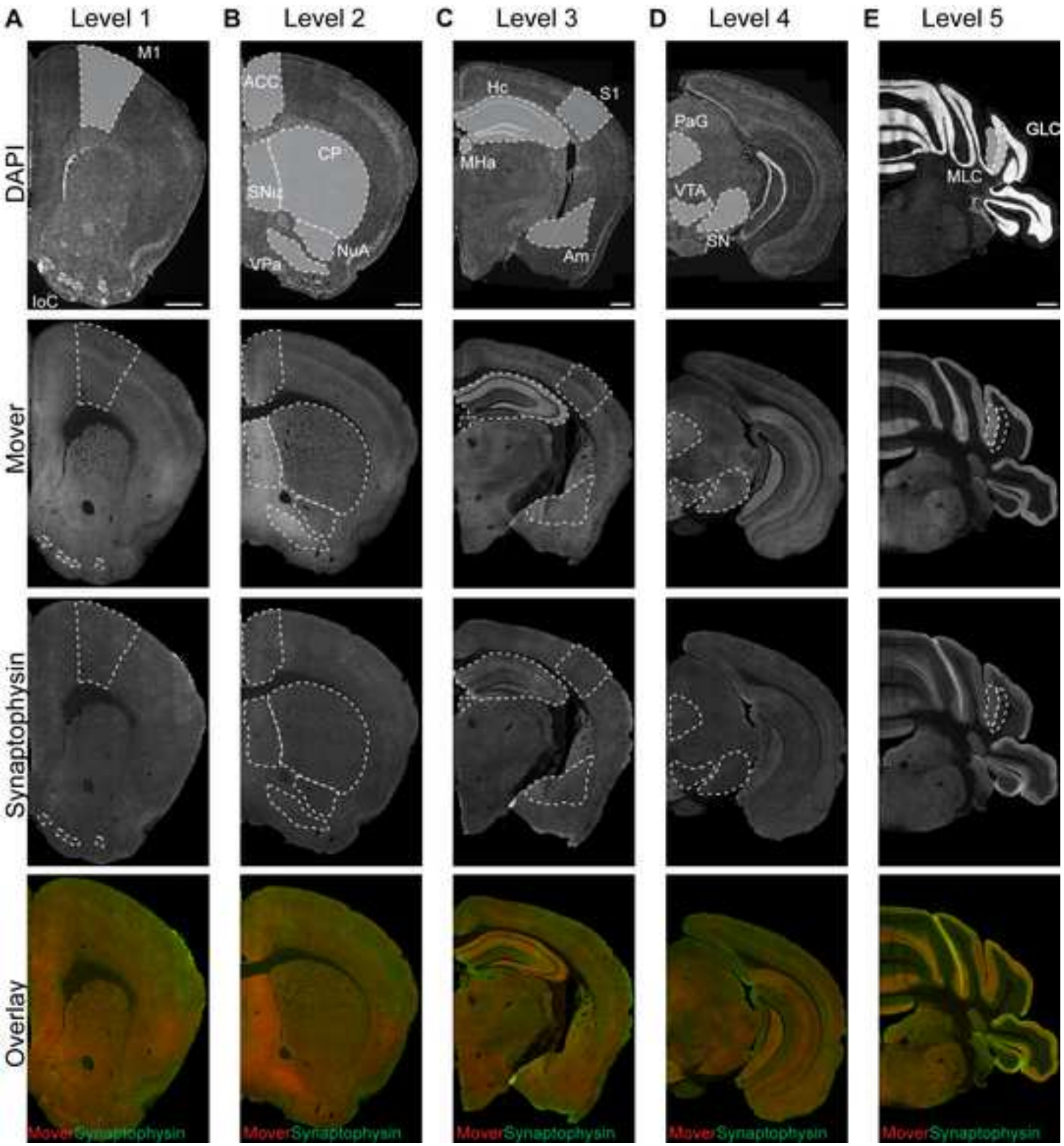

Figure 2

Click here to access/download;Figure;Figure 2 Distribution in the mouse brain-01.tif

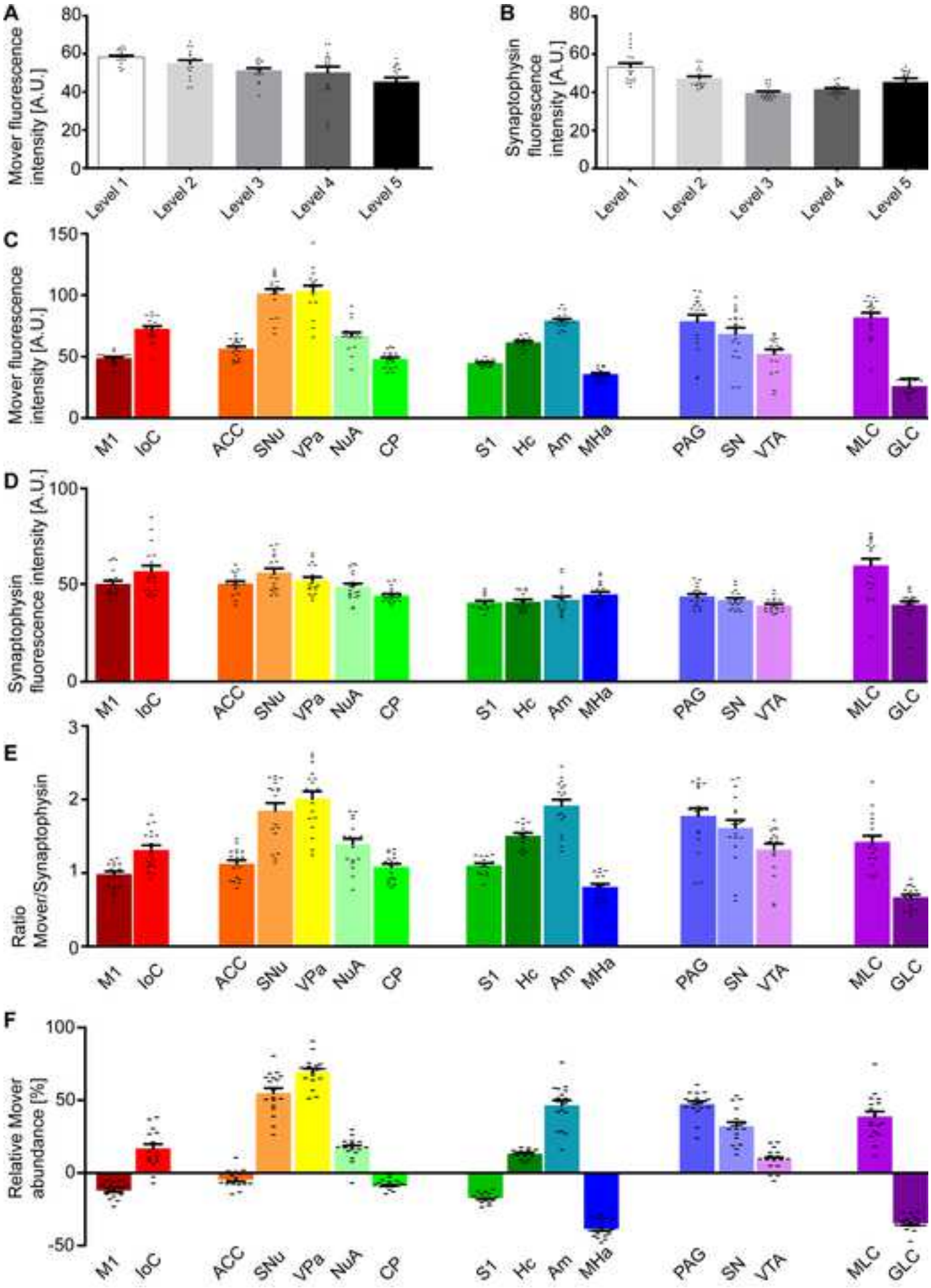

Figure 3

Click here to access/download;Figure;Figure 3 Mover distribution in the Hippocampus-01.tif

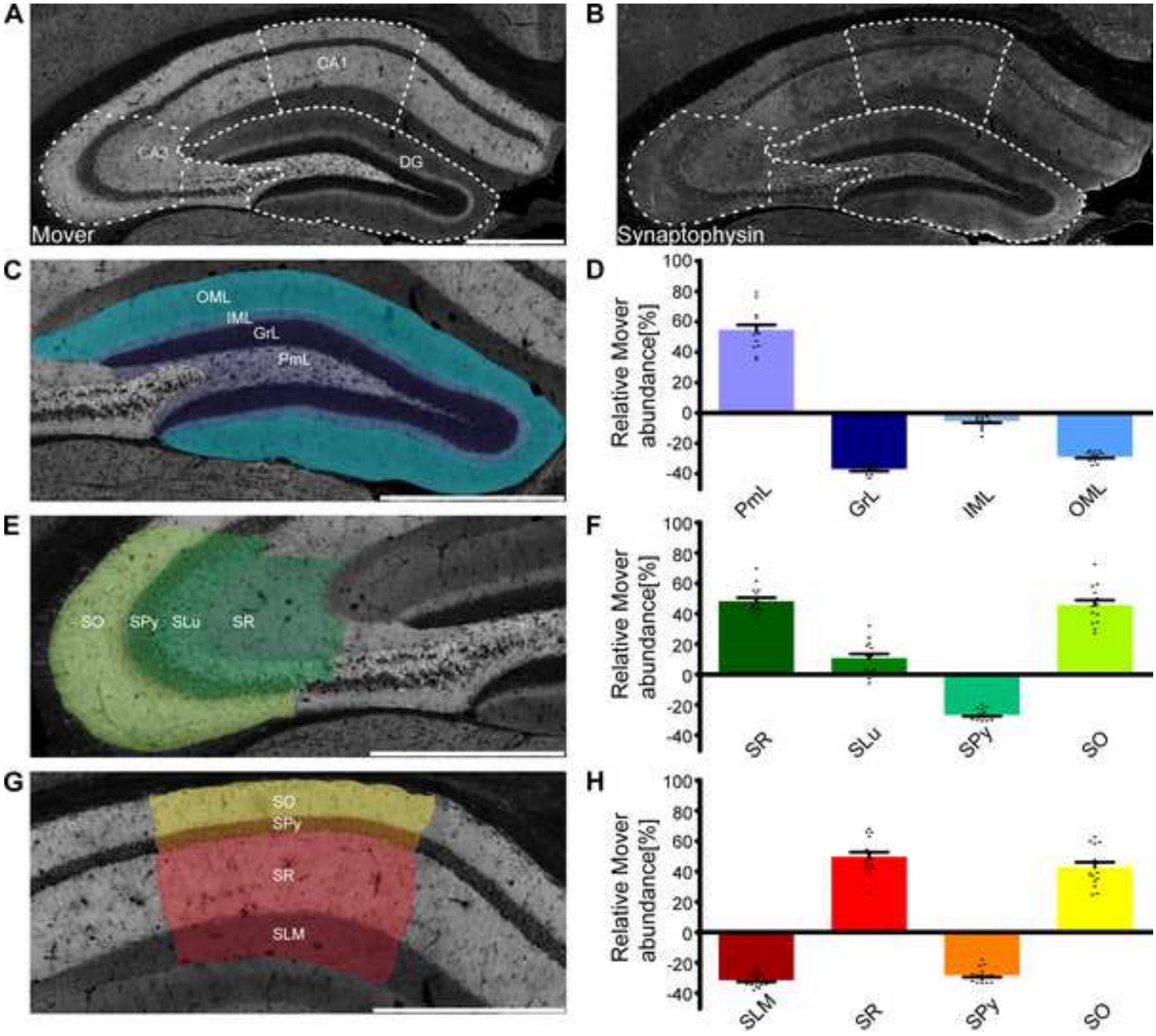

Table 1

Fixative (500 mL)

Mix 20 g paraformaldehyde (total conc.: 4%

50 mL 10x PBS stocksolution (total con

450 mL bidest H_2O

Adjust pH to 7.4 with NaOH

Note: To solve the paraformaldehyde in PBS, h
temperatures higher than 70 °C.

Caution: PFA is toxic, potentially carcinogenic
under the fume hood. Avoid ingestion.

0.1M PB (1 L)

Stocksolution X

35.61 g $Na_2HPO_4 \bullet 2\ H_2O$ in 1 L bidest H_2O

Mix 385 mL stocksolution X

115 mL stocksolution Y

500 mL bidest H_2O

Blocking buffer (50 mL)

Mix 1.25 mL normal goat serum (total conc

	1.25 mL normal donkey serum (total cc
	0.5 mL nonionic surfactant (Triton-X10(
	47 mL 0.1 M PB
Antibody buffer (50 mL)	
Mix	0.25 mL normal goat serum (total conc
	0.25 mL normal donkey serum (total c(
	0.1 mL nonionic surfactant (total conc.
	49.4 mL 0.1 M PB
Washing buffer 1 (50 mL)	
Mix	1 mL normal goat serum (total conc.: 2
	49 mL 0.1 M PB
Washing buffer 2 (50 mL)	
Mix	0.5 mL normal goat serum (total conc.:
	49.5 mL 0.1 M PB

ɓ) c.: 1x) ıeat the solution. Do not heat over 70 °C, as PFA disintegrates at and teratogenic. Wear gloves when working with PFA and work
Stocksolution Y 27.60 g $NaH_2PO_4 \bullet H_2O$ in 1 L bidest H_2O
.: 2,5%)

ɔnc.: 2,5%) 0, total conc.: 1%)
ɔ.: 0,5%) onc.: 0,5%) .: 0.2%)
?%)
1%)

Primary antibodies			
Directed against	Host species	RRID	Concentration
Mover	Rabbit	AB_10804285	1:1000
Synaptophysin	Guinea pig	AB_1210382	1:1000
Secondary antibodies			
Target species	Host species	Fluorophore	Concentration
Rabbit	Donkey	AlexaFluor 647	1:1000
Guinea pig	Goat	AlexaFluor 488	1:1000

Table 3

	A	B	C	D	E	F
1	**Hemisphere**					
2	**Hemisphere #**	**Mean fluorescence intensity Synaptophysin (A.U.)**	**Mean fluorescence intensity Mover (A.U.)**	**Ratio Mover/Synaptophysin**		
3	1	29.134	22.810	=C3/B3 0.783		
4	2	31.008	24.046	=C4/B4 0.775		
5	3	38.641	29.324	=C5/B5 0.759		
6	4	30.775	25.444	=C6/B6 0.827		
7	5	21.658	18.091	=C7/B7 0.835		
8	6	27.277	23.364	=C8/B8 0.857		
9	**Hippocampus**					
10	**Hemisphere #**	**Mean fluorescence intensity Synaptophysin (A.U.)**	**Mean fluorescence intensity Mover (A.U.)**	**Ratio Mover/Synaptophysin**	**Ratio to hemisphere**	**Relative Mover abundance (%)**
11	1	35.26	29.889	=C11/B11 0.848	=D11/D3 1.083	=(E11-1)*100 8.269
12	2	33.955	27.825	=C12/B12 0.819	=D12/D4 1.057	=(E12-1)*100 5.673
13	3	41.231	31.978	=C13/B13 0.776	=D13/D5 1.022	=(E13-1)*100 2.200
14	4	39.853	31.787	=C14/B14 0.798	=D14/D6 0.965	=(E14-1)*100 -3.528
15	5	30.129	27.817	=C15/B15 0.923	=D15/D7 1.105	=(E15-1)*100 10.530
16	6	28.737	25.861	=C16/B16 0.900	=D16/D8 1.051	=(E16-1)*100 5.064

Table of Materials

Click here to access/download;Table of Materials;Materials_Wallrafen et al.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL reaction tubes	Eppendorf	30120094	
50 mL reaction tubes	Greiner Bio-One	227261	
multiwell 24 well	Fisher Scientific	087721H	
plastic pipette (disposable)	Sarstedt	861,176	
1000 µL pipette	Rainin	17014382	
2 µl pipette	Eppendorf	3123000012	
Vortex Genius 3	IKA	3340001	
Menzel microscope slides	Fisher Scientific	10144633CF	
Stereoscope	Leica		
LSM800	Zeiss		Confocal microscope
freezing microtome	Leica		
PBS (10X)	Roche	11666789001	
PFA	Sigma	P6148-1kg	
NaCl	BioFroxx	1394KG001	
sucrose	neoFroxx	1104KG001	
Tissue Tek	Sakura	4583	OCT
Na2HPO4	BioFroxx	5155KG001	
NaH2PO4	Merck	1,063,460,500	
normal goat serum	Merck Millipore	S26-100ML	
normal donkey serum	Merck	S30-100ML	
Triton X-100	Merck	1,086,031,000	
rabbit anti-Mover	Synaptic Systems	RRID: AB_10804285	
guinea pig anti-Synaptophysin	Synaptic Systems	RRID: AB_1210382	
donkey anti-rabbit AF647	Jackson ImmunoResearch	RRID: AB_2492288	
goat anti-mouse AF488	Jackson ImmunoResearch	RRID: AB_2337438	
Mowiol4-88	Calbiochem 475904		
ZEN2 blue software	Zeiss		Microscopy software
FIJI	ImageJ		Analysis software
Microsoft Excel	Microsoft		

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Author(s): Rebecca Wallrafen, Thomas Dresbach, Julio Santos Viotti

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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 extremities[27]. 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 blockage[29]. 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 NaN_3 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.

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