**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.

We have carefully proofread the manuscript.

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 obtained the copyright permission to reuse data, specifically relating to Fig. 6. We have updated the text in the figure legend to reflect this.

3. Please provide an email address for each author.

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4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

The introduction starts with giving an overview of the purpose and the possible applications of the method. As requested, we have now further added a statement at the end in order to summarize the intentions of the paper and the overall goal of the method:

“The overall goal of this paper is to provide technical, methodological and scientific details on the autoradiography technique for informing about tissue distribution and pharmacological analysis of protein targets.”

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. adjusted

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc. adjusted

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. Revised

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:

1.1: What animal and tissue are used? Please specify the euthanasia method. What is used to dissect tissue?

1.1. Euthanize mouse by cervical dislocation and immediately dissect out the brain using scissors and forceps. Directly proceed to the next step to avoid tissue damage.

1.2. Snap-freeze tissue by submersion in powdered dry ice, gaseous CO2 or isopentane. Directly transfer frozen tissue to a cryostat with the temperature set to -20 °C, or store tissue at -80 °C until processing.

1.3: Please specify the temperature.

More details about temperature have been added.

1.4 Cover tissue holder with embedding medium outside the cryostat and quickly place frozen tissue specimen in the desired orientation while the embedding medium is still liquid. For instance, mouse brain is placed vertically onto cerebellum in order to achieve rostral coronal sections. Transfer tissue holder back to the cryostat and expose embedding medium to temperatures below -10 °C for hardening.

2.3: Please break up into sub-steps.

2.3. Pre-incubate sections mounted on slides in assay buffer adjusted to target in question (for GHB protocol 50 mM KHPO4 buffer, pH 6.0 is used) by carefully applying an appropriate volume onto the slide (700 µL for 3-4 rodent coronal sections).

Note: Make sure that every section is covered completely with liquid.

2.3.1. Cover plastic trays with lid in order to avoid evaporation and pre-incubate at relevant temperature (for GHB protocol pre-incubate for 30 min at RT under constant gentle (20 rpm) shaking on a plate shaker).

2.3.2. For the determination of non-specific binding, supplement assay buffer with relevant concentration of unlabelled compound (for GHB protocol, 1 mM GHB).

Note: Pre-incubation may not be necessary.

3.6: Please describe how to stain tissue sections with cresyl violet.

**4. Optional: Cresyl violet staining of tissue sections**

4.1. Prepare 1% cresyl violet solution by mixing 5 g cresyl violet acetate in 500 mL deionized water (dH2O) until dissolved (approximately 2 h). Filter through a filter paper using a funnel into a new 500 mL bottle. Adjust pH to 3.5–3.8.

4.2. Position slide staining set under fume hood. Prepare trays with the following solutions in white polypropylene trays (except for xylene):

a. 50% ethanol:50% dH2O

b. 70% ethanol: 30% dH2O

c. 100% ethanol

d. 100% ethanol

e. 100% dH2O

f. 1% cresyl violet

g. 0.07% acetic acid (add 175 µL acetic acid to 250 mL dH2O).

h. 100% xylene in green solvent-resistant trays

i. 100% xylene in green solvent-resistant trays

4.3. Transfer slides to fume hood and place them in a slide rack.

4.4. Dissolve lipids through increasing graded series of ethanol in dH2O into 100% ethanol (tray a-d) by dipping slides for 1 min.

4.5. Rehydrate specimens to dH2O through descending concentrations of ethanol (tray a-d in reverse order, followed by tray e) by dipping slides for 1 min.

4.6. Immerse specimens in cresyl violet solution for 10 min.

4.7. Rinse the specimens in 0.07% acetic acid by lifting the slides up and down gently for 4-8 s. Wash slides by dipping in dH2O for 1 min.

4.8. Dehydrate specimens by immersion of slides for 30 s in ascending concentrations of ethanol (tray a-d).

4.9. Transfer specimens through two trays of 100% xylene (tray h and i) to quench the ethanol.

4.10. Rehydrate specimens to dH2O through descending concentrations of ethanol (tray a-d in reverse order, followed by tray e) by dipping slides for 1 min.

4.11. Remove slides from saline with forceps. Add a few drops of organic solvent mounting media per slide and add a 24 x 60 mm coverslip on top to protect specimens. Remove air bubbles between the specimen and coverslip by gently pressing onto the coverslip.

Note: Keep remaining slides in xylene during mounting to prevent drying.

4.12. Dry slides overnight in fume hood at RT.

4.13. Obtain a picture of specimen with a microscope and 1.25 x objective.

4.1-4.2: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

We added more steps on how to proceed with the digital analysis. Nonetheless, specific details (like button clicks) are hard to describe precisely as they depend on the specific software used. If it is important to describe the specific details we are happy to give an example using our favourite software.

5.1. Measure optical densities (ODs) of each calibration standard from the [3H]microscale with an image analysis software. First, select an area of equal size for each point of the [3H]microscale according to the instructions of the proprietary software. Second, export OD values for each point of the calibration standard and perform linear regression to obtain a standard curve for further densitometric analysis.

5.2. Perform quantification of autoradiograms using the proprietary imaging software by selecting the region of interest (ROI) in every section and measuring its ODs. Select the same region in every section by creating a template, which is manually adjusted to minor variations in brain anatomy for each autoradiogram. Identify the anatomy of the ROI by comparison of autoradiograms with a brain atlas (e.g.18). When multiple treatments are compared, perform the analysis blinded and randomized in order to avoid biased selection of ROIs.

5.3. Export OD values and sizes of selected areas into a spreadsheet.

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

10. 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.  
11. 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. Please do not highlight any steps describing anesthetization and euthanasia.  
12. 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 highlighted the important steps of the protocol (yellow in the revised manuscript).

13. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We removed trademark names, added all catalogue numbers and material.

14. References: Please do not abbreviate journal titles. adjusted  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
  
Manuscript Summary:  
Very nice overview of the methodology, with solid examples using multiple radioligands  
  
Major Concerns:  
The most significant concern I have is a 6 page discussion. Does seem a bit long and would be appreciated by the reader(s) if it could be streamlined I suspect

We agree that the discussion may seem lengthy. However, we do think that all considerations are relevant to get the full understanding of the technique and potential caveats. We have carefully read through the text and think that deleting a part may cut out relevant information. We also considered if additional headlines could streamline more but it is not common practice of the journal. We hope this is acceptable.

Minor Concerns: Several minor comments, with highlight to the line of the article:  
Line 32-what do the authors imply with regard to an "adequate" concentration

How to choose the adequate radioligand concentration is addressed in the discussion. This would be too extensive to mention within the abstract, hence we rephrased this sentence:

“Therefore, frozen tissue sections are incubated with radioligand solution, and the binding to the target is subsequently localized by the detection of radioactive decay for example by using photosensitive film or phosphor imaging plates.”

Line 56-radioisotope

“Autoradiography is easily implemented in a standard *radioisotope* laboratory given the availability of a suitable radioligand with the required pharmacological specificity…”

Line 93-determination of non-specific binding Spelling corrected  
  
For the CAUTION segment, perhaps change legislation to regulations.

We changed it to ‘regulations’.

Segment 2.1-a pencil seems risky, not a non-erasable pen or marker?

Because the slides are dipped in ethanol baths during cresyl violet staining, the writing of a marker would disappear. This has now been clarified in the protocol:

“2.1. Thaw sections for at least 30 min at room temperature (RT). Label slides with experimental conditions. Use a pencil because slides will be bathed in ethanol during subsequent staining.”

Segment 2.9-eliminate moisture Spelling corrected

Segment 3.1-are currently no longer commercially available Adjusted

Segment 3.2-it is a bit confusing and there may be a better way to word this.

We have rephrased this segment from the original:

Erase tritium sensitive phosphor imaging plate immediately before usage following instructions on the phosphor imager by exposing to visible/infrared light.

To the new version:

“Erase tritium-sensitive phosphor imaging plate immediately before usage in order to remove accumulated signals from storage and to eliminate background signals. Therefore, load plate into phosphor imaging machine and expose it to visible/infrared light according to the instructions of the manufacturer”

Line 274-is performed to permanently establish the ligand-protein interaction

We have exchanged ‘fixate’ with ‘permanently establish’

Segment 4.5, line 298 and line 310: In the first instance, it is described as technical replicates, then four biological replicates. Perhaps clarify and be consistent.

We clarified what is meant by technical and biological replicates within in the protocol.

5.6. Average the ODs of technical replicates, *i.e.* section replicates using tissue from the same animal.

5.10. Average the binding of every biological replicate by using the average of the technical replicates of each animal (obtained in step 5.6).

For any of the tritiated substrates, is there risk of exchange with non-tritiated hydrogen?

This generally depends on the compound and the stability of the 3H-labelling. We added a comment in the discussion in order to point out the possibility:

“Moreover, it should be considered that 3H-ligands have the possibility to undergo hydrogen exchange with water depending on the stability of the 3H-label.”  
  
Line 365-what might be the case for inverse agonists? Perhaps almost as good as antagonists (affinity, no efficacy).

Inverse agonists would also be suitable as radioligands but as they preferably bind to and stabilize the inactive conformation they are not equal to neutral antagonists. Thus, we have not changed the text markedly. The only addition is the word ‘neutral’ to avoid confusion:

“In contrast, neutral antagonists most often display affinity for all receptor states27,30.”

Line 470-consider changing setup to approach –

We have changed the wording to ‘approach.’

Line 542-consider changing depicts to represents

This has likewise been changed.

Line 544-"a relatively fast and simple protocol". Just an editorial comment on that, it is fast and straightforward once you have done all the troubleshooting and optimization of course.

We agree with the reviewer and have adjusted the sentence accordingly:

“The method produces reproducible results by the employment of a relatively fast and simple protocol *once optimal assay conditions have been established*.”  
  
  
**Reviewer #2:**  
  
Manuscript Summary:  
This manuscript provides a thorough account of the use of autoradiography and I expect it will be of interest to the community.  
  
Major Concerns:  
None  
  
Minor Concerns:  
  
In the Tissue Preparation section, I would suggest adding a note to limit repeated freeze/thawing of tissue. Also, including dessicant material inside the slide boxes helps to minimize moisture that can build up on the tissues.

Added to 1.1: Note: Avoid repeated thawing/freezing to reduce tissue damage.  
  
Added to 1.5: Note: Addition of desiccant material to slide boxes minimizes moisture build up on tissue sections.

Line 100: Consider using an isopentane instead of powdered dry ice. Cleaner?

We have added isopentane as another option for tissue freezing:

“Snap-freeze tissue by submersion in powdered dry ice, gaseous CO2 or isopentane.”  
  
Line 140: Consider aspirating slides

Aspirating the liquid with a pipette infers a bigger risk of damaging tissue sections. We thus prefer pouring off the liquid. No changes have been made.

Line 151: Can air dry in 5 min with blower set on cold Added

“Position slides vertically in racks for air-drying for at least one hr at RT *or dry slides for 5 min with blower set to cold temperature.”*  
Line 159: I think it should say "moisture" not moist. Corrected  
  
Line 166-169: Consider using carbon-14 standards as an alternative

We just discovered that the tritium standards are commercially available again, so there is no need for alternatives. The product number etc has been added to the material list.   
  
Line 186: densitometric is misspelled Corrected  
  
Line 189: Figure 2, Label panels with letters or numbers

We thank for the good suggestion. We have added numbers to the figure and updated the figure legend accordingly.  
  
Line 222: Figure 4, Use anatomically similar images for mouse, rat, pig

We totally agree with the reviewer that the different anatomical levels may be misleading. We have therefore updated the figure to include similar images for mouse, rat and pig.



Line 230: The [3H] standards look different from each other

Regarding figure 5 – the scales look a bit different because the data was obtained in different experiments and sections were exposed to different plates, each time together with one scale.

Line 243: are expressed "at" Corrected  
  
Line 429: should be moisture Corrected  
  
Line 538: should be "radionuclides" and "fluorine-18" Corrected