We thank the editor and the reviewers for their detailed examination of the manuscript. We have considered every one of their comments and made corrections accordingly when possible. Please find below our reply (in red, and preceded by “Re:”) to each of these comments. We hope that you will find this much improved manuscript suitable for publication.

Sincerely,

Arlette Kolta (Senior author)

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Re: Done

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.

Re: Done

3. Please define all abbreviations before use.

Re: All the abbreviations are defined at first use

4. Please use focused images of uniform size/resolution (at least 300 dpi).

Re: Ok

5. 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.

Re: Done

6. Please provide an email address for each author.

Re: Done

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

Re: The summary has been rephrased accordingly

8. Please ensure that the Abstract is between 150-300-word limit.

Re: The abstract contains 300 words.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Re: The numbering of the protocol has been modified

10. Please leave a single line space between each numbered step, sub-step and notes of the protocol.

Re: Done

11. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Re: Done. There are no personal pronouns in the Protocol section of the text

12. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Re: The text has been modified accordingly

13. The Protocol should contain only action items that direct the reader to do something.

Re: The Protocol have been modified accordingly

14. Please add more details to your protocol steps. 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.

Re: More details have been added to the Protocol

15. 110: How do you anesthetize? What concentration? What animal is used? Age, sex strain specific bias? How do you perform the brain removal?

Re: These precisions are now given in the text

16. 112: Do you isolate whole brain? Do you remove extra tissues surrounding it?

Re: Details were added to the text.

17. 112: What is the area of interest that is being dissected and how? Please provide details.

Re: Here, the area of interest is the brainstem. Details were added to the text, but it should be kept in mind that the described method is valid for all other areas as well.

18. 162-172; 250-272: We cannot have paragraphs of text in the protocol section. Please consider moving to the discussion section or convert it to substeps in imperative tense.

Re: The text has been converted to substeps in imperative tense.

19. After all the formatting and changes, please ensure that the protocol is no more than 10-page.

Re: The protocol does not exceed 10 pages

20. Please ensure that the 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. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Re: Done

21. 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].”

Re: This information will be uploaded as a doc file as requested

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Re: Those points are now covered in the discussion.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

Condamine and colleagues describe a method to assess astrocytic connectivity through dye-coupling and Image-J. Indeed, a proper measurement of functional connectivity would be very beneficial to the scientific community.

Major Concerns:

No major concerns

Minor Concerns:

-Page 2, line 50 "astrocytes .....and are activated by K+ released during neuronal firing." –

Astrocytes are not "activated" by K+, rather clear it from the extracellular matrix once concentration exceeds a certain limit, see Bellot-Saez et al

<https://doi.org/10.1016/j.neubiorev.2017.03.002>

Re: This part of the sentence has been deleted

-page 3, line 106, "slice holder", should be "slice incubation chamber" or "slice recovery holding chamber".

Re: Text corrected in pages 2-3, lines 123 to 138

The authors should also relate to recent development in the field that allow prolonged incubation.

Re: A note was added at 1.12 regarding this

-The authors apply the biocytin for 30 min, which probably diffuse into astrocytes that are not directly connected to the recorded astrocytes. This should be discussed.

Re: The 30 min application starts after the cell is patched. It is unclear why biocytin should diffuse into unconnected cells. In Condamine et al., Glia (2018), we compared labelings obtained with biocytin applications for 30min and 1 hour and found no difference arguing against the point raised by the reviewer. Nevertheless, text was added to the discussion with regard to this comment (page 15, lines 642-645).

The "subtract background" tool is fundamental to this process, and must be constant, otherwise it may lead to bias of the results... please discuss

Re: the "subtract background" tool is constant; it is only adjusted in cases of very strong deposits of biocytin in the area of the patched cell.

**Reviewer #2:**

Manuscript Summary:

Condamine et colleagues detail a method to describe spatial organization of astroglial network within a specific brain structure using brain slices and image processing with ImageJ, Excel and Adobe Illustrator. They use their own ex vivo data to illustrate the procedure : experiments were performed in slices from the dorsal part of the trigeminal main sensory nucleus (NVsnpr), an area involved in masticatory movements, which is a well studied by A. Kolta's laboratory (Morquette et al. 2015, Condamine et al. 2018, etc).

While part of the procedures is relatively classic (detection of astroglial network using dye coupling in slices, count of loaded cells, detection of 2D size of the astroglial networks), Condamine et colleagues also developed a quantification allowing comparisons of putative preferential orientation of astroglial networks, which is a new and a very interesting tool allowing the study of astroglial coupling dynamics. This approach could be useful in various conditions, ranging from physio- to physiopathological states.

The paper is well written and the procedure is clear. Step-by-step instructions can be followed with no major difficulties. Of note, experimental data used to illustrate the procedure have been already published in Glia (2018).

Major Concerns (relative to the yellow section):

\*A summary is needed for the "image analysis" section.

Authors should summarize briefly image processing at the beginning of the "image analysis" section (l. 173) to favor understanding of the global procedure.

Re: It is against JoVE’s instructions to have paragraphs of text in the protocol section. The protocol has to be described step by step in bullet form.

\* Condamine et al. Glia (2018).

It should be clearly mentioned, in the main text, that data and image processings are already published in Glia in 2018. Authors only mention it in figure legends.

Re: Condamine et al. Glia (2018) is now mentioned at the beginning of the representative results section.

\* Free software.

It would be great to avoid the use of commercial software (illustrator or excel) and promote the use of free softwares (such as inskape, Calc, …). First of all, formulations such as "vector image editor" and "spreadsheet application" should be preferred to commercial software names, and commercial names should be only cited as examples. Secondly, might authors translate adobe illustrator commands in inscape commands?

Re: We corrected and used the formulations proposed and cited the commercial software names in example. As for the command regarding Adobe Illustrator, we are not aware of how to proceed with the reviewer’s suggestion.

\*Simplified processes.

Authors should improve some procedures.

First, image processing in section 6.5 can be performed on image J. (Thus, the use of Adobe Illustrator seems not required).

-4x image can be resized in Image J (process=> Adjust => size), and save in .TIFF in image J.  
-the coordinate called "20x referential point" can then be determined in image J: when you deplace the cursor mouse in the resized 4x, the coordinates of the cursor mouse position (x =, y =, and also the intensity value of the pixel = …) are written on the left-bottom corner in the main Image J toolbar.

Re: The procedure suggested by the reviewer does not allow us to determine the left top corner of the 20X image named "20X referential point" in the 4X image. The main interest to use a vector image editor was to superpose the 20X image on the 4X image and align them by using an opacity tool like the one in Adobe Illustrator CS4 to adjust the transparency of the 20X image and align it on the 4X image.

Secondly, could authors explain the relevance of determining both the coordinates of the 20x image in the "4x resized image" and the coordinates of the 20x image in the brain structure image (after the use of rectangle bounding option) for the positioning of the loaded network in the brain structure. Is the step "positioning in the "4x resized image" really needed (section 6.5)? If so, authors should explain why it is not a redundant procedure.

Re: Yes the step "positioning in the "4X resized image" is really needed because this image is used to determine the position and the coordinates of the bounding rectangle surrounding the NVsnpr. The coordinates of each cell of a network was first determined in the 20X image, but we needed to translate them into the 4X image referential in order to report them in the bounding rectangle referential (used for population data).

Finally, it might be interesting for readers to know that Image J can process calculations (using macros), and that the used of Excel is likely not required to translate cells coordinates.

Re: This is an interesting suggestion, but a spreadsheet application is more user friendly.

\*Alternatives  
Authors should give alternatives to their procedures, when appropriate.

For example,

- The "cell counter" pluggin allow manual detection on raw z-stack image. I agree that it is not an automatic detection, but sometimes "analyze particule" is difficult to perform or prone to errors. Authors might mention cell counter pluggin as a good alternative (or to double check).

Re: We tried using the "cell counter" pluggin for the work published in Condamine et al. (Glia 2018) and found that, since this is a manual method, it is really prone to experimenter bias. However, we added a sentence mentioning that a cell counter pluggin can be used when labeling is unequivocal.

- Watersheld is not always appropriate (as mentioned by authors in discussion), and can easily lead to overestimation. Drawing a white line between fused cells is some time more efficient to dissociate cells.

Re: Watershed draws a white line of 1 pixel thickness between two close objects on a binary file. We wrote to use the watershed option only when necessary: page 7 lines 292-294 “If a group of 2 or more labeled cells in the network are detected as a single cell with the Analyse Particles tool, because they are too close to each other use the Watershed tool”. Moreover, we had already added a “warning” in the discussion regarding use of this tool “Another limitation of the method described is the “watershed” tool used to discriminate cells that are too close to each other. Visual inspection of detected cells should be done when using this tool.”

- It might be mentioned that SR101 loading is not required to p identify astrocyte in slices for patch-clamp recordings.

Re: A ramp and a step IV protocol were used to identify astrocytes on the basis of their electrophysiological properties, but SR-101 loading (Kafitz et al., 2008) is really useful to target astrocytes for patch-clamp recording. A sentence was added at the beginning of step 3 to mention that.

\* Pixels or µm?

Is the use of images scaled "in pixel" required or could the global process being fully achieved with images already scaled in "µm"? If "pixel scaling" is required, then authors might indicate how to switch between pixel and µm in the section "preparation of data" section (l. 174). (image => properties: choose then "pixel" with "1" for pixel dimension).

Re: We use pixel unit in all the process and convert the measure in µm at the end. We choose this method to avoid conversion problems if the data set is not imaged with the same microscope for example. We added a sentence about pixel scaling into the section "preparation of data".

\*Relevance of vector size: Is a small vector really indicate a preferential orientation?  
The size of the vector (for each network) indirectly informs on the strength of the preferential orientation of the astroglial network, independently of the cells number within the network. Thus, the size of the vector matter, and can give substantial informations. However, this is not mentioned by authors. To my opinion, this is a major limit of the detailled method in the manuscript.  
Which factors influence vector size (density of cells, spatial orientation...) ? What type of informations are provided by vector size ? How such information can be used for comparison of astroglial networks? What is the limit (Is a small vector really indicate a preferential orientation?)? Authors should clarify this in the main text and/or ideally discuss its importance in the discussion section and/or add a complement of procedure for a comparison between astroglial networks.

Re: The reviewer is correct by assuming that a small vector indicates that there is no clear “dominant” or preferential orientation (independently of the cells number within the network). It will be mostly influenced by the spatial location of each cell in the network in relation to the patched astrocyte. We agree that smaller vectors do not reflect a strong preferential orientation, but still they reflect one direction. When is it too small to count? We prefer to not set limits and instead use the mean values. This information is still useful when comparing 2 conditions (like sensory stimulation vs Ca2+-free medium, for example). A sentence was added to the discussion to stress the importance of vector size.

Minor Concerns:

- L.31 Authors referred to "density of the network" in the introduction, but never quantify it further. this should be clarified.

Re: Due to this misunderstanding, we removed this expression.

- For figures: Scale bars are in µm on figures. This is confusing, because all the procedure is performed in pixel. Authors should remove the "100 µm" in scale bars from figures, calibration of scale bars could however be indicated in the figure legend (pixels or equivalent in µm).

Re: All the analysis was performed in pixel and all the numeric data was converted in µm at the end. To us, it makes much more sense to provide the reader with a measure in µm, which is more meaningful. However, the figure has been corrected as requested.

- L.32: "4/ repositioning of the network within the area of interest" is not clear.

Re: Area of interest means a nucleus or a studied area of the brain. We use a general term to illustrate the use of this protocol.

- L. 53: Authors should to choose between « etc » or « … ».

Re: Corrected

- L.105: The "perfusing buffer" is also used for slice storage (not only for patch-clamp and dye coupling).

Re: Corrected

- L.109: the tubing in the storage chamber is required for carbogen (95 % O2 -5% CO2), not only for oxygen delivery.

Re: Text corrected (page 3 lines 113, 120 and 126)

- L.116: Authors should indicate how long slices have to stay in storage condition (aCSF, RT) before 34°C bath (for SR101 Loading). ~ at least 1 hour ? less ?

Re: Text corrected

- L.134: It should be also mentioned that astrocytes display, in general, a Vm around -80mV.

Re: In some brain structures like in NVsnpr astrocytes are more depolarized and showed a Vm around -70mV. It’s the reason why we don’t mention a general Vm.

L.141: Is the IV protocol (every 5 min) required to follow variation of Resistance input or is it performed to facilitate dye diffusion? This question should be addressed, considering the very low (leaky) membrane resistance of astrocytes.

Re: The IV protocol every 5 minutes is only required to facilitate biocytin diffusion.

- L.147: add "then" before "discard the case", to facilitate the reading.

Re: Corrected

- L.154: in the "4.biocytin relevation" section. One should always pay attention to the side of the slice, when mounting slices on coverslips. Indeed, the use of astrocyte patch-clamp for dye coupling experiments are usually performed in "surface" of the 350 µm-slice. The "loaded side" of the slide should be accessible for scanning confocal acquisition. If true for authors, this should be mentioned in the "4. biocytin revelation" section.

Re: Corrected. We added sentences to the text (in the “Astrocytes patching and filling with biocytin” section and the Biocytin revelation section) with regard to that comment.

- L.156: streptavidin without "-e"

Re: Corrected

- L.195: it's "make binary", not "make a binary".

Re: Corrected

- L.202: "In the setting "show"" is not clear (and not reported adequately in fig 3C). "Display results" will be probably more appropriate.

Re: Corrected.

- L.212: for a real unbiaised analysis, the "Watersheld" tool should be systematically used before the "analysis particule".

Re: ‘’Watershed’’ process is only used when some cells are too close to be considered by the counting process. We didn’t use this process systematically because it can increase the number of cell detected by dividing some cell in two different parts. We recommend visual inspection every time it is used.

- L.245: "draw a ROI at the patched cell location and add it to the ROI manager." might be clearer.

Re: Corrected

- L.268: it should be mentioned that the coordinate of the "reference cell" is not included. The length of the main vector is then divided by : the number of cells in the network - 1.

Re: Corrected

- L.286: "Select and hold the "pipette tool" to see "the measure tool" in the tools panel" might be clearer.

Re: Corrected

- L.385: Calcium free condition also increases neuronal activities (Morquette et al. 2015) and increase of neuronal activities might increase astroglial coupling. Thus, can the calcium free condition only be used to increase connexin permeability? Reformulation of this part could be required.

Re: We don’t see how a stimulus can increase astroglial coupling without having an effect on connexins. The sentence states that we have used the Ca2+ free condition as a massive stimulus to open up the syncytium and maximize tracer diffusion through gap junctions (whether it’s indirect through an effect on neurons or not is irrelevant).

- L432-438: there are not enough data (7C and 7D) to clearly access to sample distributions without over-interpretation. A "peak" can not be determined with n=2 networks. Student test cannot be used here (inequal variance, low sample size, Gaussian distribution not so clear…). Is there really a preferential orientation in 7B3 if you take into account the size of vectors? Is there a preferential orientation when data are distributed from 0 to 180°? Authors should reformulate with caution this part.

Re: These data have been published as they are and have already been reviewed for publication in Glia. We do not think there is over-interpretation. The data is not distributed equally from 0 to 180° as stated by the reviewer. In fact 6 of the 8 networks (with sensory stimulation) have a preferential orientation above 80°. The important point here is that an orientation towards the center would have been closer to zero as with the networks obtained under Ca2+-free conditions. Obviously, all the networks obtained with sensory stimulation will not have a single dominant preferential orientation since the position of their starting point (the patched astrocyte) varies. We removed the reference to “peaks”. As for the statistics, we systematically verify when a test can be used according to data distribution and sample size. In this case the normality test and the equal variance test were passed (with p = 0.273 and p = 0.478 respectively) and allowing for use of the student test. Both groups passed the Kolmogorov-Smirnov test assess normality (Sensory Stim: K-S Dist. = 0,174, P > 0,200; and Ca2+-free: K-S Dist. = 0,272, P = 0,053).

- L.532: Why do authors believe that "the number of cells detected with this method is largely underestimated"? references with patch-clamp of neurons can be misunderstood. Thus, this first paragraph is confusing and should be clarified.

Re: The reasons why we believe that the number of cells are underestimated is clearly stated in the text. In many types of coupled cells (not just neurons) there are evidence on the basis of electrophysiological recordings that dye coupling does not always occur between coupled cells. Many examples are from studies on neurons, but it is also the case of other types of cells and there is no reason to believe why it would be different for astrocytes.

- L.532: To my opinion, It should be clearly stated that the number of loaded cells depend, at first, of the time used during experiment to load the patched cell.

Re: To avoid variability, 30 minutes was the time systematically used for loading, and as stated above, in Condamine et al, Glia (2018) we compared loadings of 1 hour and 30 min and found no differences.

- Authors should precise what is the Iman-Conover methods (2 way ANOVA on ranks) and which software has been used for statistics.

Re: There is no section for statistics in JoVE. We do not know where to add that.

- material : change "VT100S" with "VT1000S".

Re: Corrected

**Reviewer #3:**

Manuscript Summary:

In the study entitled "Methods to analyze, shape and directionality of networks of coupled astrocytes" a ImageJ/FIJI-based analysis of gap junctional coupling of astrocytes in the trigeminal nucleus is described. Sulforhodamine 101-labeled astrocytes in acute tissue slices were patch-clamped and dialyzed with biocytin to mark coupled cells. After labeling of coupled cells the cell number, the network area and the preferential orientation of dye spreading was determined using ImageJ/FIJI. The semi-automated analysis provides more objective results compared to earlier studies that rely on manually evaluated networks. However, this study suffers from a couple of weaknesses (see comments below) and needs to be thoroughly revised. In particular, the post hoc identification of the patched cell and the lack of a critical discussion of advantages/disadvantages of this method compared to other methods is a matter of concern.  
  
Major Concerns:

1. In chapter 3 (Astrocyte patching) fundamental information is missing:

A paragraph has been added to describe the patch procedure in details. This was first omitted because the paper’s focus is on methods to assess astrocytic networks, rather than electrophysiological recordings.

a. What is the sampling rate of the whole-cell recordings?

Re: The sampling rate was 10kHz. This has been added to the text.

b. Was the liquid junction potential corrected?

Re: We added a sentence with a reference for a method to correct for LJPs when needed. Liquid junction potentials will vary greatly with internal and external solutions used for recordings which are likely to differ with the type of experiment being conducted. They are important to determine for experiments involving ion channel selectivity or when precise voltage commands are administered, and importantly, they are likely to change in the course of a recording session if the bath solution changes or during a prolonged recording from a cell. Therefore, we did not put emphasis on correcting LJP here since voltage measurement and commands were not strictly important. In the experiments described here we corrected the offset before seal formation and subtracted any value that appeared at the end of the recording after retracting from the cell.

c. Were traces online or offline filtered?

Re: Traces were filtered online at 1KHz.

d. Were patches compensated for fast and slow capacitance and series resistance?

Re: Yes

2. Yes, "in most cases, the patched astrocyte shows the stronger labelling than the adjacent the adjacent cells stained through tracer coupling" (L235-236). This is a rather shaky way of determining the origin of the network. It is better to use a predominantly gap-junction impermeable and fixable fluorescent dye that does not interfere with the biocytin labeling fluorophore. In this case, Alexa Fluor 488 would be suitable. Then you can be sure that you can unerringly identify the patched cell. The corresponding discussion section (L564-569) suggests that this improvement is somehow difficult, which is not the case (cf. Augustin et al., 2016, Glia).

Re: The reviewer is correct about the fact that adding a dye to the recorded cell. Alexa 488 crosses gap junctions in our hands and is not fixable. However, we changed the text and now propose to add Dextran, which resist fixation and do not cross GJ when large enough.

3. As there are patched cells lost eventually upon withdrawal the pipette, the capability to determine reliably the origin of the reconstructed network is lost as well. In order to save the maximum of results/data the authors calculate the putative position of the patched cell (section 6.4.1). However, you cannot be absolutely confident about the position of the lost cell thereby challenging all analyses that depend on that information. It is better to omit such experiments from e.g. the vector analysis. However, it is still possible to use respective experiments for the analysis of network area and cell number (if you add +1 in the latter case to account for the lost cell).

Re: The location of the initial biocytin deposit is very clear most of the time and is usually quite limited so that it will not affect the vector analysis dramatically. Since, loss of the patched cell is common, removal of all cases where it happens would reduce the sample size significantly. However, the reviewer’s suggestion is a possibility that can be considered and is now added as an alternative option in the text.

4. Astrocytes and oligodendrocytes form panglial networks in many brain regions (Maglione et al., 2010, Glia; Griemsmann et al., 2015, Cereb Cortex; Augustin et al., 2016, Glia; Moshrefi-Ravasdjani et al., 2017, Neurochem Res; Claus et al., 2018, Cereb Cortex). Are all coupled cells really "labelled astrocytes" (L387)? If it cannot be proven somehow, it must they must be called "labelled cells" (L378). See also L509.

Re: The reviewer is absolutely correct about this. Corrections have been made accordingly.

5. In section 4 "Biocytin revelation", fluorescent labeling of biocytin via streptavidin is described (L154-161). However, Figure 6C middle (0 Ca2+ experiment) shows DAB labeling. The latter method is not mentioned in section 4. Regarding the shown quantification and comparison of coupled cells and covered area (Figure 6D-E) it is fundamental to use only one method. It is likely, that DAB reaction stronger labels cells that would have been below detection threshold using streptavidin conjugated with a fluorophore. Comparison with other experimental configurations (Ctrl, fiber tract stimulation and CBX in 0 Ca2+) is not valid.

Re: We disagree that the DAB reaction gives stronger labeling. In fact, we used DAB in the beginning for that reason specifically, but on comparisons with labelings obtained with Streptavidine, we found no differences. Details about the DAB revelation are now added to the methods and an advice against using different methods is also given. However, we think it remains an interesting option that experimenter should be aware about if they cannot use fluorescence.

6. It is stated that preferential direction of dye spread is only addressed in one other study (Houades et al., 2008; J Neurosci). However, that is not the current knowledge. Preferential dye spreading has been investigated in the hippocampus (Houades et al., 2006, Neuron Glia Biol; Rouach et al., 2008, Science; Anders et al., 2014, Philos Trans R Soc Lond B Biol Sci), the cortex (Houades et al., 2006, Neuron Glia Biol), the glomerulus (Roux et al., 2011, Proc Natl Acad Sci U S A), the thalamus (Claus et al., 2018, Cereb Cortex), and the lateral superior olive (Augustin et al., 2016, Glia).

Re: Again, the reviewer is absolutely right about these previous findings. These are now mentioned in the introduction and the discussion.

7. It should be discussed what the advantage/disadvantage of the present method is compared to the methods used in the literature listed above (#6).

Re: The main advantage of our method is that it provides for a mean to describe the organization and orientation of astrocytic networks in structures like the dorsal trigeminal main sensory nucleus that are not known to have a precise organization. In all of the above studies, the networks orientation is described in relationship to the shape of the structure itself which is already documented (e.g. the barreloid in the thalamus, the barrels in the cortex, the layers in the hippocampus and cortex, the glomeruli in the olfactory bulb...etc). In addition, our vectorial analysis enabled us to detect that coupling orientation may vary with conditions (e.g. when comparing orientation preference observed with zero Ca2+ vs with sensory fiber stimulation). This advantage is now added to the discussion.

Minor Concerns:

1. Stick to one name of the software: ImageJ vs. ImageJFIJI (e.g. L29+240 vs. L175).

Re: Corrected with ImageJFIJI

2. In L37-38, why not define the (trigeminal) nucleus instead of staying unspecific?

Re: Corrected

3. The list of putatively benefitting nuclei (L42-43) should be extended by the auditory brainstem, which is spatially rather close to the trigeminus. In the lateral superior olive glial networks exhibit a specialized topography (Augustin et al., 2016, Glia).

Re: Corrected

4. Consider adding the review by Verkhratsky and Steinhäuser, 2000, Brain Research Reviews (L50).

Re: Added

5. Unconventional list. Please modify and add a suitable citation preferentially another review (L53).

Re: References added

6. Specialized gap junctional coupling is named for barrel cortex and olfactory bulb (L60-68). In addition, a link to another sensory system, i.e. the lateral superior olive within the auditory brainstem (Augustin et al., 2016, Glia), which is spatially close to the trigeminal nucleus, should be given.

Re: Corrected

7. Consider citing Ref. #6 at the end of sentence (L71).

Re: Corrected

8. The x/y ratio as a mean to describe network topography was not exclusively used in the barrel cortex (L82-85), but as well in the hippocampus and the lateral superior olive (Houades et al., 2006, Neuron Glia Biology; Augustin et al., 2016, Glia). Consider addition of this information for a more comprehensive view.

Re: Corrected

9. Typo: "SR-101" (L121).

Re: Corrected

10. Room temperature was defined as "RT" (L115). Use it accordingly (L122).

Re: Corrected

11. Typo: "SR101 labelled" vs. "SR101-labeled" (L130). See also L448.

Re: Corrected

12. Check correct usage of hyphens: e.g. "voltage-clamp" (L134), "whole-cell current-voltage protocoll" (L135), "current-clamp" (L137). See also L446 ("patch-clamp").

Re: Corrected

13. Define step size for current-clamp protocol (l138).

Re: We performed steps of 100pA. This was added to the text

14. L206: add "(3C, left part)" at the end of the sentence.

Re: Corrected

15. L226: add "Figure 4B"

Re: Corrected

16. L227: Wrong figure reference: It must read "Figure 4B".

Re: Corrected

17. Depending on the confocal system, the Meta data already contain the conversion factor for the microscope. Thus, FIJI already uses µm scaling, e.g. when processing pictures taken at a Leica LSM SP5. This possibility has to be mentioned in order to avoid confusion.

Re: we mention that we only work in pixels with imageJFIJI and check the setting for this (line 259)

18. In section 6.4.2 the new referential coordinates are given as " x', y' " (L255-258). In section 6.4.3 the coordinates of the cells are given as " x1, x2 " etc. (L265-267). Here, one should better write " x'1, x'2 " etc. (accordingly for y coordinates) to be not confused with the coordinates " x, y " from the initial FIJI analysis.

Re: Corrected

19. In Figure 5 the labeling of axes is heterogeneous (regarding spaces). At the arrows indicating orientation it must read "Medial". In L344 it must read "lateromedial" and "dorsoventral". See also Figure 7.

Re: Corrected

20. L275: Specify Adobe Illustrator version. There arrangement of controls might be heterogeneous across different Adobe Illustrator versions.

Re: We used CS4 version. Added to the text.

21. To account for left-right orientation it is suggested to recalculate x coordinates (L327-331). Why not just mirroring the picture in FIJI before starting the analysis? Then one has not to perform another calculation.

Re: Interesting suggestion. We added it to the text.

22. L353: To avoid confusion specify which part of the formula is covered by the square root, e.g. by adding another pair of brackets.

Re: Corrected

23. Consider indicating that CBX was used in an independent set of experiments (L374-378).

Re: Specified as requested

24. Figure7: Bars in panels C and D are not aligned and do not optically fit to the binning mentioned in the text (L434+436). I suspect that the bars were misaligned during the design process in the graphic software. This should be corrected.

Re: Figure has been corrected and the text was modified du to another comment.

**Reviewer #4:**

Manuscript Summary:

Methods are given for measuring the extent of tracer coupling of astrocytes and applied to the MesV sensory nucleus. The coupling is blocked by carbonoxylone, a well know blocker of gap junctions. Extent of coupling is altered by sensory stimulation and by placing in low Ca solution. That's good, but haven't they already published that?

Re: The reviewer is clearly aware of our very recent publication (in Glia) regarding astrocytic networks in the Main (and not MesV) sensory nucleus. The present article was written following an invitation from JoVE to describe in greater detail the methods used in this recent publication.   
  
Major Concerns:

A) The depth dimension of the coupled domains is not analyzed. B) If the labeled cells constituted a compartment, the vector of the coupling would depend on which cell was injected. C) There is no convincing evidence that the limit of tracer spread is not simply a matter of drop off with distance of concentration to the threshold of their method.

Re: A) The depth dimension beyond the limit of the Z stack is not analyzed because of the limits imposed by the fact of working in slices. We assume that cells at the surface of the slice (on each side) and their processes are damaged, leaving only the middle part of the slice intact. Cells in that region are presumably encompassed in networks revealed with Z-stacks, but given the narrowness of this region and the slight variability in cutting angles from one animal to the other, we collapsed the Z stack in 2D.

B) Indeed, the vector of coupling is expected to depend on the position of the injected cell within the network. This is exactly why we developed these elaborate calculations.

C) If this were the case, tracer spread would be similar under all conditions and we would not have more labeling under one condition (low calcium) vs the other (sensory stimulation). In addition, in experiments using the exact same methodology, but in the visual cortex for a different project, we reveal much larger networks with much more extensive tracer spread (unpublished results), suggesting that it is not a limit of the method but rather a limit of the networks being investigated.

We have no idea whether coupling domains overlap or whether the whole nucleus could be one domain. You could develop and use a technique for multiple injections (at least 2) and tracers of different colors. Are the domains volume filling of the space between neurons? There is reason the think that single astrocytes interdigitate to only a small degree.

Re: The reviewer’s comment is relevant for interpretation of the data rather than the methods. Whether astrocytic coupling domains overlap or not is important to understand the function of the circuitry. Here we only report about the methods used to describe a domain. The question raised by the reviewer is beyond the scope of this methodology paper. Secondly, according to some, introduction of a tracer in a cell often interferes with labelling by another tracer; although this is something that we have not validated ourselves.

One would have liked some consideration of the dye coupling as a function of GJ permeability. Re: A sentence regarding tracers permeating GJ have been added to the discussion

Admittedly not easy to measure in networks, but fundamental to the observations. If the tracer, e.g., biocytin is not destroyed in the cells and does not leak out, the coupling coefficient is unity given enough time. The vector of tracer coupling always points away from the injected cell. That makes it a not very important parameter functionally. Indeed coupling might drop off towards the edge of the nucleus (and it would have been interesting to make some measurements just outside of the border of the nucleus.

Re: We do not understand the point that the reviewer is trying to make with this comment and do not know how to address it clearly. Of course the vector of tracer coupling will always point away from the injected cell, but if the network is not confined within a structure, it will point in any direction and there would be no clear preferential orientation, even when the injected cell is near the border of the nucleus. This is not the case here, when cells near the border are injected, the preferential orientation of the network points towards the center of the nucleus and very rarely do we get tracer spread outside of the nucleus. In addition, we did carry out injections of cells in the ventral part of the nucleus (which correspond to a different area functionally) and obtained large networks that spread outside of the nucleus. These results are not presented here because again this is a method paper and not a paper to discuss the concept of astrocytic network domains.

The literature contains lots of pictures of dye filled cells with labeled processes. Why not here?

Re: The labelling reported here is comparable with that reported in most other articles using the same techniques. Moreover, visualization is done at low magnification to capture the entire network and this does not allow visualization of fine processes.

The techniques are of some interest and the change in tracer coupling with CBX and low Ca also. But we are left with many questions about the extent of coupling, questions that could be resolved with not much refinement of the methods.

Re: The reviewer did not spell out the questions to be addressed exactly. These questions are maybe addressed in our previous publication in Glia which reports about the findings and their functional significance. Here we limited the content to the methods used.

Minor points:

31. Define density? What about the z axis dimension?

Re: Density is the number of cells per μm2. See reply above regarding the Z axis dimension

32. Explain somewhere how GJs can have a preferred orientation. "repositioning" means what?

Re: We never said that GJs have a preferred orientation. What we say is that there is a preferential orientation to tracer spread. This can happen because either: 1) only cells connected in a particular configuration are coupled through GJs, or 2) GJs are dynamically regulated. If the input regulating coupling is itself oriented (like is the case with the sensory fiber terminals for instance), then tracer spread across the regulated junctions will appear to have a preferential orientation.

Repositioning means to place back the reconstructed network within the nucleus or area of interest.

41. Are there functional domains? Hard to tell from single injections. Multiple injections might or might not show an edge of a domain.

Re: See reply above

50. activated ???

Re: This sentence was changed with regard to a comment raised by Reviewer 1 as well.

56. Are there restricted networks?

Re: Yes, according to our findings in Condamine et al, Glia, 2018, and to those of many others in the barrel cortex, the olfactory glomeruli, the barreloids in the thalamus, the lateral superior olive and the hippocampus (see reply to the comment below).

63. I don't think you mean "overlap neural compartments". They fill in lots of the space between neurons. The neurons may or may not be coupled by gap junctions to form compartments. In the barrel cortex, there is more astrocytic coupling within a barrel and less between barrels. Generally, astrocytes interdigitate very little with each other. How are the astrocytic networks oriented towards the center of the glomerula or of the barrel?

Re: We mean that in areas where neurons have a topographical organization, like in the Barrel cortex, astrocytic networks also display a structural segmentation that corresponds perfectly to the neural segmentation. The point that the reviewer is trying to make with the 2nd, 3rd and 4th sentences of this paragraph is unclear. As for the last sentence, we only report about what has been previously published by others.

79. synchronized not very precise?

Re: We say synchronized and coordinated. What else could be more precise?

84. parallel to the surface is two axes.

Re: We changed the sentence.

87. preferred ???

Re: Preferred as in the dominant orientation reflected by the sum of vectors

90. a method to replace each network in reference to the boundaries of the nucleus ???

Re: See reply regarding “repositioning” the network above.

98. pretty low rate of cooling

Re: It may depend on the volume put to freeze. In any case it’s an approximate

113. plane of cutting? It's a long time before we find out.

Re: We do not understand what the reviewer wishes to address with this comment

123. How long can you keep the slices at room temperature?

Re: We keep brainstem slices easily for at least 8 hours. Others working on cortical or hippocampal slices can keep them for longer.

140. not positive to -60 mV

Re: Corrected

144. 15 min in addition to the 30 min for injection

Re: Yes. This is now specified

157. Alexa

Re: Corrected

167. "resolution of 800 by 800 pixels" What is the pixel size; what is the z dimension?

Re: The pixels size was 0.749μm.The z dimension was adjusted for all networks to allow getting the entire signal properly.

173 - 360. Who highlighted all this and why?

Re: This is requested by the journal for the video

185. Selection of the white outliers may be rather arbitrary.

Re: We are not sure to fully understand the question here. We remove « bright » outliers because we’re working against a black background.

195. The binary image obviously throws away a lot of data. Then there is the discard of small spots. What is the criterion for a cell? What has happened to the cell processes that connect the cell somata?

Re: The binary image may throw away some data, but we think it is safer to underestimate the number of cells rather than counting artifacts and deposits as cells. The criterion for a cell are given in the section 6.2 just below this one. As for the processes, we do not consider that they should be counted as cells to avoid counting the same cell twice.

223. The surface area of the network as calculated excludes the cell processes on the edge of the network. What about the z axis extent of the network? Does this depend on the plane of section of the brain? Network area x/y extent can be close to section thickness.

Re: The surface area may be slightly larger if considering the processes, but the difference should not be significant and in any case, it can only be based on what is visible in the images. See the comment above regarding the Z axis. Depending on the area and the organization of astrocytic networks, their surface may vary with the plane of cutting and will obviously be limited by section thickness.

242. I see no strongly stained processes. Looks more like leakage. This measurement is before making the binary image.

Re: The sentence has been removed.

380. How do you get an area for a line connecting two cells?

Re: The area value given is an average value for all networks and not only those having 2 cells; and in the latter case, we draw an ellipse around the 2 cells and not a straight line.

390. In Fig. 6C 0 Ca image, why not biocytin fluorescence? Lots of leak?

Re: Just because, these were the first experiments conducted and we used DAB then. Later, we confirmed that equivalent results could be obtained in fluorescence, so we switched to fluorescence. There is not more leak on that image than on other images.

408. Fig. 6 D. Electrical stimulation still has an effect in the presence of CBX. Less marked for 0 Ca.

Re : It is probable that some labelling result from cells pumping biocytin that has leaked in the extracellular space. It is known that more active cells will pump more of the biocytin. An explanation for the data could be that electrical stimulation is more effective to activate the cells, whereas 0 Ca2+ only favors GJ opening.