**Rebuttal document** for“2-photon imaging of microglial processes attraction toward ATP and serotonin in acute brain slices of young and adult mice” by Etienne et al.

**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
2. Figure 7: Please include a space between numbers and their corresponding units (e.g., 500 µm, 5 µm, etc.). Should “Mean + SEM” be “Mean ± SEM”? DONE  
3. Movies S1-S3: Please define scale bars in the movie legend. DONE  
4. Table 1: Please provide units for the numbers in the table. Table 1 has been removed  
5. Please revise the title to be more concise if possible. DONE  
6. Please provide an email address for each author. DONE on the first page of the manuscript.  
7. Please shorten the Short Abstract to 10-50 words. DONE (42 words)  
8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. DONE  
9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. DONE  
10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Pyrex, Chameleon Ultra2, etc. DONE  
11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). DONE  
12. 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. DONE  
13. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. DONE  
14. The Protocol should contain only action items that direct the reader to do something. Please move material information (e.g., 3.1.1, 3.2.1, etc.) to the Table of Materials. DONE  
15. 3.2.2: Please specify the age, gender and strain of mouse used. DONE (in the Material List)  
16. Step 5: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. DONE  
17. Please include single-line spaces between all paragraphs, headings, steps, etc. DONE (space 12 pts after each paragraph)  
18. 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.  
19. 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.  
20. 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.  
21. Discussion: As we are a methods journal, please also discuss critical steps within the protocol and any limitations of the technique. DONE  
22. Reference 26 is not quoted in the manuscript but in the reference list. Please check. DONE

Reviewer #1:

Manuscript Summary:

In their manuscript Etienne et al. describe a protocol for the analysis of microglial processes attraction towards a source of ATP or serotonin gradient using acute brain slices. They also give some important clues to keeping acute brain from slices in optimal conditions, providing the files for the 3D printing of suggested incubation chambers. This is a relevant protocol and I recommend its publication after minor revision.  
  
Major Concerns: None

Minor Concerns:

Please, indicate whether it could be necessary to adapt the proposed 3D printed chamber when using a different microscope.

The recording chamber dimensions are those of a “standard” bathchamber insert, i.e. 62 mm outer diameter. We added these sentences in the Results, 1st paragraph: “**Our recording chamber dimensions are set to fit standard microscopes as they are similar to classical bathchamber inserts (62 mm outer diameter) but as their models are downloadable from supplemental material, the design can be adapted to fit in slice holders of other dimensions**.”

Why should the bit depth of the images be 12 bits or more?

We added a note in the protocol (in 4.1.3) to better explain this: “**NOTE: Higher-bit images allow to distinguish smaller differences in fluorescence intensity than lower-bit images: a change of one gray value in a 8-bit image would correspond to a change of 16 gray values in a 12-bit and of 256 gray values in a 16-bit image. Therefore, higher-bit images are more appropriate for quantitative analysis, but as their size increases with bit depth, storage capacity and computing power can become limiting**.”

Authors indicate that "The frequency of acquisitions is 1 image every 2 minutes". Please, discuss how the frequency of acquisition may affect the temporal resolution of the analysis and the accuracy of obtained data.

The choice of the acquisition frequency is a compromise between the accuracy of the kinetics, the size of the files, and the risk of photobleaching and phototoxicity. We observed that this moderate frequency of acquisition of 1 image every 2 minutes was sufficient to quantify the attraction of processes toward the source of attractant. This frequency has already been used by other groups to measure attraction of microglial processes (e.g. Davalos et al, 2005: 1 image every 2 min; Haynes et al, 2006: 1 image every 5 min) or microglial surveillance - also called “motility” - (e.g. Wu et al, 2008 and Paris et al, 2017: 1 image every 2 min).

However, imaging to measure surveillance is most often performed at 1 image per min (e.g. Madry et al, 2018; Wu et al, 2007; Gyoneva et al, 2013), or faster (e.g. Fontainhas et al, 2011 and Pagani et al, 2015: 1 image every 10 sec).

Indeed, whereas increasing the rate of image acquisition is not critical to measure attraction, which is a net increase of fluorescence over time, it is relevant to improve the accuracy of motility quantification, which is a measure of the turn-over of microglial processes. We thus added a sentence at the end of the discussion : “**However, to quantify the fast extension and retraction rates of microglial processes, and detect potential variations, it would be relevant to increase the acquisition frequency. For example, Pagani et al. use a frequency of one image every 10 seconds28**.”

Authors also suggest reducing the number of Z planes to decrease scanning time, please discuss how reducing the number of Z-planes may affect the resolution/quality of the analysis.   
In addition, it is indicated to "Select the scan mode XYZT with a Z-interval range at 2 microns". Why could this be relevant? Is this Z-step enough to assure an appropriate 3D resolution? In the same way, is it necessary to use a XY resolution of 1024x1024 (295,07 x 295,07 microns)? Please, briefly discuss this.

A Z-interval range of 2 microns is used in numerous studies performed in 2D (Madry et al, 2018; Davalos et al, 2005; Wu et al, 2007), although a smaller z-interval has been used in recent studies in 3D (1 µm in Paris et al, 2017; 0,4 µm in Heindl et al, 2018). However, Paris et al show in their article that acquisitions with 1 or 2 µm z-intervals give similar quantification of processes and tips velocity. Therefore, the 2 µm z-step interval is appropriate for 3D resolution, at least with the ProMolJ tool (Paris et al 2017). A 3 µm z-interval can also be used to measure attraction (as in Eyo et al, 2014), but this impacts some 3D analysis (Paris et al, 2017), therefore this setting should be the same for all the acquisitions to be compared. Regarding the 1024x1024 XY resolution, it allows a more detailed observation of the processes, which can be relevant for some analysis, but it can be decreased if necessary.

In order to clarify these points in the manuscript, **we changed some explanations in the Note in 4.1.3 in the Protocol.**

Moreover, at the end of the discussion, we added: “**Finally, recent publications described methods to quantify morphological changes or individual processes motility in three dimensions. For such analysis, although a z-step interval of 2 µm is enough for some programs (ref 27), other require a better axial resolution (i.e. z-step 0.4 µm in ref 33.)**”.

Indicate how to exclude z-planes when necessary (first 30 microns).

This has been better explained in the protocol (5.1.2)

Please, indicate the type of Z-stack projection you used (mean, maximum, sum, etc.).

We apologize for this oversight, it is a “Max Intensity Projection”. This is now specified in the protocol (5.1.3).

Indicate whether, when comparing slices, it could be important to analyze 2D images containing information from a similar number of z-slices (similar z-stack thickness). Considering this, in Figure 7A, did the analyzed images of each slice contained information of the same z-thickness?

We perform the z-projection using all the z-slices where fluorescence is visible. This “useful” z-stack thickness may vary but as all the fluorescence is taken into account, we observed no link between this z-stack thickness and the quantification. In addition, the deep z-slices exhibit low fluorescence and contribute scarcely to the signal in the z-projection (that’s why they could be excluded at the imaging step, as indicated in the second Note of 4.1.3 and our answer above).

We now mention this point in the Representative Results, and compare with our methods of quantification and Z-projection: “**To note is that in our protocol the thickness used for the max z-projection encompasses all the z-slices where fluorescence is visible (usually 180-220 µm, see 5.1.2). Therefore, variations in the absolute number of z-slices does not impact on the quantification of the response. In contrast, some studies use thinner z-stacks (40-60 µm) for z-projection (Ref 6,7,11,27). This is another option, with the risk to exclude some z-slices which exhibit a response as we observed that the attractant effect was visible as far as 70 µm (in z) away from the pipette tip in some experiments. If the thinner option is preferred, it is thus critical to center the z-stack on the pipette tip in z and, importantly, only z-projections done in the same manner (i.e., using all fluorescent z-slices or using a thin z-stack) can be compared.**”

In Figure 7A, the z-thickness in the initial figure were from 180 to 280 µm but considering the point raised by the reviewer, in order to standardize the protocol and make it clearer, we updated the Figure 7A using values obtained from 180-200 µm z-thickness projections (i.e. excluding eventually the deepest z-slices, which had indeed very little fluorescence).

In figures, please, indicate the z-thickness of the images shown.

They are now indicated in the figure legends.

Please, discuss the possibility of analyzing images in 3D, without using Z stack projections, maybe using a sphere (circular ROIs of decreasing radius from the central plane where the tip of the pipette is detected) as a 3D ROI for quantification.

The advantage of a morphological analysis in 2D as the one proposed in this manuscript, *versus* in 3D, on z-stacks, is that it requires less computer power and can be performed straightforward using simple image analysis tools. A limitation is that, by definition, the z-projection leads to underestimation of the growth of the microglial processes, by masking the movements in the z dimension. The suggestion of using circular ROIs with decreasing radius (taken into account the distance between two consecutive z-plans) is very interesting and should logically lead to a slightly higher, and more accurate, estimation of the fluorescence increase near the pipette tip. Nevertheless, this would require a specific macro, in order to apply different ROI on the different z planes, and a very precise localization of the pipette tip in the 3 dimensions. Therefore, we believe that this goes beyond the aim of this manuscript, which is to provide a simple technique for researchers who would like to start investigating the morphological dynamics of microglia, even though they are not familiar with image analysis. For researchers who would like to pursue on this, we provide references on 3D analysis tools in the Discussion part.

Mean intensity may be affected by changes in the expression of the fluorescent protein after treatment, thus a process already included in the initial ROI may increase the intensity of its fluorescence. Please, indicate how this could affect the interpretation of the data and suggest at least an alternative approach to correct or overcome this problem.

We agree with this remark, in theory, but in the experiments we describe, the increase of fluorescence is visible in the first minutes after injection. Because of this rapid effect, increased expression of the gene encoding the fluorescent protein could not contribute to the increase in fluorescence. This could be an issue for long-term imaging, but it is unlikely when imaging lasts no more than 30 minutes after treatment.

An alternative approach to overcome this problem is to draw manually and measure at each time point the area not covered by microglial processes. This method was mentioned in a note of the protocol, but taking into account the remark of the reviewer, we have rephrased the sentence to briefly presented the advantage and disadvantage of this method, and moved it to the Discussion part: “**an alternative method which does not depend on the fluorescence level of the microglial processes, but requires more actions of the experimenter for the image analysis, is to measure the reduction of the empty space around the pipette tip after compound application ref 11,32**.”

Reference 26 is not mentioned in the manuscript.

This has been solved (formatting problem).

Please, discuss the quantification method and compare your data with those from other studies.

Our quantification method is inspired by the method used by Davalos et al 2005.

As suggested, we compared, in the Representative Results, our data with those from other studies using the same kind of quantification method: “**The effect of ATP is in the same range than those obtained by other groups with a similar method in vivo (Davalos et al, 2005: 0.53; Haynes et al, 2006: 0.413) and in slices (Dissing-Olesen et al, 2014: 0.8 ref5 if normalized as here; Pagani et al, 2015: 0.6 ref28). Differences can come on one hand from biological parameters: the slice preparation method, the amount of ATP injected, the age or the brain region, and on the other hand from analysis parameters: the diameter of the ROI and the thickness of the z-stacks used for the z-projection (i.e., the whole thickness where fluorescence is detected, or only the 40-60 µm around the pipette tip, where the maximal response is expected).."**

Reviewer #2:

In this study, Etienne et al. characterized a detailed protocol to study microglial processes chemotaxis towards ATP or serotonin in acute brain slices. Microglial dynamics are useful information to understand microglial function in health and disease. The protocol is very well described and would be valuable for the microglial research. Here are some minor concerns need to be addressed:

1. When introducing ATP-induce microglial process chemotaxis, the authors omitted the earliest characterization of this phenomena using ATP-pipette in acute brain slices (Wu et al., Glia, 2007). In addition, the paper is the first to combine microglia electrophysiology and time-lapse live imaging to study mechanisms underlying ATP-induced microglia process chemotaxis.

We apologize for this oversight and this princeps study is now cited in the introduction.

2. Also, it is not correct to state that microglia process attract to glutamate (in abstract and introduction). Many studies have shown that glutamate cannot directly induce process chemotaxis, but most indirectly caused by glutamate-triggered ATP release (Wu and Zhuo, J Neurophysiol, 2008; Eyo et al., J Neurosci, 2014; Dissing-Olesen et al., J Neurosci, 2014; Wendt et al., J Neurosci, 2016).

For sure we know that the effect of glutamate is not direct, and we regret that the sentences were confusing.

In the abstract, we don’t mention glutamate anymore (indeed, by shortening the sentence, we had ended with a wrong statement). In the introduction, we have added this sentence (l.67): **“These effects either are directly mediated by microglial receptors (for ATP and norepinephrine) or require ATP release from neurons (for NMDA).”** as well as the reference to Eyo et al., J Neurosc. 2014 in addition to Dissing-Olesen et al, 2014.

3. Line 93, page 3. The authors cited Gan's paper about imaging microglia using YFP. This is actually a wrong statement, because the mice do not have strong fluorescent signals for live imaging.

It is true that the fluorescent signal in these mice is much lower than in the CX3CR1GFP/+ mice, and that microglia imaging in their brain slices is challenging. However, with some changes in the acquisition parameters, we were able to record microglia processes attraction toward ATP and 5-HT and to perform the analysis described in the protocol. A movie performed on these mice is provided for the reviewer (not to be included in the manuscript). As these mice are available and are useful tools to combine genetic deletion and imaging, we think they deserve to be mentioned, but we changed the text of the Note in 4.1.3 to strengthen the fact that it is more difficult than with the CX3CR1GFP/+ line and requires optimization of the acquisition parameters : **“NOTE: It is possible to perform similar experiments on slices from CX3CR1creER-YFP mice, a mouse line used to induce genetic deletion in microglia only, and which microglia constitutively express YFP. However, the expression level of YFP is very low compared to GFP in CX3CR1GFP/+ mice, thus imaging is possible but challenging and require optimization of the acquisition parameters. We propose to adjust them as follows:…” .**

4. For ATP delivery, no microinjection is needed to induce microglial process chemotaxis. As soon as ATP-containing pipette is inserted into the brain slices, it forms the ATP gradient that can induce process chemotaxis towards the pipette. This should be discussed.

We sometimes observed such effect, but it was moderate in comparison with the effect of injection. To note is that we use an ATP concentration of 500 µmol.L-1, which is less than in many studies (e.g. Wu et al, 2007 and Eyo et al, 2014: 3 mmol.L-1; Dissing-Olesen et al, 2014 : 4 mmol.L-1; Haynes et al, 2006 : 20 mmol.L-1), and may explain our moderate attraction in absence of injection.

Nevertheless, this issue is now discussed in the Protocol, 4.2.6: “**NOTE: leakage of ATP out of the pipette can attract microglial processes even before injection (if this occurs, it will be visible at the analysis step). Although this should be moderate with the ATP concentration used (500µmol.L-1), if it is an issue, consider prefilling the micropipette with 2 microliters of ACSF prior to add the ATP (or other compound) solution at 4.2.6**.”.

5. The analysis of microglial chemotaxis is a bit simple. The authors should discuss recent studies using 3D analysis of microglial process dynamics (Paris et al., Glia, 2018; Heindl et al, Front Cell Neurosci, 2018). Also, why 35um diameter was used for analysis?

We agree that the quantification is simple, but the aim of our manuscript is to provide experimenters genuine to the field with an easy-to-implement procedure. Actually, in the lab we initially used a more complex quantification of the processes growth (see in Kolodziejczak et al 2015), but finally moved to the method presented here, which is more straightforward. The studies mentioned for 3D analysis have been developed to quantify microglial surveillance (Paris et al, 2018), and the complexity of microglial ramification (Heindl et al, 2018), but not specifically the chemoattractant effect. We nevertheless agree that 3D analysis provide more detailed data and these two studies are now presented in the Discussion (l. 743-746).

Regarding the diameter of the ROI used for analysis, it is the same than the one used in Davalos et al, 2005. Smaller (20 µm diameter in Pagani et al, 2015) or larger diameters (70 µm diameter in Haynes et al, 2006; 100 µm diameter in Dissing-Olesen et al, 2014) are also found in the literature. In order to address quantitatively this question of the reviewer, we reanalyzed a series of experiments with ROI of different sizes and the results are now discussed in Figure 7B (l. 551): “**Figure 7B shows how the size of the ROI also impacts on the quantification, here of ATP-induced processes growth. Increasing the diameter from 35 (the diameter used in Figure 7A and for all our analysis) to 50 or 70 µm reduces variability among experiments (slices) by suppressing the issue of the small R1 positioning. However, it also decreases accuracy and the magnitude of the detected response. Indeed, with larger ROIs, there is more background due to processes or cell bodies not affected by the treatment, and a growth of processes can be partially blunted by the concomitant retraction of microglial branches more distant from the pipette but nevertheless inside the ROI. In conclusion, it can be relevant to use ROIs different than a 35 µm diameter circle, but it is fundamental that the ROI is always the same in all the data-sets to be compared**.”

Reviewer #3:

2-photon imaging of microglial processes attraction toward ATP or serotonin in acute brain slices of young and adult mice.

The manuscript by Etienne et al. focuses on the imaging and analysis of microglial motility towards a source of ATP or 5HT in acute brain slices. The topic is of high interest and the methods described will be useful to researchers in the microglial field. The method is well explained and touches upon different ways to improve slice health. The manuscript, however, could be improved by addressing the following comments.

The authors describe the injury induced-like movement of microglia towards an ATP or serotonin source, but do not address why an experimenter would use one chemotactic source versus the other. A slightly more detailed introduction covering the benefits of imaging this type of motility and what sort of biological questions that could be answered would help the reader decide on the exact methodology to use.

We have modified the introduction following these recommendations. Notably, the different forms of morphological plasticity are presented in the introduction.

The interface slice chamber and the dual perfusion imaging chamber the authors describe are quite interesting. They claim that using these significantly improves slice health and extends their use compared to using other standard aCSFs, maintaining the slices submerged prior to imaging and imaging in a bath with the slice touching the coverslip. Could they provide an estimate of by how much the health of the slice is improved and which of the modifications they believe to be the most important?

Unfortunately, it is difficult to tell quantitatively by how much the health is improved because our final protocol results from several modifications that were tested and added over time, and which finally allowed us to achieve more robustness and a better reproducibility in our experiments than at the beginning. Several modifications probably contribute synergistically to the global improvement, and it would be difficult to establish a hierarchy among them. Nevertheless, in the Discussion, we highlighted the mechanisms by which the specificities of the protocol may improve the viability and indicated references of previous studies addressing them separately (l.696-729).

With respect to making the chambers, it is unclear how the different parts come together- are they glued together? More information on building the chambers would be helpful.

They don’t need to be glued because they fit perfectly together. This is now explained in the Protocol (3.1.3.2, 4.2.1) and in the Results (l.442-444).

The slices are maintained in an interface chamber until ready for recording- more details on the amount of liquid above the nylon mesh and how the slices are prevented from drying should be provided.

This is now detailed in the Protocol (3.1.3).

The authors state the infrared laser is tuned to 920nm and used with 5-15% power- what does this translate to in terms of actual power at the objective (in mW/cm2)?

This information has been added (4.1.2): “**This corresponds to a power of 3 - 5 mW under the objective**”.

The image analysis is done in 2D, which I understand is a common limitation in this field of research. However, more information should be provided on things that should be taken into consideration when comparing different datasets- eg. the size of the z stack, the positioning of the pipette tip, the size of the ROI, etc.

Image analysis in 2D is a limitation if the aim is to quantify the motility of individual processes - or, more exactly, it does not allow to measure the real distance traveled by the tips of microglia, but we believe is not the case when quantifying a global attraction toward a local source of a compound, as we do here.

If 3D analysis has to be performed, the z-interval can have an impact, depending of the analysis plugin. If the aim is to measure the speed of the changes affecting the processes, either in 2D or 3D, the sampling rate has a strong impact. We added informations on these two points in the Discussion:

(l.740): “**to quantify the fast extension and retraction rates of microglial processes, and detect potential variations, it would be relevant to increase the acquisition frequency. For example, Pagani et al. use a frequency of one image every 10 seconds”.**

(l. 743): “**Finally, recent publications described methods to quantify morphological changes or individual processes motility in three dimensions. For such analysis, although a z-step interval of 2 µm is enough for some programs27, other require a better axial resolution (i.e. z-step 0.4 µm in 33)”**.

In addition, we thank the reviewer for raising the point of things to be taken into consideration to compare datasets. We now discuss the different parameters along the Representative Results and Discussion, and have notably added a Figure (7B) to compare the impact of the ROI size. We hope these comments will be helpful to design and compare experiments.

Similarly, no information is given on how to deal with photobleaching over time. If photobleaching occurs then it is possible that the mean intensity ROI plot will give a flatline despite clear processes moving towards the source.

We agree with the reviewer that photobleaching is an important issue which may lead to underestimation of the processes attraction. For our experiments, we checked that our excitation and acquisition parameters did not induce any photobleaching and it is confirmed by the stable fluorescence over time of the slices treated with ACSF (Figure 7C). However, as photobleaching could create a serious bias, in the new version of the Results we gave more information about photobleaching, how to check for it and how to limit it (l. 575): **“As it can bias measurements, it is important to rigorously check that there is no photobleaching in the experimental conditions. To do this, we recommend to acquire a XYZT series on a slice with GFP-expressing microglia, for 30 minutes (ACSF can be injected, but actually no stimulation is needed), with the excitation and acquisition parameters set as in the experimental conditions. Then, a quantitative measure of the fluorescence over time in different regions of interest, including microglia cell bodies or processes, will reveal if there is a gradual loss in emission intensity, usually an exponential decay, indicating photobleaching. If it is the case, some adjustments can be performed: re-alignment of the laser, reduction of the laser power and increase of the detector gain, reduction of the number of z-planes and increase of the interval between them to limit illumination. Photobleaching is favored by high power or long (ex: repeated illumination for line averaging) excitation, one must thus pay particular attention to it if a sustained illumination is used to image cells with low fluorescence”.**

In the representative results the authors show high variability in results between slices- the highest response is approximately 3-fold greater than the lowest. A discussion of this and limitations of the methodology when a small effect size is predicted should be included

We now discuss possible sources of variability in the Representative Results. We chose not to address here the question of the number of samples to use depending of the variability and the expected effect size, as this is a concern for statistics in general, not specific for this technique.

In addition, we would have liked to compare the variability in Figure 7A with those in other studies using similar techniques, but it was not possible as the raw results are generally not shown in publications. The SEM however is in the same range than those found in similar studies.

The paper would benefit from a troubleshooting section to cover the above points

To our knowledge, the format of JoVE publication does not include a troubleshooting section. We therefore used Notes in the protocol, or comments in the Results and Discussion parts, to cover these important points and hope that the reader will find the relevant informations

Minor comments

The grammar should be reviewed throughout

We have now reviewed the manuscript to our best.

The second paragraph of the introduction has no references beyond the first sentence

Two references (Stence et al, 2001 and Kurpius et al, 2006) have been added.

Becher I believe is beaker in English

Thank you, we changed this.

Figure 3B the lettering is difficult to see

Lettering size has been increased.

Figure 6 the arrows and arrowheads are difficult to distinguish from the cells. This figure would benefit from being in colour.

They are now bigger and in red.

Are the micropipettes used made from thin or thick walled glass?

They are made from thin wall capillaries. This was said in the material list but has been added in the protocol.

Reviewer #4:

The manuscript provides an experimental protocol for analysing the morphological dynamics of microglial cells in acute brain slices.

Overall, it is fairly well written and organized, providing practical information on how to maintain / improve the quality of the biological sample and the imaging data. While it mostly rehashes previously published information, it does provide useful insights and reminders on how to prepare and maintain live brain tissue sample, which is very sensitive.

Thus, the protocol could be a handy resource for researchers entering the microglia field, as 2-photon microscopes and fluorescently labelled mouse lines have proliferated.

Comments and suggestions to improve the manuscript:

\* Stating the % values of laser power is useless; absolute IR power after the objective should be provided. This is important for comparing the threshold for inducing phototoxic effects between different experimental conditions, mice, users, labs etc.

This information has been added (4.1.2): “**This corresponds to a power of 3 - 5 mW under the objective**”.

\* There is no point in Table 1, which lists the settings for a particular type of pipette puller, as many labs likely have a different one. Instead, they could show images of the tip of the pipette and its best geometry.

Table 1 has been removed.

\* A pixel resolution of 295 nm is a bit low, given the optical resolution of their 2-photon microscope and the thin size of microglial processes. I would recommend at least 200 nm to comply with the sampling theorem and not to throw away information needlessly.

Indeed, having a better xy resolution could be interesting for a detailed reconstruction of microglia morphology as in Heindl et al, 2018, which uses a resolution of 200 nm, and we will consider this suggestion for future experiments. However, for our global analysis of fluorescence, it was not a problem, and indeed we prefered to image larger fields with lower xy resolution in order to see microglia affected and not affected by the injection. In addition, in the literature, when the information is available, similar or lower resolution (i.e. larger pixel dimension) is used to quantify microglia motility (Madry et al, 2018 : 400-450 nm) and even to track processes (Paris et al, 2018 : 497 nm), so we chose not to comment this specific point in the manuscript.

\* A temporal resolution of 1 stack of images every 2 minutes seems too low to capture the rapid dynamics of microglial processes.

The choice of the acquisition frequency was a compromise between the accuracy of the kinetics, the size of the files, and the risk of photobleaching and phototoxicity. We observed that this moderate frequency was sufficient to quantify the attraction of processes. This frequency has already been used by other groups to measure attraction of microglial processes (e.g. Davalos et al, 2005: 1 image every 2 min; Haynes et al, 2006: 1 image every 5 min) or microglial surveillance - also called “motility” - (e.g. Wu et al, 2008 and Paris et al, 2017: 1 image every 2 min).

However it is true that whereas increasing the rate of image acquisition is not critical to measure attraction, which is a net increase of fluorescence over time, it is relevant to improve the accuracy of motility quantification, which is a measure of the turn-over of microglial processes. Consistent with that, imaging to measure surveillance is most often performed at 1 image per min (e.g. Madry et al, 2018; Wu et al, 2007; Gyoneva et al, 2013), or faster (e.g. Fontainhas et al, 2011 and Pagani et al, 2015: 1 image every 10 sec). We thus now comment in the Discussion (l. 740): **“to quantify the fast extension and retraction rates of microglial processes, and detect potential variations, it would be relevant to increase the acquisition frequency. For example, Pagani et al. use a frequency of one image every 10 seconds”.**

\* The authors claim that a choline-based ACSF aids the health of the brain slices and microglia. This should be supported by data or literature references.

Two references addressing this point have been added in the Discussion (l.700-701).

\* The paper by Pagani et al (Front Cell Neurosci. 2015) should be cited, where similar methodological information was presented in detail.

This study quantify attraction with another method and we agree that it is interesting to both. Pagani et al, 2015 is thus now cited and commented, notably in the Discussion (l.742).

\* The first paragraph of the Discussion should be compressed and made more relevant.

We followed this advice and shortened this paragraph.

\* Usually, after cutting brain slices are left to recover for at least one hour before the start of any recordings, according to the consensus in the electrophysiology field. So, it is unexpected that they used a much shorter period.

Re-reading the manuscript, we realized that indeed we had not provided proper information on the recovery step, and it resulted confusing, thank you for raising this point.

We have completed the protocol in 3.2.9 to explain that after the first 10 min recovery in cool choline-aCSF, the slices are transferred to and maintained in the interface chamber for a least 30 minutes before imaging. It is still less than the standard time in electrophysiology, but we didn’t observed changes in the microglia response to compounds when slices had recovered for 1h, instead of 30 minutes, in the interface chamber. To note is that several groups let the slices recover less than 1 hour for microglia imaging (e.g. : Eyo et al, 2014 : 30 min of recovery ; Madry et al, 2018: 30 minutes of labeling after slicing and before imaging).

However, as we cannot exclude that a longer recovery would be relevant in certain conditions, we mention in the Discussion that (l. 712): “**Afterwards, slices were transferred into the interface holding chamber to recover for an additional minimum time of 30 minutes. The duration of this second recovery period could be optimized according to the brain area of interest, the Na+ substitute, and the age of the animal, and it should last at least 1 hour if electrophysiology has to be performed in parallel with imaging**”.

\* To obtain more reproducible results, the use of automated pressure devices (e.g. picospritzer) are preferred over manual operation of syringes.

This was mentioned in the protocol, but we have rephrased the Note for better clarity (4.2.12) and commented on this point in the Results (l.548): “**Therefore, to detect small effects or variations, it may be interesting to use an automatic device for the compound injection**”.

\* It is stated that the injection pipette is placed just on top of the slice, but it is hard to imagine how a local effect can be induced inside the slice unless the pipette really penetrates into the slice. For this a finer tip (<2 microns) may be necessary.

We apologize if this was unclear, but the tip of the pipette actually penetrates 80-100 µm below the surface of the slice, as shown in Figure 3B. Relevance of this point and parameters that may influence it are now commented in the Results (l.451): “**…its thin extremity entered slightly into the tissue, down to 80-100 µm from the surface. It is important that the pipette is not too superficial because it may not deliver the solution correctly onto the cells, nor enter too deep because it may reach a region where fluorescence signal is too low. Parameters that may affect the depth reached by the pipette tip are the angle of the pipette, which can be adjusted with the 3-axis micromanipulator, and the pipette mouth diameter**”.

\* Figure 4: the panels A and B are too redundant, consider merging with finer overlay of the graphics.

We followed this advice.

\* Figure 5: consider showing more time points and zooming in on ROI to illustrate better the directional motility effect after compound injection.

Insets zoomed on the ROI have been added on each picture. We did not add more time points here to avoid having a too crowded figure, but an intermediate time point for ATP is shown in Figure 4.