Point-by-point response to the Reviewers

We thank the reviewers for their critical reading of our manuscript and we are grateful for their feedback. Please find below our point-by-point response to each reviewer. We hope that it will convince the reviewers that our article, in its current form, will be of significant interest for the scientific community by providing a detailed description of the protocol for multicolor labeling of cortical astrocytes using *in utero* electroporation of MAGIC Markers and subsequent image analysis pipeline to single out of astrocyte volume and morphology.

Reviewers' comments:

Reviewer#1:
Manuscript Summary:
Current Manuscript named "Multicolor labeling using in utero electroporation of MAGIC Markers to individualize cortical mouse astrocytes" displays an application of the combinatorial labeling method (MAGIC Markers) used to label and trace the lineage of multiple progenitors in a simultaneous way, previously described by the same group. Specifically, the authors explain with detail the whole procedure to label cortical progenitors during embryonic development by in utero electroporation to later focus on the analysis of morphological aspects of protoplasmic astrocytes including arborization or the territorial volume occupied by them using appropriated software. Overall the manuscript is well written, the protocol is presented with appropriated detail and the effectiveness of the protocol is proven, as shown by the provided figures. I believe the application of in utero electroporation described here is: 1) interesting because the study of morphological aspects of astrocytes is not easily achieved by conventional methods, 2) helpful to instruct researchers in the use of the image analysis software and 3) definitively suitable for its publication in JoVE, however I have some concerns that I believe should be addressed.

Major Concerns:
-Magic Markers approach makes possible to label complete clones with a particular and unique signature, nevertheless, tracing experiments to conduct clonal analysis are not conducted in the current manuscript. In this manuscript the authors work identifying labeled isolated astrocytes (Line 304, step 7.1.1) to study its morphological aspects. The authors need to clarify the advantages of using multicolor tracing to individualize isolated astrocytes versus the use of single labeling achieved by electroporation of one transposon expressing one fluorescent protein or electroporation of low concentrations of plasmids expressing Cre in Cre-Reporter mice like Ai6 or Ai9. For example is the multicolor method in combination with the image analysis software helpful to sort out the arbor of one astrocyte from other when they partially overlap?

We agree with Reviewer#1 that *in utero* electroporation of MAGIC Markers can be used to single out cortical astrocyte clones and study cortical astrocyte development, as demonstrated by us recently (Clavreul 2019). However, we decided to focus here on the anatomical use of this approach that may interest a larger audience and be more easily implemented.
As explicated in the introduction and discussion, the advantage of multicolor markers is to permit to single out astrocytes even in cases of apposition to labeled neighbors. In monochrome approaches (such as electroporation of single-color transposons or Cre electroporation in Ai9 mice), the ability to single out individual astrocytes is dependent on labeling sparseness, itself directly linked to the concentration of injected reporter (high

concentration will lead to high number of labeled astrocytes and therefore higher probability to mark clustered astrocytes). Instead, the MAGIC Markers strategy proves effective for such purpose with a wide range of labeling densities. In the Figure R1 below is presented a clear example of how color helps to single out touching astrocytes (A). Whereas it is not possible to extract astrocyte morphology when touching astrocytes are single colored (as seen when using only the green channel, B-B'), this limitation is alleviated when we use the blue channel (C-C'). (See Figure 3A for another example). To clarify this, we have added in the discussion a sentence on the advantage of color vs. monochrome labels. Thanks to Reviewer #1 comments, we also realize that our previous formulation of step 7.1.1 may lead to understand that we analyze only isolated astrocytes. We rephrased it in the following way: « Load the image stack and search for isolated astrocytes or nearby astrocytes displaying distinct colors ».

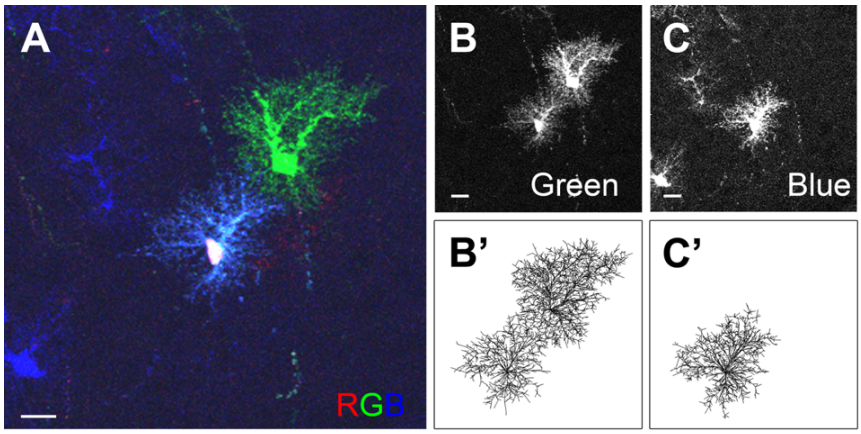

Figure R1. Arbor segmentation of neighboring cortical astrocytes. Example of two adjacent astrocytes both expressing EYFP but efficiently singled out using the expression of mTurquoise2. Maximal intensity projections of cropped z-stack showing adjacent green and cyan astrocytes at P7 (A, B, C) and their arborization reconstruction after segmentation with Vaa3D using the green (B') or blue (C') channel. Scale bar: 40 μm.

-In the discussion the authors mention as a main limitation of the method the existence of dense labeling of pyramidal neurons that prevents semi-automated segmentation. This limitation could be easily solved conducting electroporation at early postnatal ages, like P1, instead of embryonically. The number of astrocytes born from neural stem cells at P1 is elevated, allowing the analysis described in present manuscript without the interference of pyramidal neurons. The authors also state that electroporation at postnatal ages prevents labeling of early-born astrocytes however it looks to me the scope of this work is not to label all of the astrocytes if not some of them. The authors need to further discuss about appropriated timing for electroporation and success of the analysis.

We agree with Reviewer#1 that electroporation of neural stem cells at early postnatal stages could be used to prevent the labeling of cortical pyramidal neurons as in PALE. We now mention this possibility in the discussion of our manuscript. Yet, embryonic electroporation of MAGIC markers enables to target astrocytes issued from embryonic progenitors, thus labeling a larger complement of astrocytes and avoiding potential biases linked to their origin. We therefore believe that the protocol that we present here offers more flexibility and an access to a broader set of biological questions regarding cortical astrocyte anatomy, development and physiology. In addition, the labeling of pyramidal neurons is not that inconvenient as it can be restricted to the upper cortical layers (layers 2-3) if the *in utero* electroporation is performed at E15.5, as stated in the first paragraph of the discussion on the timing of electroporation.

Minor Concerns:

-When comparing MAGIC markers to other methods used to label cortical astrocytes the authors state that other method, Star Track rely on the use of GFAP promoter as a possible problem, however new versions of the Star Track method use the Ubiquous promoter Ubiquitin C (Figueres Oñate et al, 2016).

We agree with Reviewer#1 that an updated version of StarTrack relies on the Ubiquitin C promoter. We now have corrected this and added the paper published by Figueres- Oñate et al in 2016 in our reference list.

-The authors may want to discuss if this analysis could also be applied to study morphological aspects of other cell types like oligodendrocytes.

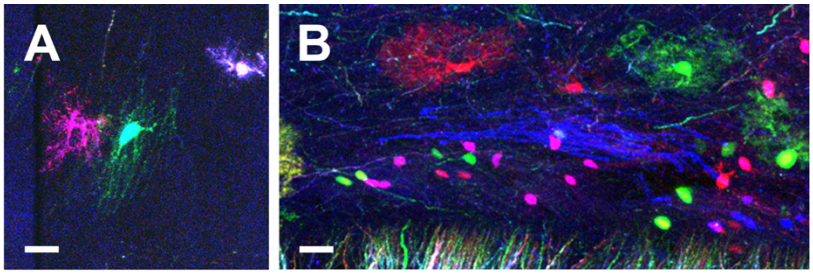

Figure R2. Examples of two isolated oligodendrocytes at P21 labeled with MAGIC Markers. Scale bar: 20 μm.

We thank the Reviewer for suggesting this possibility. As oligodendrocytes present a branched architecture comparable on many aspects to that of astrocytes, we anticipate that a similar pipeline may be applicable to study these cells. Indeed, cells presenting all hallmarks of oligodendrocytes are found in brains electroporated with MAGIC Markers, opening the way to the development of such analysis pipeline. We are now mentioning this idea in the Discussion.

-Which mouse strain is employed?

We performed the protocol described here on RjOrl:SWISS (outbred) mice and we added this previously missing information to the revised version of the manuscript. However, this protocol can be successfully applied to inbred (C57BL/6) mice provided modifications of a few electroporation parameters (number of pulses, voltage, analgesic solution concentration and injection volume), and this information is also now added to the Discussion of the revised version of the manuscript.

-In step 2.2.4, why do the authors place a drop of Fast Green in the lid of the dish plate?

Using a micropipette, we place a 1 μl drop of Fast green in the lid of the dish plate to use it as a reference volume for approximately 1 μl. The sentence has been clarified.

-How do they adjust the diameter of the micropipette's tip?

We set up the diameter of the micropipette's tip by breaking its tip with fine forceps and by adjusting the pressure parameter and injection duration of the INJECT+MATIC micro injector in order to obtain equivalent size drops between the drop delivered by the micro injector and the reference 1 μl –drop of Fast green placed previously on the lid of the dish plate. This enables delivery of approximately 1 μl of DNA solution per injection.

-Could the authors provide further information about the measurements that can be

done with the Vaa3D software? For example explain how to quantify number of branches or its complexity.

Following the suggestion of Reviewer #1, we have added a last step in the protocol for Arbor segmentation indicating how to retrieve measurements using Vaa3D (7.2.12). However, we must emphasize, as mentioned explicitly in the manuscript that the limited resolution offered by confocal microscopy can only provide a partial rendering of astrocyte morphological complexity. While images acquired with higher resolution (63x 1.4 NA oil objective) and deconvolution algorithms can be used to reconstruct finer details of astrocyte arbors, the finest processes cannot be resolved with conventional optical imaging, ultimately preventing absolute quantification of number of branches or complexity. We rather aimed here to provide an easy method to quickly assess astrocyte volume and morphology in a semi-quantitative manner useful for an efficient screen for potential phenotypes affecting cortical astrocyte volume or morphology in various experimental conditions (gain or loss of functions, mouse models of neurological diseases….).

-Regarding the highlighted text authors go straight from the histology to the arbor tracing using Vaa3D software without providing any information about acquisition of the images. I believe some information about the complex acquisition of the multicolor images will help to have a smoother transition during the video recording.

We thank Reviewer #1 for this excellent suggestion that we took into account in the updated version of the manuscript. We now present the acquisition of the multicolor images, instead of the classical perfusion and histological techniques that are most commonly used.

Reviewer#2:
Manuscript Summary:
In this manuscript, the authors reported a MAGIC markers (MM) strategy, which is an in utero electroporation-based multicolor labeling method, for labeling individual protoplasmic astrocytes. Expression constructs, including CAG-piggyback/Tol2 transposases and self-excisable Cre recombinase, with reporter constructs, containing multiple fluorescent proteins, including eBFP, mTurquoise2/mCerulean, EYFP, and tdTomato/mCherry, were electroporated into cortical ventricle at embryonic stages to label cortical progenitors with distinct fluorescent proteins. The progeny of cortical progenitors, including astrocytes, could be tracked during development, based on their expression of distinct sets of fluorescent proteins. This manuscript also provides a detailed description of imaging analyzing pipelines for analyzing the volume and morphology of astrocytes.

Major Concerns:
The usage of in utero electroporation with reporter constructs containing multiple fluorescent proteins in embryonic stages is the major advantage of this method, when compared with previous methods for labeling astrocytes. However, the major disadvantage of this method, as mentioned in the discussion, is that it does not label astrocyte specifically. However, as labeled astrocytes and neurons distribute differently and have different morphology, it is still a useful tool for studying astrocyte development.

The major concern of this method is that cells derived from the same clone/progenitor, labeled with the same color, are likely to be distributed close to each other (as the cell clusters seen in Figure 2). Although this method is ideal to analyze clonal relationship of astrocytes, it might be difficult to analyze cellular processes of individual astrocytes from the same clone, when they are of the same color and

distributed close to each other. However, as shown in the authors' recent manuscript (Clavreul, et al., 2019), they were able to analyze both the clonal organization and arborization pattern of individual astrocytes.

We thank Reviewer #2 for adequately pointing the advantages and disadvantages of our multicolor labeling strategy and subsequent image analysis pipeline to single out cortical astrocyte volume and morphology. We have added a note in the discussion on the possibility to electroporate MAGIC Markers postnatally to suppress neuronal labeling. We also agree that an important limitation of our technique is to individualize cortical astrocytes belonging to a clonal cluster because by definition, all sister cells will display identical color combination. However, as pointed in our recently published paper (Clavreul et al, 2019), the scattered architecture of astrocyte clones often permits to bypass this issue, and our multicolor strategy is helpful to single out touching astrocytes belonging to distinct clones as shown in Figure R1 below (also see Figure 3A).

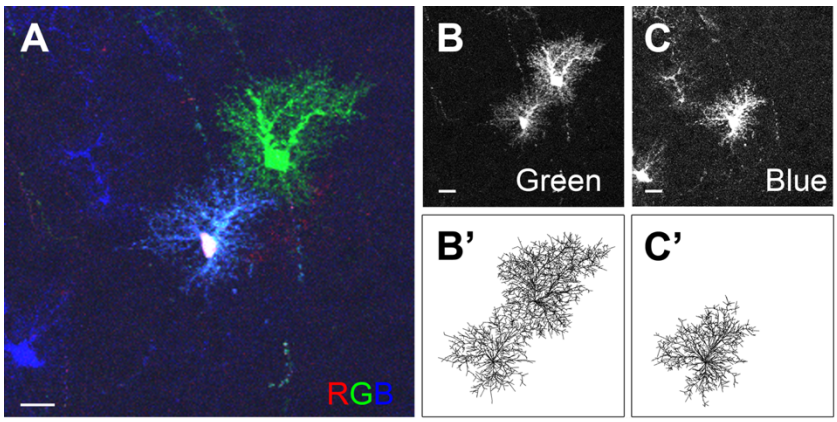

Figure R1. Arbor segmentation of neighboring cortical astrocytes. Example of two adjacent astrocytes both expressing EYFP but efficiently singled out using the expression of mTurquoise2. Maximal intensity projections of cropped z-stack showing adjacent green and cyan astrocytes at P7 (A, B, C) and their arborization reconstruction after segmentation with Vaa3D using the green (B') or blue (C') channel. Scale bar: 40 μm.

Minor Concerns:
1. As the usage of the self-excisable Cre is important for this method, it should be mentioned in the introduction, instead of only mentioned in the figure legend.

We agree with Reviewer #2 and have now mentioned the self-excisable Cre in the introduction of the revised version of our manuscript.

2.As electroporation condition varies depending on the embryonic stages, authors should indicate the embryonic stage (e.g. E15.5) they used in the method.

We apologize for the omission and the E15.5 embryonic stage of electroporation is now clearly mentioned in the revised version of the manuscript.

Reviewer#3:
Manuscript Summary:
This manuscript describes a useful technique that can be harnessed for labeling of subsets of astrocytes in the developing cortex. While the electroporation technique is common, where this paper makes an important contribution is in the imaging of sparsely and multi-colored astrocytes in the cortex.

The manuscript is well written and describes this technique is great detail, such that most scientists familiar with the basic electroporation technique and basic confocal microscopy should be able to utilize this approach.

Major Concerns:
None. This paper is ready to be published.

We thank Reviewer #3 for his/her appreciation of our work and his/her validation of our manuscript that has been even further improved in the revised version.