Journal of Visualized Experiments

In Utero Electroporation of Multiaddressable Genome-Integrating Color (MAGIC) Markers to Individualize Cortical Mouse Astrocytes

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61110R1
Full Title:	In Utero Electroporation of Multiaddressable Genome-Integrating Color (MAGIC) Markers to Individualize Cortical Mouse Astrocytes
Keywords:	cerebral cortex; astrocytes; development; cell volume; morphology; transgenes; electroporation; gene transfer techniques; Microscopy
Corresponding Author:	Karine Loulier Institut des Neurosciences de Montpellier Montpellier, FRANCE
Corresponding Author's Institution:	Institut des Neurosciences de Montpellier
Corresponding Author E-Mail:	karine.loulier@inserm.fr
Order of Authors:	Laura Dumas
	Solène Clavreul
	Jason Durand
	Edwin Hernandez-Garzon
	Lamiae Abdeladim
	Raphaëlle Barry-Martinet
	Alicia Caballero-Megido
	Emmanuel Beaurepaire
	Gilles Bonvento
	Jean Livet
	Karine Loulier
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Montpellier, France

Institut des Neurosciences de Montpellier

Inserm U1051 : Déficits sensoriels et moteurs

Montpellier, December 10th, 2019

Manuscript submission

Title: Multicolor labeling using *in utero* electroporation of MAGIC Markers to individualize cortical mouse astrocytes

Authors: Laura Dumas*, Solène Clavreul*, Jason Durand, Edwin Hernandez-Garzon, Lamiae Abdeladim, Raphaëlle Barry-Martinet, Alicia Caballero-Megido, Emmanuel Beaurepaire, Gilles Bonvento, Jean Livet, Karine Loulier.

Dear Dr Myers,

It is my pleasure to submit the manuscript by Dumas et al. to your consideration for publication in *Journal of Visualized Experiments*.

I have been invited by Isabel Martinez Garay, guest editor of "Standard and novel methods in the study of corticogenesis" to contribute to this method collection with a methods video featuring the techniques we use to study lineage tracing during cortical development.

For this purpose, my co-authors and I have put together a manuscript that details the key steps of multicolor labeling based on *in utero* electroporation (IUE) to single out astrocytes in the mouse cerebral cortex, and analyze their volume and morphology with a user-friendly image analysis pipeline.

Therefore, we present here a protocol to label cortical astrocytes with the multicolor MAGIC Markers strategy, which relies on piggyBac/Tol2 transposition and Cre/*lox* recombination to stochastically express distinct fluorescent proteins (FP) in nearby cortical progenitors using IUE. This results in combinations of color markers expressed by cortical progenitors prior to the start of gliogenesis, which enable to track their descent, including astrocytes, at the individual cell level, from embryonic to adult stages. Semi-sparse labeling is achieved by adjusting the concentration of electroporated MAGIC Markers vectors while color contrasts resulting from distinct FP expression in astrocyte processes permit to collect key information about their territorial volume (using IMARIS) and complex morphology (using Vaa3D). In summary, our present work provides a comprehensive experimental workflow including the details of the electroporation procedure, multichannel imaging with confocal image acquisition and computer-assisted 3D segmentation.

Importantly, this convenient and robust multicolor strategy helped us to uncover key features of cortical astrocyte development (Clavreul et al, 2019). Indeed, this technique gives an easy and quick access to individualized cortical astrocyte surface and morphology in the mouse cerebral cortex at various developmental stages. Finally, this powerful approach will be useful to the research community as it offers an alternative way to analyze cortical astrocyte anatomical properties in various experimental conditions without resorting to complex crosses with transgenic reporter lines.

Given the above elements, I thank you for considering our manuscript for publication in *Journal of Visualized Experiments.*

Sincerely yours,

Karine Loulier, Ph.D,
Institut des Neurosciences de Montpellier
80 avenue Augustin Fliche
34091 Montpellier Cedex 5 – France
Phone: +33 (0)4 99 63 61 09
Email: karine.loulier@inserm.fr

TITLE:

In Utero Electroporation of Multiaddressable Genome-Integrating Color (MAGIC) Markers to Individualize Cortical Mouse Astrocytes

AUTHORS AND AFFILIATIONS:

Laura Dumas[1]*, Solène Clavreul[2]*, Jason Durand[1], Edwin Hernandez-Garzon[3], Lamiae Abdeladim[4], Raphaëlle Barry-Martinet[2], Alicia Caballero-Megido[1], Emmanuel Beaurepaire[4], Gilles Bonvento[3], Jean Livet[2], Karine Loulier[1]

[1]Université de Montpellier, INSERM, Institut des Neurosciences de Montpellier, Montpellier, France

[2]Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France

[3]Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Département de la Recherche Fondamentale, Institut de biologie François Jacob, Molecular Imaging Research Center (MIRCen), CNRS UMR 9199, Université Paris-Sud, Université Paris-Saclay, Fontenay-aux-Roses, France

[4]Laboratory for Optics and Biosciences, Ecole polytechnique, CNRS, INSERM, IP Paris, Palaiseau, France

*These authors contributed equally.

Corresponding Author:

Karine Loulier (karine.loulier@inserm.fr)

Email Addresses of Co-authors:

Laura Dumas (laura.dumas@inserm.fr)
Solène Clavreul (solene.clavreul@helmholtz-muenchen.de)
Jason Durand (jason.durand@inserm.fr)
Edwin Hernandez-Garzon (ehernandezg4@gmail.com)
Lamiae Abdeladim (labdeladim@berkeley.edu)
Raphaëlle Barry-Martinet (raphaelle.barry-martinet@univ-tln.fr)
Alicia Caballero-Megido (alicia.caballero@inserm.fr)
Emmanuel Beaurepaire (emmanuel.beaurepaire@polytechnique.edu)
Gilles Bonvento (gilles.bonvento@cea.fr)
Jean Livet (jean.livet@inserm.fr)

KEYWORDS:

cerebral cortex, astrocytes, development, cell volume, morphology, transgenes, electroporation, gene transfer techniques, microscopy

SUMMARY:

Astrocytes tile the cerebral cortex uniformly, making the analysis of their complex morphology challenging at the cellular level. The protocol provided here uses multicolor labeling based on in utero electroporation to single out cortical astrocytes and analyze their volume and morphology

with a user-friendly image analysis pipeline.

ABSTRACT:

Protoplasmic astrocytes (PrA) located in the mouse cerebral cortex are tightly juxtaposed, forming an apparently continuous three-dimensional matrix at adult stages. Thus far, no immunostaining strategy can single them out and segment their morphology in mature animals and over the course of corticogenesis. Cortical PrA originate from progenitors located in the dorsal pallium and can easily be targeted using in utero electroporation of integrative vectors. A protocol is presented here to label these cells with the multiaddressable genome-integrating color (MAGIC) Markers strategy, which relies on piggyBac/Tol2 transposition and Cre/*lox* recombination to stochastically express distinct fluorescent proteins (blue, cyan, yellow, and red) addressed to specific subcellular compartments. This multicolor fate mapping strategy enables to mark in situ nearby cortical progenitors with combinations of color markers prior to the start of gliogenesis and to track their descendants, including astrocytes, from embryonic to adult stages at the individual cell level. Semisparse labeling achieved by adjusting the concentration of electroporated vectors and color contrasts provided by the Multiaddressable Genome-Integrating Color Markers (MAGIC Markers or MM) enable to individualize astrocytes and single out their territory and complex morphology despite their dense anatomical arrangement. Presented is a comprehensive experimental workflow including the details of the electroporation procedure, multichannel image stacks acquisition by confocal microscopy, and computer-assisted three-dimensional segmentation that will enable the experimenter to assess individual PrA volume and morphology. In summary, electroporation of MAGIC Markers provides a convenient method to individually label numerous astrocytes and gain access to their anatomical features at different developmental stages. This technique will be useful to analyze cortical astrocyte morphological properties in various mouse models without resorting to complex crosses with transgenic reporter lines.

INTRODUCTION:

Astrocytes play numerous vital functions in brain development and physiology[1]. Beside their role at the blood-brain barrier where they regulate nutrient uptake and blood flow, they actively contribute to synapse formation and function while producing neuromodulators that can alter neuronal activity and behavior[2]. Furthermore, astrocyte dysfunction contributes to a variety of neurological disorders[3]. Astrocytes located in the cerebral cortex display an elaborate morphology enabling extensive contact with neuronal processes. These contacts, essential for circuit function, also control astrocyte morphogenesis and synaptogenesis through cell-adhesion proteins[4]. Neuroscientists need convenient and robust tools to investigate astrocyte development and morphogenesis in their neurological models of interest. However, due to the astrocyte's close apposition to its neighbors and the uniform three-dimensional tiling of the gray matter, it is challenging to single out cortical astrocytes and comprehensively assess their morphology using immunomarkers.

Currently, two main genetic engineering strategies enable labeling and individualization of cortical astrocytes in situ: sparse reporter activation in transgenic mouse lines or somatic transgenesis using electroporation of reporter plasmids. The first strategy relies on breeding a

floxed reporter mouse line with mice expressing an inducible form of Cre recombinase activated specifically in astrocytes upon tamoxifen delivery (e.g., *Aldh1l1-CreERT2*[5]). Several disadvantages are associated with this strategy. First, breeding transgenic mice requires a large number of animals and multiple assays are typically needed to determine the proper dose of tamoxifen to provide adequately sparse labeling of cortical astrocytes. Analyzing cortical astrocyte phenotypes in a genetic mouse model of interest will require even more breeding and mouse consumption. Furthermore, in utero tamoxifen injection is known to interfere with parturition, making this strategy difficult to apply to the study of the earliest stages of astrocyte development. In vivo DNA electroporation is an alternative tamoxifen-free strategy that relies on a minimum number of animals[6]. Performed either at embryonic or postnatal stages, this approach consists of injecting reporter plasmids in the lateral ventricles of rodents followed by electric pulses that create pores in the cell membrane, hence allowing DNA to enter progenitor cells lining the ventricle. Subsequently, the reporter transgenes carried by the electroporated plasmids are processed by the targeted cell machinery and expressed[7]. Two electroporation methods have been previously described to label mouse cortical astrocytes: 1) Postnatal Astrocyte Labeling by Electroporation (PALE), which relies on the electroporation of 1–2 single-color episomal reporter plasmids at early postnatal stages[4]; 2) The StarTrack strategy based on in utero electroporation (IUE) of multiple single-color integrative reporter plasmids[8–10]. Although these two techniques efficiently label PrA in the cerebral cortex, they also present some limitations. In their initial version, both methods rely on a glial fibrillary acidic protein (GFAP) promoter to drive expression in astrocytes, which may bias the labelling toward radial glia as well as pial and reactive astrocytes that express GFAP more strongly than normal resting PrA[11,12]. Regarding PALE, other disadvantages are the late stage of electroporation, which prevents labeling of early-born PrA (or those originating from early delaminating progenitors) and analysis of early stages of astroglia development, and the use of episomal vectors that become diluted through successive divisions during the massive proliferation that PrA undergo during the first postnatal week[13,14]. In contrast to PALE, StarTrack is based on the embryonic electroporation of integrative reporter plasmids that allow tracking the contribution of both embryonic and postnatal progenitors to PrA. An updated StarTrack scheme relying on the ubiquitin C promoter (UbC-*StarTrack*) achieves broader expression of fluorescent reporters in both the neuronal and glial descent (astrocytes included) of neural progenitors[15–17]. However, in its current version, implementation of this approach is complex, as it relies on an equimolar mixture of 12 distinct plasmids expressing six fluorescent proteins (FP) with partial excitation and emission spectra overlap.

Presented here is a straightforward in utero electroporation-based multicolor labeling method using integrative reporter constructs driven by a strong and broadly active promoter to single out cortical astrocytes[14]. In addition, an easy image analysis pipeline using both licensed (e.g., Imaris) and open access (Vaa3D[18–20]) image analysis software is provided to segment astrocyte territorial volume and arborization, respectively. Compared to the previously described methods, this strategy relies solely on 1–2 multicolor integrative transgenes Multiaddressable Genome-Integrating Color Markers (MAGIC Markers or MM[21]) directed to the cytoplasmic and (optionally) nuclear cell compartment whose expression is driven by a synthetic *CAG* promoter comprised of a cytomegalovirus enhancer, chicken β-actin promoter, and rabbit β-globin splice acceptor site[22]. This enables labeling and tracking of cortical astrocytes, from embryonic to late postnatal stages,

independent of GFAP expression[14,23]. Each of these transgenes bears the following four distinct FP: eBFP, mTurquoise2/mCerulean, EYFP, and tdTomato/mCherry, which display minimal spectral overlap that can be easily circumvented with 1) Sequential channel acquisition; 2) Optimized excitation power and collection gain; and 3) Specific dichroic filters to collect narrow FP emission windows. The MM strategy uses Cre/*lox* recombination with a self-excisable Cre recombinase (seCre) to drive stochastic expression of FP in a cellular population. A single copy of MM transgene expresses FP in a mutually exclusive manner, while multiple transgenes give rise to FP combinations, creating dozens of distinct hues. Genomic integration of the transgenes is driven by the piggyback (PB) or Tol2 transposition system[24–26]. Therefore, through in utero electroporation, the MM toolkit and the multicolor 'mosaic' that it generates enable simultaneous marking of multiple adjacent cortical progenitors and the tracking of their glial descent, including cortical astrocytes, over long periods. Color contrasts resulting from the expression of distinct FP permit delineation of the contour of PrA and subsequently extract key information about their territorial volume (using IMARIS) and complex morphology (using Vaa3D). The multicolor strategy presented in detail here is a convenient and robust method that gives quick and easy access to the cortical astrocyte surface and morphology in wild type mice at various developmental stages, and is easily adaptable to investigate astrocyte anatomical features in mouse models of neurological diseases without using transgenic reporter lines.

PROTOCOL:

All animal procedures described here were carried out in accordance with institutional guidelines. Animal protocols have been approved by the Charles Darwin animal experimentation ethical board (CEEACD/N°5).

1. Preparation of endotoxin-free plasmids for MAGIC Markers in utero electroporation

1.1. Bacterial transformation

1.1.1. On ice, thaw DH5 alpha competent cells stored at -70 °C.

1.1.2. Warm up the agar plates containing the appropriate antibiotic (100 µg/mL ampicillin or 50 µg/mL kanamycin) at 37 °C.

1.1.3. Add 1 µL of 5–50 ng of MAGIC Markers plasmid DNA in 10 µL of thawed DH5 alpha competent cells and incubate on ice for 10 min without mixing.

1.1.4. For the heat shock transformation, place the aliquot in a 42 °C water bath for 45 s, then place immediately on ice and wait 3–5 min.

1.1.5. Under sterile conditions, add 230 µL of SOC medium and incubate for 1 h at 37 °C.

1.1.6. Spread the content of the aliquot over the agar plate and incubate overnight at 37 °C.

1.2. Plasmid culture

1.2.1. The following morning, under sterile conditions, pick up a colony from the agar plate and put it in a 14 mL tube containing 2 mL of LB medium with appropriate antibiotic. Let it incubate for the day at 37 °C in a shaking incubator at 300 rpm.

1.2.2. At the end of the day, seed 300 mL of LB with antibiotic using the 2 mL preculture from step 1.2.1 and incubate overnight at 37 °C in a shaking incubator at 300 rpm.

1.3. Plasmid DNA preparation

1.3.1. The following morning proceed with the purification of MAGIC Markers plasmid DNA (i.e., PB-CAG-Cytbow and Tol2-CAG-Nucbow as well as CAG-driven plasmids expressing PB and Tol2 transposases and Cre recombinase) using an endotoxin-free maxiprep kit following the manufacturer's protocol.

1.3.2. Elute the DNA in 200 µL of sterile water and estimate its concentration using a spectrophotometer prior to storage at -20 °C.

2. Preparation for MAGIC Markers in utero electroporation (MM IUE)

2.1. Solution preparation

2.1.1. Warm 30 mL of 0.9% saline solution at 37 °C in a water bath and keep it warm during the entire duration of the surgery.

2.1.2. Prepare a plasmid mix containing *PB-CAG-Cytbow* and *Tol2-CAG-Nucbow* (final concentration, 0.8 µg/µL each), PB and Tol2 transposases (final concentration, 0.4 µg/µL each), *CAG-SeCre* (final concentration, 0.16 µg/µL), and 0.01% Fast Green dye in PBS without Ca^{2+} and Mg^{2+}.

NOTE: For astrocyte anatomical reconstruction purposes, the *Tol2-CAG-Nucbow* construct may be omitted. However, this plasmid is useful to distinguish doublets of closely juxtaposed astrocytes and when using MAGIC Markers to probe clonal relationships among astrocytes[14].

2.1.3. Prepare anesthetic solution containing 100 µL of ketamine (100 mg/mL) and 100 µL of xylazine (20 mg/mL) diluted in 2 mL of saline solution.

2.1.4. Prepare analgesic solution by diluting 0.3 mg/mL buprenorphine stock solution 1:10 in saline solution.

2.2. Preparation of the surgery material

2.2.1. Sterilize the surgical tools (see **Table of Materials**) at high temperature in a glass bead sterilizer or equivalent.

2.2.2. Place a drop of electrode gel in a 35 mm dish.

2.2.3. Insert the micropipette in the microinjector holder, break the tip of the micropipette, and aspire the DNA solution.

2.2.4. Place a 1 µL drop of Fast Green solution (0.01%) in the lid of a 3 cm dish and, using it as a reference, adjust the diameter of the micropipette's tip by breaking its tip with fine forceps. Adjust the pressure parameter so that equivalent size drops are produced by the microinjector, thereby enabling delivery of approximately 1 µL of DNA solution per injection.

2.3. Preparation of the pregnant female mouse

2.3.1. Weigh the RjOrl:SWISS pregnant mouse.

2.3.2. Perform an intraperitoneal injection with 12.5 µL per gram of body weight (BW) of anesthetic solution. Wait for 5 min and verify that the mouse is asleep by pinching its toe.

2.3.3. Once the animal is unresponsive to pinch, inject it subcutaneously with 1.6 µL/g BW of analgesic solution.

2.3.4. Add a drop of ocular gel on each eye to prevent drying during the surgery and place the animal belly up on the warming pad.

2.3.5. Gently shave its abdomen, clean it with a pad soaked with iodine, and sanitize the shaved area with an alcohol pad.

2.3.6. Arrange an operating field by placing sterile compresses around the shaved, cleaned, and sanitized area.

3. In utero electroporation (IUE)

3.1. Intraventricular injection

3.1.1. Cut a 2 cm-vertical incision along the midline starting in the lower part of the abdomen, through the skin, and then through the underlying muscle. Expose the uterine horns by gently manipulating the embryonic bags and assess the location of the cervix and the number of embryonic bags on each side of the cervix.

3.1.2. Orient the brain of the E15.5 embryo to be electroporated in order to see the bregma, , easily recognized as its location matches with the junction of the three main blood vessels that run along the cerebral fissures.

3.1.3. Imagine a virtual line between the bregma (visible through the skull) and the eye; introduce

the micropipette between the virtual line and the longitudinal fissure, then press the injector's foot pedal to deliver 1 µL of DNA solution in the lateral ventricle of the targeted hemisphere.

NOTE: When injected at the proper location, the filled lateral ventricle appears blue, indicating that it has been filled with the DNA solution.

3.2. Electroporation

3.2.1. Apply the electrodes (see **Table of Materials**), previously dipped in electrode gel, on both sides of the injected embryo with the anode covering the injected hemisphere. Press the foot pedal of the electroporator to deliver a series of four 50 ms pulses of 35 V, each separated by a 950 ms interval.

3.2.2. Dampen the embryo with warmed saline solution.

NOTE: The embryos should be kept humid using warmed saline solution during the entire surgical operation and the uterus should not dry out.

3.2.3. Repeat steps 3.1.3–3.2.2 for each embryo.

3.2.4. Once all targeted embryos have been electroporated, replace the uterine horns in the abdomen by gently pushing them with forceps back to their original position. Fill the abdominal cavity with warmed saline solution to prevent the uterus from drying while sutures are made.

3.2.5. First close the abdominal muscle with a continuous absorbable suture, and then the skin with multiple individual stitches (~10) using 4-0 suture.

3.2.6. Put the animal in a clean cage, lying on its side on a clean paper towel and turn the animal to the other side every 5–10 min until it wakes up and starts moving on its own.

3.2.7. Assess the state of the animal the following day, especially if a nest has been made.

NOTE: No postsurgery treatment is required. In the absence of a nest, any sign of pain (e.g., prostration, shaggy fur), and/or heavy bleeding, the animal should be euthanized without delay.

4. Tissue harvesting and sectioning

4.1. Tissue collection

4.1.1. Inject phenobarbital (100 mg/kg BW) intraperitoneally for terminal anesthesia at the desired harvesting time. In this case, the harvesting times were at postnatal (P) days P4, P7, and P21.

4.1.2. Perform intracardiac perfusion using cold premade paraformaldehyde-based solution.

4.1.3. Dissect out the brain and place it overnight at 4 °C in the paraformaldehyde-based solution for postfixation.

4.2. Histology

4.2.1. The following morning rinse the brain 3x for 10 min with 1x PBS.

4.2.2. Embed the brain in 3% agarose dissolved in 1x PBS.

4.2.3. Cut 80 µm sections using a vibrating-blade microtome.

4.2.4. Collect sections in a 24 well plate prefilled with 1x PBS.

4.2.5. Mount sections in the mounting medium (see **Table of Materials**) between the slide and coverslip. Keep the mounted slides at -20 °C for optimal fluorescent protein preservation and long-term storage.

5. Multichannel confocal imaging

5.1. Microscope settings

5.1.1. Set up the configuration of the confocal microscope to separately excite mCerulean/mTurquoise2, EYFP, tdTomato/mCherry using 440, 515, and 559 nm laser lines, respectively.

5.1.2. Use a 20x 0.8 NA (or higher NA) objective and adjust XY sampling and Z-step size according to Nyquist criteria.

NOTE: Images acquired with higher resolution (e.g., 60x 1.4 NA oil objective) and deconvolution algorithms will enable reconstruction of the finer details of astrocyte arbors. However, the experimenter should keep in mind that the finest astrocyte processes may not be resolved by conventional optical imaging.

5.2. Image acquisition

5.2.1. Find the brightest astrocytes in the electroporated area.

5.2.2. Because labeled cells located close to the surface might appear brighter than those deeper in the slice, adjust the acquisition settings on the surface cells to avoid saturating the images.

5.2.3. Adjust acquisition settings separately for each of the three channels while making sure to avoid pixel saturation using *HiLo* LUT, which displays zero values as blue and maximum pixel values as red.

NOTE: An adequately balanced image should display only a few blue and almost no red pixels.

5.2.4. Acquire tiled 1,024 x 1,024 pixel Z-stacks using the motorized stage of the microscope, with a 10% overlap between adjacent stacks to subsequently enable mosaic reconstructions of the electroporated area or the zone of interest.

6. Astrocyte territorial volume segmentation

NOTE: This is performed using a commercial software program (e.g., IMARIS).

6.1. Preparation of the dataset

6.1.1. Crop the labeled astrocytes within the 3D reconstruction (250 x 250 pixels) by selecting cells entirely enclosed in the imaged tissue section.

NOTE: It is also possible to work directly on larger volumes if the computer used can handle them.

6.1.2. Click on the **Blue Surface** button in the **Object** toolbar of the Surpass view to create a new **Surface** for each astrocyte.

6.1.3. To obtain better visualization contrast, first adjust the minimal and maximal color contrast values by clicking on **Edit** | **Show display Adjustment** option and dragging both handles. If needed, change the channel color by clicking directly on the channel name in the **Display Adjustment** window in order to select a new color.

NOTE: Preferentially always work with the same color display to prevent visual bias.

6.1.4. Click on **Edit | Image Properties** to manually indicate the voxel size in micrometers. If the voxel size is not accurate, the volume calculations will be incorrect.

6.1.5. Save the new settings.

6.2. Surface segmentation

6.2.1. Use the **Blue Icon** to introduce a surface. A **Surface** icon and box will appear on the **Scene** list. A **Volume** box allows to see/hide the dataset.

6.2.2. Select the surface line and click on **Skip Automatic Creation |Edit Manually**.

6.2.3. Click on **Contour | Visibility** and click **None**. Then move to the **Select** view and click on the **Draw** button.

6.2.4. Click on **Mode** to select the drawing mode.

6.2.5. Use the fast and efficient **Isoline** semiautomated drawing tool. Define the cell outline by moving the mouse pointer on it. If the **isoline** preview does not match the astrocyte outline, adjust the mouse pointer position. To validate the preview, left click on the selection. To correct potential mistakes, use the **Board** window to Delete the current Z-plane contour or press **Ctrl + Z**.

6.2.6. Using **Slice | Position** to navigate through the Z-planes, move to the next plane by changing the Z-plane number and start a new contour. Preferentially start from the middle of the cell and then move to the extremities.

6.2.7. When contours have been drawn in all the Z-planes containing the astrocyte, click on **Create Surface**. Before creating a surface, check that all the components are connected. If not, select the largest or connect relevant ones before exporting volume data.

6.2.8. Check the quantitative data displayed in the **Data Panel** and export statistics in spreadsheet format, saving volume value and unit.

6.2.9. Save the surface under a new name to access it later.

7. Tracing astrocyte arborization

NOTE: This is done using open access software program Vaa3D.

7.1. Preparation of the dataset

7.1.1. Load the image stack and search for isolated astrocytes or nearby astrocytes displaying distinct colors.

NOTE: The resolution of the reconstruction can be improved by increasing the objective NA and sampling. Ultimately, however, due to the resolution limit of confocal microscopy, the finest astrocyte processes cannot be accurately traced. Care should be taken to avoid oversegmenting beyond the resolution of the acquired images.

7.1.2. Using Fiji, crop 250 x 250 pixel image stacks around each astrocyte.

7.1.3. As Vaa3D tracing is performed on only one color, select one channel and deactivate the other channels.

7.1.4. Convert the image to RGB and save it in *.tiff* format.

7.2. Arbor tracing

7.2.1. Open the RGB image in Vaa3D. Go to **Image | Data | Geometry** then **image resampling**,

and adjust X/Y and Z voxel size values.

NOTE: While reconstructing astrocyte arbors, care should be taken not to trace details finer than the resolution provided by the images.

7.2.2. Open a 3D window by clicking on **Visualize | 3D viewer for the entire image**. Right-click on a 3D image and choose **1-right-click to define marker**. Point with the cursor the center of the astrocyte and then right-click to set a marker.

NOTE: Click on **Escape** to have access to the 3D view again.

7.2.3. Go to **Advanced|3D tracing|Vaa3D-Neuron2-auto-tracing**.

7.2.4. On the newly opened window, select the channel on which to apply the plug-in.

7.2.5. Choose a background threshold (often between 30 and 90). Adapt this value for each astrocyte if needed.

7.2.6. Uncheck **Radius** from **2D** and keep **Auto-down sample** and **auto-resample** checked.

7.2.7. Set **cnn_type** at 3, **length_thresh** at 1, and **SR_ratio** at 0.1.

7.2.8. After clicking **ok**, ensure that the plug-in automatically generates two files based on the original name. The suffix –ini.swc and -coordinateX–coordinateY-coordinateZ-app2.swc will be added. Manually add a distinctive label to these name files in order not to overwrite them while continuing to run the plug-in.

7.2.9. Open **Object Manager**, go to **Neuron | Line structure**, select the traced astrocyte, click on **Display Mode**, and select **Always line mode**.

7.2.10. Go back to the 3D window, where a skeleton of the astrocyte appears.

NOTE: If segmentation and signal do not match, repeat steps 7.2.3–7.2.10 and adjust the threshold until they do. Make sure to re-enter the other parameters as they are reset when the plug-in is restarted.

7.2.13 Right-click on **skeleton** and select first choice **neuron | line#1…APP2_Tracing** to access quantitative measurements that will appear on a new window **Surface| Object Annotation**.

NOTE: Due to the limited resolution of light microscopy, measurements displayed under the listed items **Number of branches** and **Number of bifurcations** provide only a rough estimate of astrocyte arbor complexity.

REPRESENTATIVE RESULTS:

Electroporation of MAGIC Markers in embryonic cortical progenitors allows for the labeling of astrocytes from early to late stages of cerebral cortex development (**Figure 1**). These astrocytes were found in all cortical layers at various postnatal stages (P4, P7, P21) as they dispersed widely into the entire cerebral cortex. They were assessed with tiled confocal images acquired with a 20x 0.8 NA (or higher NA) objective and assembled as Z-stack reconstructions (**Figure 2**). MAGIC Markers combinatorial labeling enabled individualization of cortical astrocytes and extraction of information regarding their volume and morphology. Using the commercial image analysis software, the contour of individual astrocytes was delineated on each individual optical section of confocal image stacks to segment and reconstruct the territorial domain occupied by each astrocyte (**Figure 3**). From the same Z-image stacks, the branched morphology of singled-out cortical astrocytes was segmented using the open access software that allows extraction of the skeleton of the main astrocyte processes (**Figure 4**). These segmentation and tracing tools provided a semiquantitative assessment of the increase of territorial volume (**Figure 3**) and morphological complexity (**Figure 4**) that occurred for individual cortical astrocytes from early to late postnatal stages[14]. These approaches also revealed the heterogeneity of volume and morphology displayed by distinct cortical astrocytes at the same stage of development, as illustrated in **Figure 2**, **Figure 3**, and **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of MAGIC Markers (MM) in utero electroporation (IUE) to label cortical progenitors and their descent during brain development. (**A**) The MM toolkit comprises several plasmids encoding *PB-Cytbow*, *Tol2-Nucbow*, PB and Tol2 transposases, and self-excisable Cre recombinase (seCre). In MM constructs, three pairs of incompatible *lox* sites (*loxN*, *lox2272*, and *loxP*) flank four distinct FP coding sequences (EBFP2, mTurquoise2/mCerulean, EYFP, tdTomato/mCherry) and create mutually exclusive possibilities of excision upon Cre recombination. Before Cre action, only the first gene (EBFP2) is expressed. After Cre-mediated excision induced by seCre, either tdTomato/mCherry (red FP), EYFP (green FP), or mTurquoise2/mCerulean (cyan FP) is expressed. Co-expression of FP from multiple MAGIC Markers copies yields color combinations in the cytoplasm (*PB-CAG-Cytbow*) or nucleus (*Tol2-CAG-Nucbow*) of labeled cells. PB and Tol2 transposition endfeet framing the MM cassettes permit their integration into the genome of cortical progenitors when MM constructs are coelectroporated along with PB and Tol2 transposases coding plasmids. (**B–G**) Graphic illustration of IUE successive steps including the laparotomy of anesthetized pregnant mice (**B**), injection of the MM plasmid mix (**C**) in the lateral ventricles of the embryos (**D**), delivery of electric pulses through carefully positioned tweezertrodes (**E**) to target cortical progenitors in one of the two brain hemispheres (**F**), and suturing of pregnant mouse abdomen (**G**).

Figure 2: Following in utero electroporation of MAGIC Markers toolkit, multicolor astrocytes were found scattered in the entire cerebral cortex at postnatal stages. IUE of plasmids driving MM, seCre, PB, and Tol2 transposases expression in E15.5 mouse cortical progenitors resulted in labeling of layer 2–3 neurons and astrocytes at P4, P7, and P21. Expression level and color palette depended on the number of MM transgenes integrated in the genome of cortical progenitors. Montage of maximal intensity projections from tiled confocal image stacks acquired on 80 µm sagittal brain sections. Scale bar: 200 µm.

Figure 3: Segmentation of cortical astrocyte territorial domain at distinct developmental stages. Maximal intensity projections of cropped confocal Z-stack framing individual astrocytes (**A–F**) and their associated territorial domain segmented with the commercial software (**A'-F'**) at three distinct developmental stages: P4 (**A-B, A'-B'**), P7 (**C-D, C'-D'**), and P21 (**E-F, E'-F'**), respectively. Scale bar: 20 µm.

Figure 4: Tracing of cortical astrocyte arborization. Examples of two distinct astrocytes cropped from confocal Z-stacks (**A-D**) and reconstruction of their arborization coarsely segmented with Vaa3D (**A'-D'**) collected at two distinct developmental stages: P7 (**A-B, A'-B'**) and P21 (**C-D, C'-D'**), respectively. Scale bar: 20 µm.

DISCUSSION:

In utero electroporation (IUE) of MAGIC Markers in cortical progenitors (**Figure 1**) enabled labeling of astrocytes throughout the postnatal cerebral cortex at different postnatal stages (P4-P7-P21, **Figure 2**). Interestingly, the stage of IUE is not critical, as electroporation performed from E13.5 to E15.5 yields similar labeling patterns concerning cortical astrocytes[14]. However, the location of labeled pyramidal neurons in the cortical parenchyma varies with the electroporation stage. Indeed, IUE performed at E15 marks layer 2–3 neurons whereas IUE performed at E13 labels pyramidal neurons in all cortical layers, from layer 5 to layer 2–3[6,27]. This joint labeling of cortical pyramidal neurons following MM IUE is the main limitation of this method, as it prevents semiautomated segmentation of astrocyte morphology in layers where dense neuronal labeling interferes with astrocyte processes recognition. Should that be a problem, MM could be electroporated at postnatal stages as in PALE. At P4, labeling of radial glia fibers still present at that stage may also interfere with Vaa3D arbor segmentation. While cumbersome, a solution if one wishes to proceed with astrocyte arbor reconstruction at this stage is to manually remove radial glial fibers by progressively replacing the radial fiber signal with black pixels around the astrocyte of interest using Adobe Photoshop.

Despite this limitation, MM IUE is a powerful technique when adequately performed. A few critical steps need to be handled with care: 1) embryos must be kept humid during the entire surgery procedure and carefully manipulated to increase their survival; 2) using glass capillaries with a large diameter or squeezing embryonic bags too tightly can lead to bag rupture and therefore embryos' death; 3) during DNA injection, blood vessels must be avoided to prevent bleeding; 4) the whole procedure should not last more than 40 minutes from anesthesia to suture in order to maximize embryo survival; 5) stress plays a critical role in IUE success and therefore extra sources of stress such as change of cage, transportation, noises, and vibration must be avoided from 5 days prior surgery to 7 days after birth in order to prevent abortion and cannibalism.

Of note, experimenters wishing to specifically target cells born at a given stage can use the MM toolkit without adding the piggyBac and Tol2 transposases such that only the cells born at the time of the electroporation express the combinatorial labels. Another advantage of the method is the flexibility that it confers in terms of the density of labeled cells and their location in various

brain regions. Indeed, denser labeling of cortical astrocytes can be achieved by increasing the total concentration of MM transgenes while keeping plasmids ratio constant (1:10 ratio for Cre recombinase/MM constructs and 1:2 ratio for transposases/MM constructs). Contrary to monochrome approaches, such as electroporation of single-color transposons or Cre electroporation in Ai9 mice, where the ability to single out individual astrocytes requires sparse labeling, color contrasts offered by the MAGIC Markers strategy enable the individualization of astrocytes over a wide range of labeling densities. In addition, positioning the electrode probes in distinct orientations permits targeting distinct brain regions such as the prospective striatum (anode in the ventral position, opposite to the dorsal position required to achieve electroporation in the cerebral cortex), or hippocampus (anode in medial position)[28]. Finally, IUE can be performed in different mice strains such as outbred (OF1, Swiss) and inbred (C57BL/6J or N) mice, which opens the way for use of the MM toolkit in transgenic animal disease models. However, to successfully achieve IUE in inbred mice, one should adapt the number of pulses (three pulses for C57BL/6 mice versus five pulses in Swiss), voltage (30 V instead of 35 V), and analgesic dose (0.15 mg/mL buprenorphine stock solution and an injected volume of 0.8 µL/g BW).

In comparison with transgenic animal breeding or the PALE and StarTrack approaches, this method offers several advantages. To begin with, in contrast to the breeding strategy, it uses few animals. It also allows labeling of cortical astrocytes since the earliest stages of their development, including embryonic stages, unlike PALE[4], which relies on postnatal electroporation. Furthermore, in comparison to the twelve reporter constructs used in the Star Track approach[8], this strategy relies on only two multicolor transgenes, thus making DNA mix preparation and imaging simpler. Moreover, the balance between the different colors stochastically expressed by MM is intrinsically determined by the structure of the transgenes and does not depend on the mixing of different components by the experimenter. In addition, this strategy can extend beyond simple anatomical consideration and can be successfully applied for multiclonal analysis of astrocyte development, as demonstrated in previously published work[14]. This work, using rare color combinations of cytoplasmic and nuclear markers to define cortical astrocyte clones, demonstrated that they display extensive variability in terms of spatial distribution, structural organization, number, and subtype of generated cells.

Cortical astrocytes born from MM-labeled cortical progenitors displayed significant color contrast and dispersed widely across the entire cerebral cortex (**Figure 2**). Simple multichannel Z-stack acquisitions using confocal microscopy were used to access key astrocyte features such as their territorial volume (**Figure 3**) and their morphological complexity (**Figure 4**) at several postnatal stages. Beyond astrocytes, this methodology may be adapted to study the morphology of other glial cells such as oligodendrocytes. However, it should be kept in mind that the limited resolution offered by confocal microscopy can only provide a partial rendering of astrocyte morphological complexity. While images obtained with higher resolution (e.g., 63x 1.4 NA oil objective) and deconvolution algorithms can be used to reconstruct finer details of astrocyte arbors[14], the finest processes cannot be resolved with conventional optical imaging. Nevertheless, the strategy presented here will be of interest to screen efficiently for a potential phenotype affecting cortical astrocyte volume or morphology in mouse models of neurological diseases.

ACKNOWLEDGMENTS:

We thank S. Fouquet and the imaging and animal core facilities of Institut de la Vision and Institut des Neurosciences de Montpellier for technical assistance. This work was supported by fellowships from Région Ile-de-France and Fondation ARC pour la Recherche sur le Cancer to S.C, and from Université Paris-Saclay (Initiatives Doctorales Interdisciplinaires) to L.A., by funding from European Research Council (ERC-SG 336331, PI J. Valette) to E.H., by Agence Nationale de la Recherche under contracts ANR-10-LABX-65 (LabEx LifeSenses), ANR-11-EQPX-0029 (Equipex Morphoscope2), ANR-10-INBS-04 (France BioImaging), by Fondation pour la Recherche Médicale (Ref. DBI20141231328), by the European Research Council (ERC-CoG 649117, PI J. Livet) and by ATIP-Avenir program (PI K. Loulier).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Clarke, L. E., Barres, B. A. Emerging roles of astrocytes in neural circuit development. *Nature Reviews Neuroscience*. **14** (5), 311–321 (2013).
2. Ma, Z., Stork, T., Bergles, D. E., Freeman, M. R. Neuromodulators signal through astrocytes to alter neural circuit activity and behaviour. *Nature*. **539** (7629), 428–432 (2016).
3. Blanco-Suárez, E., Caldwell, A. L. M., Allen, N. J. Role of astrocyte-synapse interactions in CNS disorders: Astrocyte-synapse disease. *The Journal of Physiology*. **595** (6), 1903–1916 (2017).
4. Stogsdill, J. A. et al. Astrocytic neuroligins control astrocyte morphogenesis and synaptogenesis. *Nature*. **551** (7679), 192–197 (2017).
5. Srinivasan, R. et al. New Transgenic Mouse Lines for Selectively Targeting Astrocytes and Studying Calcium Signals in Astrocyte Processes in Situ and In Vivo. *Neuron*. **92** (6), 1181–1195 (2016).
6. Tabata, H., Nakajima, K. Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience*. **103** (4), 865–872 (2001).
7. Shimogori, T., Ogawa, M. Gene application with in utero electroporation in mouse embryonic brain. *Development, Growth & Differentiation*. **50** (6), 499–506 (2008).
8. García-Marqués, J., López-Mascaraque, L. Clonal Identity Determines Astrocyte Cortical Heterogeneity. *Cerebral Cortex*. **23** (6), 1463–1472 (2013).
9. Martín-López, E., García-Marques, J., Núñez-Llaves, R., López-Mascaraque, L. Clonal Astrocytic Response to Cortical Injury. *PLoS ONE*. **8** (9), e74039 (2013).
10. Gutiérrez, Y. et al. Sibling astrocytes share preferential coupling via gap junctions. *Glia*. **67** (10), 1852–1858 (2019).
11. Lee, Y., Messing, A., Su, M., Brenner, M. GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia*. **56** (5), 481–493 (2008).
12. Yoon, H., Walters, G., Paulsen, A. R., Scarisbrick, I. A. Astrocyte heterogeneity across the brain and spinal cord occurs developmentally, in adulthood and in response to demyelination. *PloS One*. **12** (7), e0180697 (2017).
13. Ge, W.-P., Miyawaki, A., Gage, F. H., Jan, Y. N., Jan, L. Y. Local generation of glia is a major

astrocyte source in postnatal cortex. *Nature*. **484** (7394), 376–380 (2012).
14. Clavreul, S. et al. Cortical astrocytes develop in a plastic manner at both clonal and cellular levels. *Nature Communications*. **10** (1), 4884 (2019).
15. Figueres-Oñate, M., García-Marqués, J., López-Mascaraque, L. UbC-StarTrack, a clonal method to target the entire progeny of individual progenitors. *Scientific Reports*. **6** (1), 33896 (2016).
16. Figueres-Oñate, M., García-Marqués, J., Pedraza, M., De Carlos, J. A., López-Mascaraque, L. Spatiotemporal analyses of neural lineages after embryonic and postnatal progenitor targeting combining different reporters. *Frontiers in Neuroscience*. **9**, 87 (2015).
17. Figueres-Oñate, M., Sánchez-Villalón, M., Sánchez-González, R., López-Mascaraque, L. Lineage Tracing and Cell Potential of Postnatal Single Progenitor Cells In Vivo. *Stem Cell Reports*. **13** (4), 700–712 (2019).
18. Peng, H., Ruan, Z., Long, F., Simpson, J. H., Myers, E. W. V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. *Nature Biotechnology*. **28** (4), 348–353 (2010).
19. Peng, H., Bria, A., Zhou, Z., Iannello, G., Long, F. Extensible visualization and analysis for multidimensional images using Vaa3D. *Nature Protocols*. **9** (1), 193–208 (2014).
20. Peng, H. et al. Virtual finger boosts three-dimensional imaging and microsurgery as well as terabyte volume image visualization and analysis. *Nature Communications*. **5** (1), 4342 (2014).
21. Loulier, K. et al. Multiplex Cell and Lineage Tracking with Combinatorial Labels. *Neuron*. **81** (3), 505–520 (2014).
22. Niwa, H., Yamamura, K., Miyazaki, J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*. **108** (2), 193–199 (1991).
23. Abdeladim, L. et al. Multicolor multiscale brain imaging with chromatic multiphoton serial microscopy. *Nature Communications*. **10** (1), 1662 (2019).
24. Chen, F., LoTurco, J. A method for stable transgenesis of radial glia lineage in rat neocortex by piggyBac mediated transposition. *Journal of Neuroscience Methods*. **207** (2), 172–180 (2012).
25. Siddiqi, F. et al. Fate Mapping by PiggyBac Transposase Reveals That Neocortical GLAST+ Progenitors Generate More Astrocytes Than Nestin+ Progenitors in Rat Neocortex. *Cerebral Cortex*. **24** (2), 508–520 (2014).
26. Yoshida, A. et al. Simultaneous expression of different transgenes in neurons and glia by combining in utero electroporation with the *Tol2* transposon-mediated gene transfer system. *Genes to Cells*. **15** (5), 501–502 (2010).
27. LoTurco, J., Manent, J.-B., Sidiqi, F. New and Improved Tools for In Utero Electroporation Studies of Developing Cerebral Cortex. *Cerebral Cortex*. **19** (suppl 1), 120–125 (2009).
28. Pacary, E. et al. Visualization and Genetic Manipulation of Dendrites and Spines in the Mouse Cerebral Cortex and Hippocampus using in utero Electroporation. *Journal of Visualized Experiments*. (65), e4163 (2012).

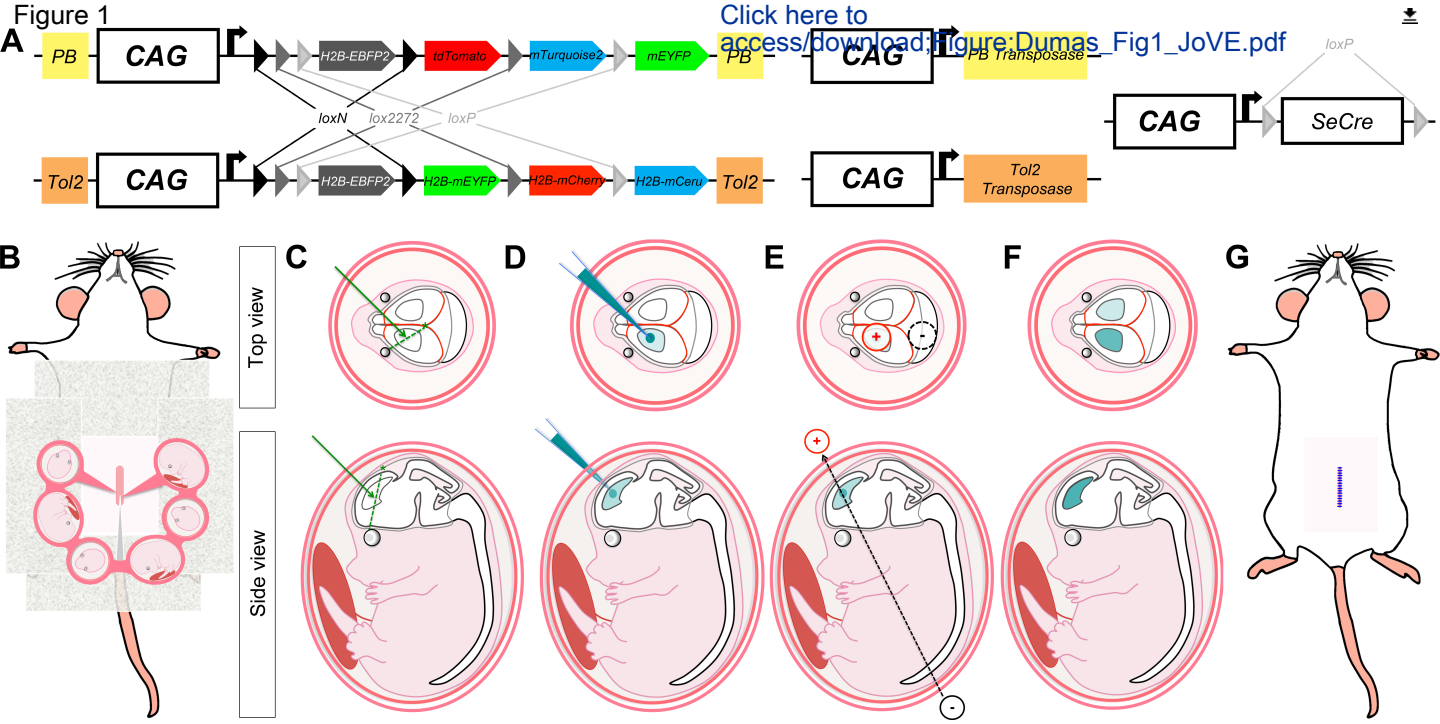

Figure 1. Dumas et al.

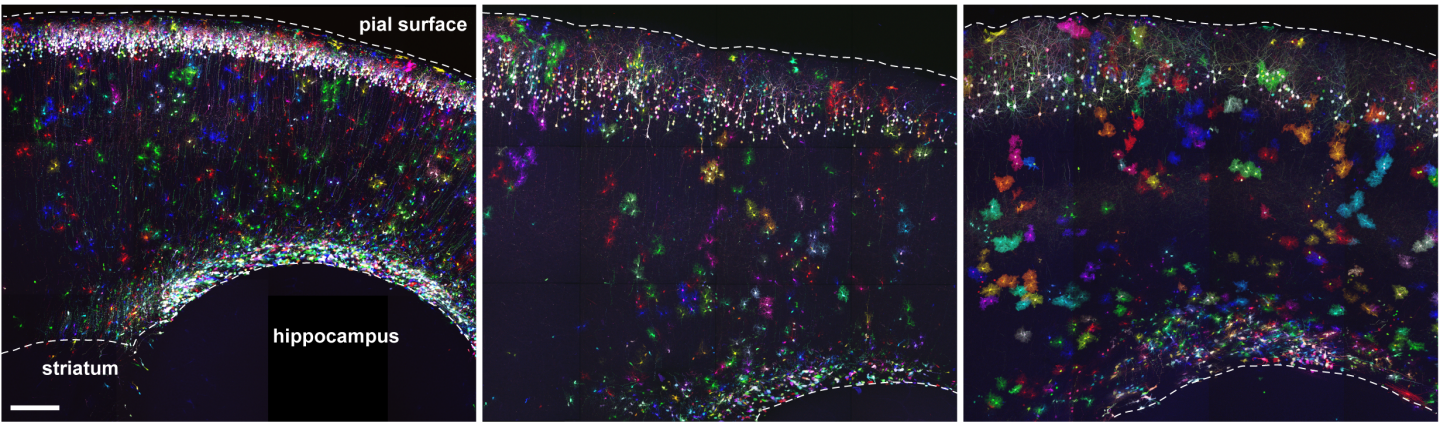

Figure 2. Dumas et al.

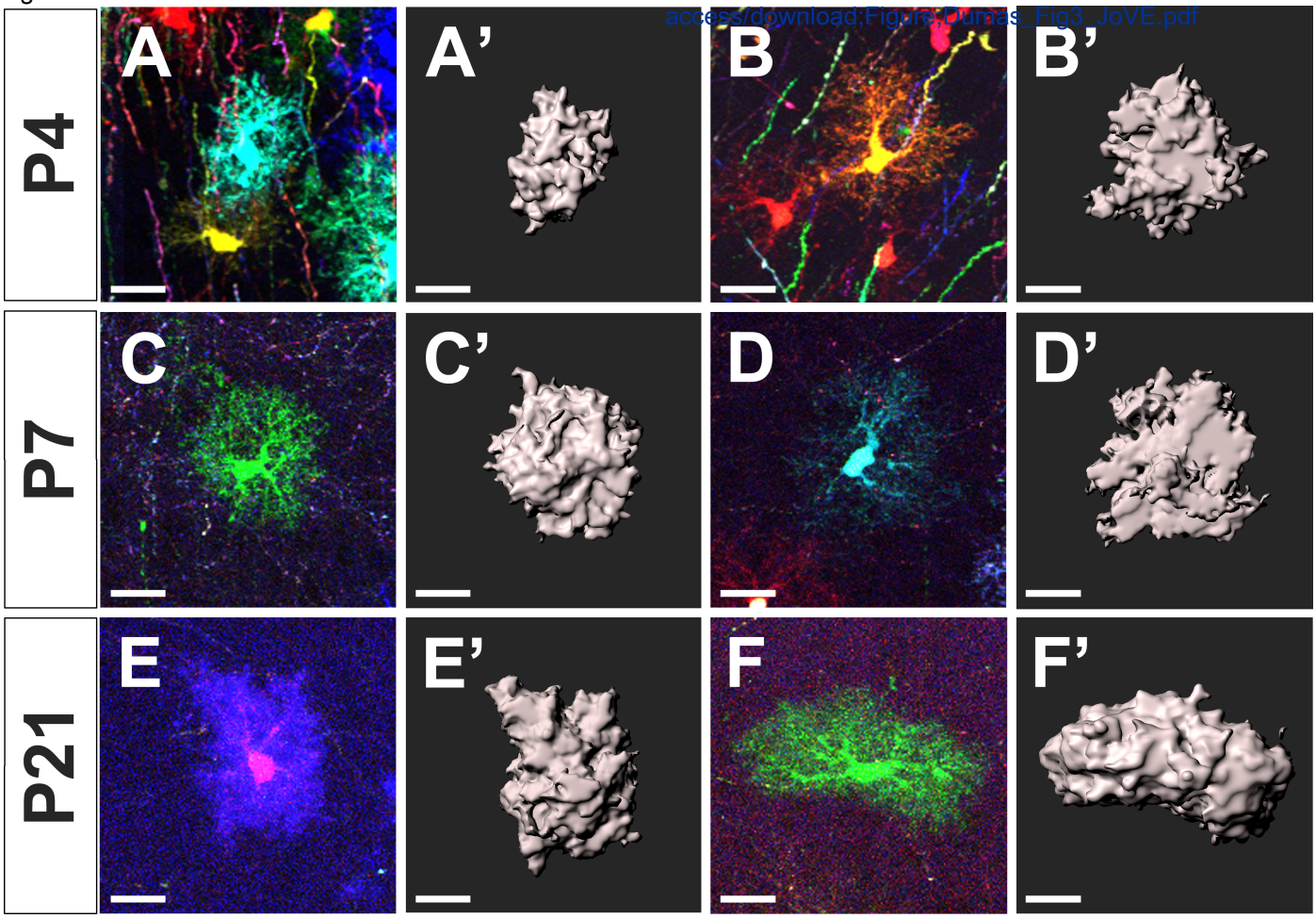

Figure 3. Dumas et al.

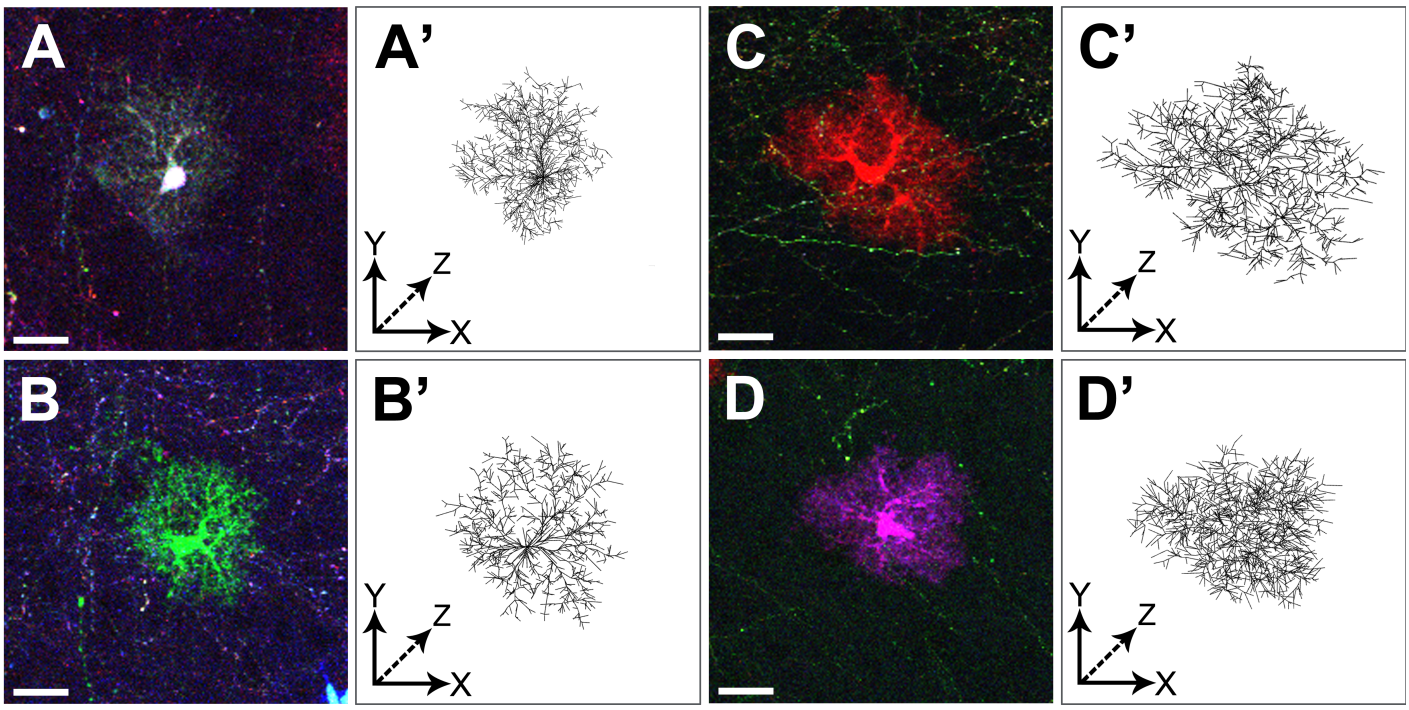

Figure 4. Dumas et al.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Dumas.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1.1 Bacteria transformation			
Ampicillin	Euromedex	EU0400-C	
DH5 alpha competent cells	Fisher Scientitic	11563117	
Ice box	Dutscher	139959	
Kanamycin	Sigma	60615	
LB Agar	Sigma	L2897	
SOC medium	Fisher Scientitic	11563117	
Sterile petri dish- 10 cm	Thermo Fisher	150350	
Water bath	VWR	462-0556H	
1.2 Plasmid culture			
14 ml culture tube	Dutscher	187262	
Glass erlenmeyer- 2L	Fisher Scientitic	11383454	
LB medium	Sigma	L3522	
1.3 Plasmid DNA preparation			
NucleoBond Xtra Maxi Plus EF	Macherey-Nagel	740426.10	
2.1 Preparation of the solutions			
26 G x 1/2 needle	Terumo	8AN2613R1	
30 G x 1/2 needle	Terumo	8AN3013R1	
Fast Green	Sigma Aldrich	F7272	
NaCl	VWR	27810.295	
Single-use polypropylene syringe, 1 mL	Dutscher	50002	
2.2 Preparation of the surgery material			
Adson Forceps - DeBakey Pattern- 12.5 cm	FST	11617-12	
Arched tip Forceps- 10 cm	FST	11071-10	
Glass bead sterilizer Steri 250	Sigma	Z378569	
Glass micropipette 1 mm diameter	FHC	10-10-L	
Graefe Forceps - Titanium 1 mm Tips Slight Curve- 10 cm	FST	11651-10	

Graefe Forceps - Titanium 1 mm Tips Straight- 10 cm	FST	11650-10
Iris Scissors - Delicate Straight- 9 cm	FST	14060-09
Laboratory tape	Fisher Scientitic	11730454
Microinjector	INJECT+MATIC	No catalog number
Olsen-Hegar Needle Holder - 12 cm	FST	12002-12
Optical fiber	VWR	631-1806
Plastic-coated white paper	Distrimed	700103
Signagel electrode gel	Free-Med	15-60
Sterile Petri dish- 35 mm	Dutscher	056714
Sterilizer, glass dry bead, Steri 250	Sigma	Z378569

2.3 Preparation of the pregnant female mouse

Alcohol pad	Alcomed	1731000
Buprecare	Axience	0.3 mg/ml
Compress	tRAFFIN	70189
Ketamine	Merial	Imalgene 1000
Ocular gel	tvm lab	Ocry-gel
RjOrl:SWISS mice	Janvier Labs	
Vetadine, 10% solution	Vetoquinol	4337400113B
Warming pad	Harvard Apparatus	72-0493
Xylazine	Bayer	Rompun 2%

3.2 Electroporation

Absorbable suture Size 4-0 45 cm Suture 1-Needle 19 mm Length 3/8 Circle Reverse	Novosyn	C0068220
Electroporateur Sonidel	Sonidel	NEPA 21
Sterile transfer pipets (individually wrapped)	Dutscher	043202S
Tweezers with 3 mm platinium disk electrodes	Sonidel	CUY650P3

4.1 Tissue harvesting and sectioning

24-well plate	Falcon	353047

Agarose	Lonza	50004
Antigenfix	Microm Microtech	U/P0014
Coverslip	Dutscher	100266
Dolethal	Vetoquinol	DOL202
DPBS (10X), no calcium, no magnesium	Fisher Scientific	11540486
Nail polish	EMS	72180
Slide	Dutscher	100001
Vectashield	Vectorlabs	H-1000
Vibratome	Leica	VT1000S

5. Multichannel confocal imaging

20X oil NA 0.85	Olympus	
Confocal Laser Scanning Microscope	Carl Zeiss	LSM880
Confocal Laser Scanning Microscope	Olympus	FV1000
Plan Apochromat 20x/0.8 M27	Carl Zeiss	

6. Astrocyte territorial volume segmentation

IMARIS 8.3 and later versions	Bitplane

7. Astrocyte arborization tracing

3D Visualization-Assisted Analysis software suite (Vaa3D)	HHMI - Janelia Research Campus /Allen Institute for Brain Science

Point-by-point response to the editorial comments

We thank JoVE editor for his/her editorial comments on our manuscript. All suggested corrections were taken into account and added accordingly to the revised version of the manuscript, where the main modifications appear highlighted in clear blue to ease editor's proofreading.

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.

We thoroughly proofread the manuscript and corrected any spelling and grammar issues.

2. Please define all abbreviations during the first-time use. e.g., MAGIC markers. Please do not use abbreviations in the title of the manuscript.

We replaced the « MAGIC » acronym of our method by its full-length meaning in the title.

3. Please provide an email address in the manuscript for each author.

We added below the list of authors the email address of each author on the first page of the revised version of the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. 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: Imaris, Bitplane, Fast Green dye (Sigma), Parker Laboratories, 15-60, INJECT+MATIC holder, Sonidel, CUY650P3, Sonidel, NEPA21, Antigenfix (Diapath), Sigma, VT1000S, Leica, Vectashield (Vector labs), IMARIS 8.3 (Bitplane), piggy Bac transposon, Germinator 500 glass bead sterilizer, SignaGel electrode gel, Parker Laboratories, Sonidel, CUY650P3, Sonidel, NEPA21, etc.

We carefully removed all commercial language from our manuscript and used generic terms instead when it was relevant.

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

The revised version of the manuscript is now formatted according to editor's recommendations.

6. 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.

We ensured that all text in the protocol section is written in the imperative tense and that any text that cannot be written in the imperative tense was added as a "Note".

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). Our protocol text has been revised accordingly.

8. Please ensure you answer the "how" question, i.e., how is the step performed? Each step is written according to this requirement.

9. 2.3: Please include the strain of the mouse used for this experiment. Also, age of embryo

used. Both mouse strain (RjOrl:SWISS) and embryonic stage (E15.5) are included in the revised version of our manuscript.

10. 3.1.5: How is this done? Further details have been added to this section to help the experimenter to perform this step.

11. 3.2.4: How is this done? Further details have been added to this section to help the experimenter to perform this step.

12. 3: please include post-surgery treatments. No post-surgery treatments are required. 12hr-lasting analgesia is induced during the surgery. If the surgery goes well, rapidly after waking up, the operated mouse moves, eats, drinks and quickly gets busy to build up its nest. If the operated pregnant mouse does not prepare its nest after waking up from anesthesia and displays additional signs of pain and/or excessive bleeding, it is euthanized right away as its health status will not be improved by any medication and will most likely result in abortion a few hours later.

13. 4: After how many days, tissue harvesting is performed? Tissue harvesting is performed at postnatal days P4, P7 and P21 as described now in step 4.1.1.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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. Following editor's and Reviewer#1's comments, we revised the protocol steps included in the filmable content in order to highlight the essential and original aspects that ensure a cohesive story of the Protocol.

15. 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]." No figures from a previous publication are used in our manuscript.

16. As we are a methods journal, please ensure that the Discussion 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
We have checked that our Discussion explicitly covers all the previously listed subjects, and we further detailed additional critical steps within our protocol in the revised version of the manuscript.

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 ».

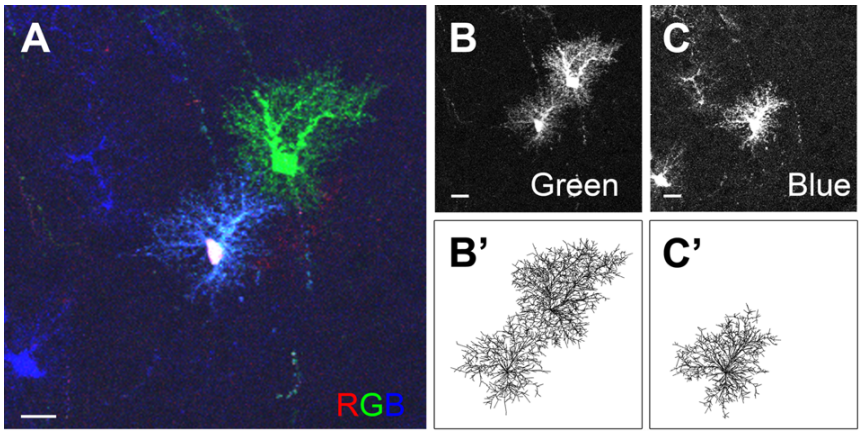

Figure R1. Arbor segmentation of neighboring cortical astrocytes. Example of two adjacent astrocytes both expressing EYFP but efficiently singled out using the expression of mTurquoise2. Maximal intensity projections of cropped z-stack showing adjacent green and cyan astrocytes at P7 (A, B, C) and their 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.

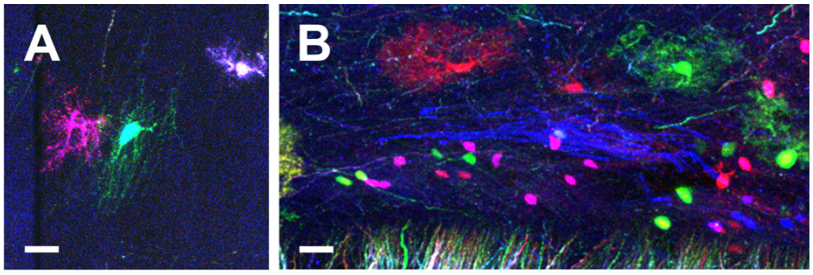

Figure R2. Examples of two isolated oligodendrocytes at P21 labeled with MAGIC Markers. Scale bar: 20 μm.

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).

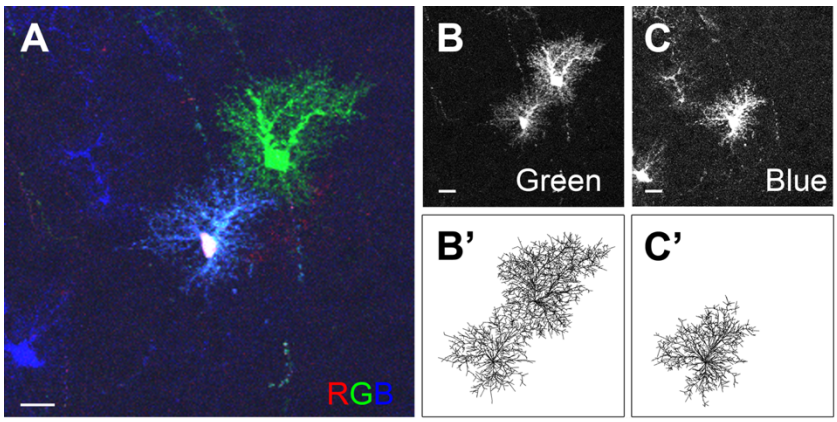

Figure R1. Arbor segmentation of neighboring cortical astrocytes. Example of two adjacent astrocytes both expressing EYFP but efficiently singled out using the expression of mTurquoise2. Maximal intensity projections of cropped z-stack showing adjacent green and cyan astrocytes at P7 (A, B, C) and their 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.