# Clonal Identity Determines Astrocyte Cortical Heterogeneity

Jorge García-Marqués and Laura López-Mascaraque

Department of Molecular, Cellular and Developmental Neurobiology, Instituto Cajal (CSIC), Madrid, Spain

Address correspondence to Laura López-Mascaraque, Instituto Cajal (CSIC), Av. Dr Arce, 37, 28002 Madrid, Spain.
Email: mascaraque@cajal.csic.es

**Astrocytes are the most numerous cell type in the brain, where they are known to play multiple important functions. While there is increasing evidence of their morphological, molecular, and functional heterogeneity, it is not clear whether their positional and morphological identities are specified during brain development. We address this problem with a novel strategy to analyze cell lineages through the combinatorial expression of fluorescent proteins. Following in utero electroporation, stochastic expression of these proteins produces inheritable marks that enable the long-term in vivo tracing of glial progenitor lineages. Analyses of clonal dispersion in the adult cortex revealed unanticipated and highly specific clonal distribution patterns. In addition to the existence of clonal arrangements in specific domains, we found that different classes of astrocytes emerge from different clones. This reinforces the view that lineage origin impinges on cell heterogeneity, unveiling a new level of astrocyte diversity likely associated with specific regional functions.**

**Keywords:** glia, gliogenesis, lineage, pial, progenitor

## Introduction

In the central nervous system, astrocytes represent a heterogeneous population of cells that fulfill a number of complex functions in the brain, related to both health and disease (Parpura and Haydon 2009; García-Marqués et al. 2010; Matyash and Kettenmann 2010; Zhang and Barres 2010). These cells originate from different types of progenitors (Cameron and Rakic 1991; Marshall et al. 2003; Hochstim et al. 2008; reviewed in Hewett 2009), although the relationship between the origin and the heterogeneity of mature astrocytes is still unclear. Indeed, it is currently difficult to gain a more precise understanding of astrocyte development due to the absence of suitable in vivo approaches to trace their lineage (Freeman 2010; Rowitch and Kriegstein 2010).

Astrocyte lineage relationships were first described by Cajal (1913) who showed the existence of the isogenic cell groups. More recent studies, partially described the clonal composition of the cortex (Luskin et al. 1988; Price and Thurlow 1988; Grove et al. 1993; Noctor et al. 2001; Zerlin et al. 2004), although there is a notable absence of reports in other regions. Furthermore, it is still unknown how astrocyte clones, composed by cells originated from the same progenitor, impact in both the astrocyte heterogeneity and the function. Lineage relationships between cells have been addressed using a variety of clonal assays mostly based on defective retroviral infection where just one progenitor acquires the fluorescent mark. Howeve, this approach has certain disadvantages related to splitting and lumping errors, retroviral silencing, as well as non-uniform targeting (Petit et al. 2005; Costa et al. 2009). More recent approaches like mosaic analysis with double markers system (Zong et al. 2005), Brainbow (Livet et al. 2007), Flybow (Hadjieconomou et al. 2011; Hampel et al. 2011) or lentiviral RGB (red, green and blue) marking (Weber et al. 2011) have taken advantage of the expression of several reporters to trace lineages in vivo. Nevertheless, these techniques rely on the combined expression of as many as four reporters and thus, they also remain prone to possible epigenetic silencing and splitting/lumping errors. To overcome these problems, we have developed a novel strategy, "Star Track", to analyze cell lineages through the combinatorial expression of 6 fluorescent proteins under the regulation of the GFAP promoter, both in the nucleus and in the cytoplasm. Stochastic expression of these 12 fluorescent proteins, after in utero electroporation, produced inheritable marks that permitted the long-term lineage of glial progenitors to be traced in vivo. An exhaustive analysis of the adult cortex revealed an unanticipated, specific, and intriguing clonal pattern. In addition to the presence of clonal arrangements of astroglial cells in specific domains, the existence of restricted pial and fibrous clones was particularly noteworthy. The positional identity of these astrocyte clones represents a new level of astrocyte diversity that may be important for their specific regional functions as an individual entity or as part of a functional unit.

## Materials and Methods

C57 mice were raised at the Cajal Institute animal facility, and they were handled in compliance with current Spanish legislation (R.D. 1201/2005 and L. 32/2007) and the European Union Council Guidelines (2003/65/CE) related to the care and use of experimental animals. The day of vaginal plug detection was designated as E0 and the day of birth was considered as P0. The embryos were anesthetized by hypothermia, whereas adult mice were anesthetized by intraperitoneal Equithesin injection (3 mL/kg body weight). After anesthesia, the embryos and postnatal/adult mice were transcardially perfused with 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB, pH 7.2). The brains were then postfixed in PF for 2 h at 4°C and coronal vibratome sections were obtained at 50–100 µm depending on the experiment.

### *Plasmid Vectors*

The promoter chosen to direct specifically the expression in astrocytes consist in a reduced version of the regulatory regions that control the in vivo expression of the human glial fibrillary acidic protein (hGFAP) gene. hGFAP promoter corresponds to a 2.2 kb fragment that spans base pairs −2163 to +47 relative to the transcriptional start site of the hGFAP gene, with the normal protein-initiating ATG codon converted to TTG codon by site-directed mutagenesis (Besnard et al. 1991). This 5′-flanking region (gfa2) of the hGFAP gene directs in vivo astrocyte-specific expression in mouse (Brenner et al. 1994).

Plasmids were generated using standard cloning methods. First, a small fragment of the hGFAP promoter was amplified polymerase chain reaction (PCR) from the LV-GFAP-EGFP (lentiviral vector encoding enhanced green fluorescent protein under GFAP promoter

regulation; kindly provided by Dr Lundberg) using primers with a 5′ XhoI site. The PCR product was subcloned into a pCRII-TOPO TA vector (Invitrogen) and then digested out with XhoI and XbaI. The remainder of this promoter was extracted by digesting with XbaI and BamHI. The hGFAP promoter was then reconstituted by inserting both fragments into a PB-UbC-EGFP vector (Piggybac transposon plasmid encoding EGFP under the Ubiquitin C human gene promoter regulation; kindly provided by Prof. Bradley) by triple directional ligation, having removed the UbC promoter with XhoI and BamHI. The resulting plasmid, PB-GFAP-EGFP was subsequently used to replace the EGFP between BamHI and EcoRI with mTSapphire, mCerulean, yellow fluorescent protein (YFP), monomeric Kusabira Orange (mKO), and mCherry. To achieve this, primers containing a 5′ BamHI and 3′ EcoRI site were used to amplify mTSapphire from pRSET-mtSapphire (kindly provided by Dr Griesbeck) by PCR, and the remaining products from CMV-Brainbow-1.1 M (Brainbow vector under the citomegalovirus promoter regulation; Addgene). The resulting plasmid was referred as PB-GFAP-XFP (to designate any of the fluorescent reporters). Finally, to generate the nuclear forms of these markers, human H2B histone (GenBank ID X00088.1) was amplified from genomic human DNA with PCR primers that included BamHI sites in their extremes. The product obtained was digested with BamHI and inserted in frame into the BamHI site of PB-GFAP-XFP, upstream of XFP. All plasmids were finally sequenced to ensure the accuracy of cloning.

### In Utero Electroporation

Pregnant mice were anesthetized with isofluorane (Isova vet, Centauro), and the uterine horns were exposed by a midline incision through the skin and the abdominal wall. The uterus was trans-illuminated with cold light and 2 μL of the plasmid mixture (2–5 μg/μL containing 0.1% fast green) was injected into the lateral ventricles (LV) of E14 embryos using a glass micropipette. After all the embryos were injected, each was placed between tweezer-type electrodes and 1 or 2 trains of 5 square pulses were applied (33 V; 50 ms followed by 950 ms intervals) using an electroporator. The uterine horns were then placed back into the abdominal cavity, filling this with warm physiological saline, and the abdominal muscle and skin were closed with silk sutures. After surgery, pregnant mice received a subcutaneous injection of the antibiotic enrofloxacine (5 mg/kg, Baytril, Bayer) and an intraperitoneal injection of the anti-inflammatory/analgesic meloxicam (300 μg/kg; Metacam, Boehringer Ingelheim). The injected embryos were allowed to survive until E18 (to test the method effectiveness), P0 (for time-lapse analysis), or P30 to adult (to analyze adult clonal arrangement).

### Tissue Staining

The sections (50 μm) were transferred to phosphate buffer saline (PBS) with 0.1% Triton X-100 (PBS-T) for 5 min, blocked in 2% bovine serum albumin (BSA; Sigma) for 30 min and incubated overnight in primary antibody solution containing 1% normal goat serum in 0.1% PBS-T and an anti-GFAP antibody (1:500; Dako) or biotinylated Tomato lectin (1:500; Sigma). In the first case, once rinsed, slices were incubated for 90 min with a biotinylated anti-rabbit IgG secondary antibody (1:200; Vector Laboratories). For both, after further rinsing, the sections were incubated with streptavidin conjugated Alexa 633 (Molecular Probes, 1:2000).

### Image Settings

The fluorescent sections were first studied under a fluorescent microscope (Nikon, Eclipse F1) equipped with filters (Semrock) that were optimized for the following fluorophores: mCerulean (FF01-405/10), GFP (FF01-473/10), YFP (FF01-520/15), mKO (ff01-540/15), and mCherry (FF01-590/20). Images were then obtained on a Leica TCS-SP5 confocal microscope, acquiring 2 or 3 different channels simultaneously. The excitation and absorption conditions for each fluorophore were (in nanometers): mT-Sapphire (Ex: 405; Ab: 525-553), mCerulean (Ex: 458; Ab: 464-481), EGFP (Ex: 488; Ab: 496-526), YFP (Ex: 514; Ab: 520-543), mKO (Ex: 514; Ab: 550-600), mCherry (Ex:

561; Ab: 601-612), and Alexa 633 (Ex: 633; Ab: 649-760). Each channel was assigned as the emission color, except for mT-Sapphire, which was assigned as dark blue. The series of images were projected maximally using the image software Image J v1.41 (NIH, USA). The projections for each channel were adjusted uniformly and overlaid using the Adobe Photoshop CS5 software.

### Time Lapse of Organotypic Slice Culture

Slices of the P0 mouse brain were cultured as described previously (Elias and Kriegstein 2007). Briefly, pups electroporated in E14 were anesthetized by hypothermia and decapitated, and their brain was dissected out in cold artificial cerebrospinal fluid (ACSF). After inclusion in low-melting point agarose (Pronadisa), vibratome slices (250 μm) were obtained and were placed in 6-well Transwell supports (Corning Incorporated) containing culture medium. Images were acquired with a ×10 objective at 40 min intervals over 48 h using an inverted microscope (Leica, DMI6000B), equipped with a CO₂ incubation system (Leica BL).

### Data Analysis

To estimate the frequency of the fluorescent marks, we analyzed clones defined as those cells sharing the same fluorescent mark and separated between them by <300 μm.

To analyze the clonal composition at the cortical pial surface, 24 sections (100 μm) from 6 adult electroporated mice were randomly selected. First, we selected those clones found to be <100 μm below the pial surface and to calculate the proportion of pial cells per clone, we then counted the number of cells showing a tangential lamellar morphology apposed to the "pia mater".

## Results

### Star Track: In Vivo Long-Term Tracing of Astrocyte Lineages

As a first step in addressing these questions, we developed a novel strategy to trace astroglial lineages in vivo, named Star Track (Fig. 1). The method is based on the combinatorial expression of different fluorescent proteins driven by the GFAP promoter (Fig. 1A) following an in utero electroporation. We reasoned that the expression of these proteins at diverse compartments would generate many different combinations in cells, each of which would constitute a specific mark that allows the lineage of each progenitor to be tracked (Fig. 1B,C). First, we generated 12 plasmids containing the open reading frame of 6 fluorescent proteins (XFP: Fig. 1A, Supplementary Table S1), whose expression was driven by the GFAP promoter in either the cytoplasm or the nucleus. Based on their brightness and photostability, we selected fluorescent proteins spanning most of the available spectral classes (Supplementary Table S1). The plasmid mixture also included the mT-Sapphire protein, which permits 2 different yellow-emitting proteins to be used due to the large Stokes shift (Supplementary Table S1). Nuclear expression was achieved by fusing human H2B histone to each fluorescent protein.

Astrocytes are generated following multiple cellular divisions of their precursors, which prevent them from being marked by regular electroporation. For this reason, we included the GFAP-XFP sequences between the terminal repeats of the PiggyBac transposon. After co-electroporation of the plasmid mixture with mPB plasmid encoding the PiggyBac transposase under the control of the ubiquitous CMV promoter, the fragment between those terminal repeats was inserted into TTAA sites in the genome (Cary et al. 1989).
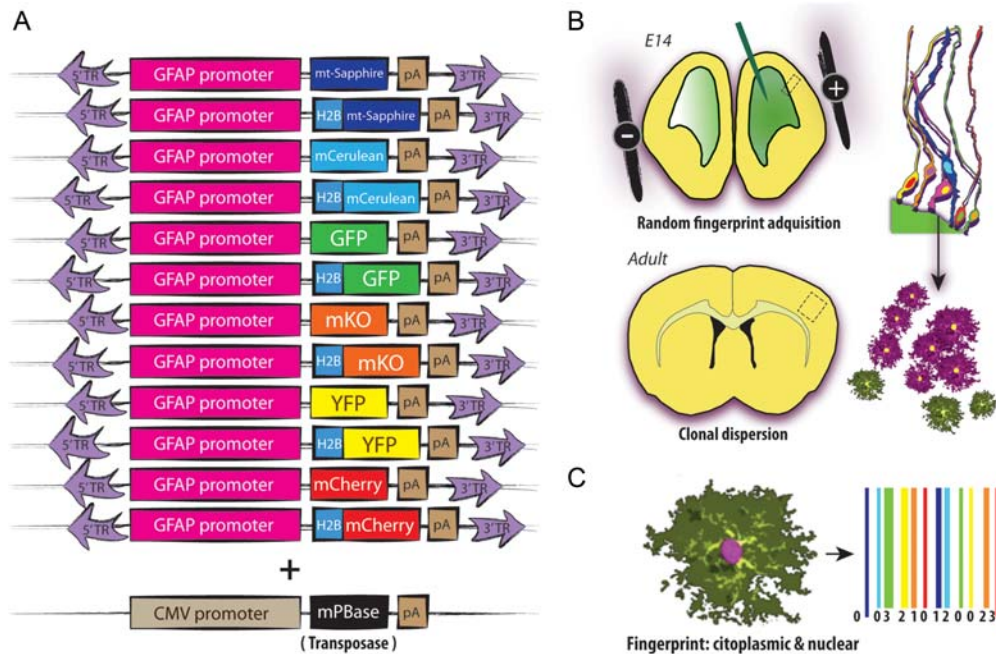
**Figure 1.** Star Track: a schematic view of the experimental procedure. (*A*) A mixture of 13 constructs comprised by twelve plasmids with the different fluorescent proteins and another one encoding the PiggyBac transposase. The twelve constructs encoded six different fluorescent proteins expressed in either the cytoplasm or the nucleus (by fusion to H2B histone). These sequences were placed in between the GFAP promoter and the bovine growth hormone poly-adenylation sequence. The construct corresponding to GFAP-XFP-pA was inserted between the terminal repeats of the PiggyBac transposon. The other plasmid encodes the PiggyBac transposase under the control of the ubiquitous CMV promoter. (*B*) After co-electroporation of the plasmid mixture at E14, transfected cells in which the GFAP promoter was activated expressed specific fluorescent proteins in both the nucleus and the cytoplasm. Progenitor cells of the ventricular wall acquire a specific combination of these proteins, inherited by the entire lineage that expands in clonal populations. (*C*) This combination constitutes a stable and inheritable fingerprint, easily traceable as the brain develops.

This generates an inheritable mark in the transfected progenitors (Fig. 1*B*), thus allowing the astrocytes they generate to be visualized (Fig. 1*C*). Genomic integration was indispensable as evidenced by an absence of fluorescent cells in electroporations without the mPB plasmid (data not shown).

### Fluorescent Fingerprints Acquired by Progenitor Cells are Inherited in Their Progeny

First, cells transfected in the LV at E14 were analyzed in the cortex at E18 (Fig. 2*A–H*) and P0 (Fig. 2*I–M*). The electroporation site was defined by an extensive cohort of cells labeled by a single color combination either in the cytoplasm, nucleus or both (Fig. 2*A,I,M*). Decomposition in separate channels from image in Figure 2*A* clearly revealed the specificity of each fluorescent mark in most cells (Fig. 2*B*). Interestingly, and despite the tremendous proliferation in this region, only some cells clonally related (same fluorescent mark) were clustered (Fig. 2*A,B,M*). When the first steps of cortical clonal dispersion were analyzed (Fig. 2*C–M*), there was less diversity of fluorescent marks after migration (Fig. 2*C,I*) than that at the electroporation site (Fig. 2*A,M*). At E18/P0, transfected cells distributed throughout the developing cortical thickness were classified into the following subtypes: 1) Pyramidal-like cortical cells mostly packed in layers 2/3 (Fig. 2*C,D,L*) displaying faint labeling due to persistence of the fluorescent protein once the GFAP promoter had been shut-off in the progenitors; 2) astrocyte clones confined to the pial surface (Fig. 2*C, E,F,J*); 3) radial glia transforming into astrocytes (Fig. 2*C,G*); and 4) clones of immature astrocytes/progenitors resembling protoplasmic astrocytes (Fig. 2*C,H,K*), characterized by thick and poorly branched processes. Subsequently, the temporal

sequence of clonal dispersion at P0 was followed in slice cultures (Supplementary Movie S1).

### Star Track Efficiency as a Method for Clonal Analysis

Accuracy of Star Track as a clonal analysis method directly resembles in the occurrence rate of each fluorescent mark. Therefore, we defined fluorescent marks exclusively attending to a qualitative criterion (presence/absence of each fluorophore) and estimated their occurrence frequency. We analyzed 132 clones (Supplementary Table S2) identifying a total of 82 different fluorescent marks. Among these, 54 marks appeared with a frequency of $7 \times 10^{-3}$ and only 1 of these marks appeared at the highest frequency value ($9 \times 10^{-2}$) as shown in Figure 3*A*. Moreover, as theoretically predicted, these frequencies were largely determined by the complexity of the mark, reflected by the number of expressed fluorophores (Fig. 3*B*). Together, these results demonstrate that a unique qualitative criterion is sufficient to define most of the clonal groups.

### Widespread Distribution of Clones Throughout the Cortex

An exhaustive analysis of the astrocyte dispersion revealed a widespread distribution of clones throughout the adult cortex (Fig. 4*A*). Electroporation of a plasmid mixture in the dorsolateral part of the LV wall produced a variety of cortical cell clones from E14 transfected progenitors that represented distinct adult astrocyte lineages, as illustrated in a representative coronal section (Fig. 4*A*). This pattern of cell dispersion followed the course of the radial glia processes (Marshall et al.
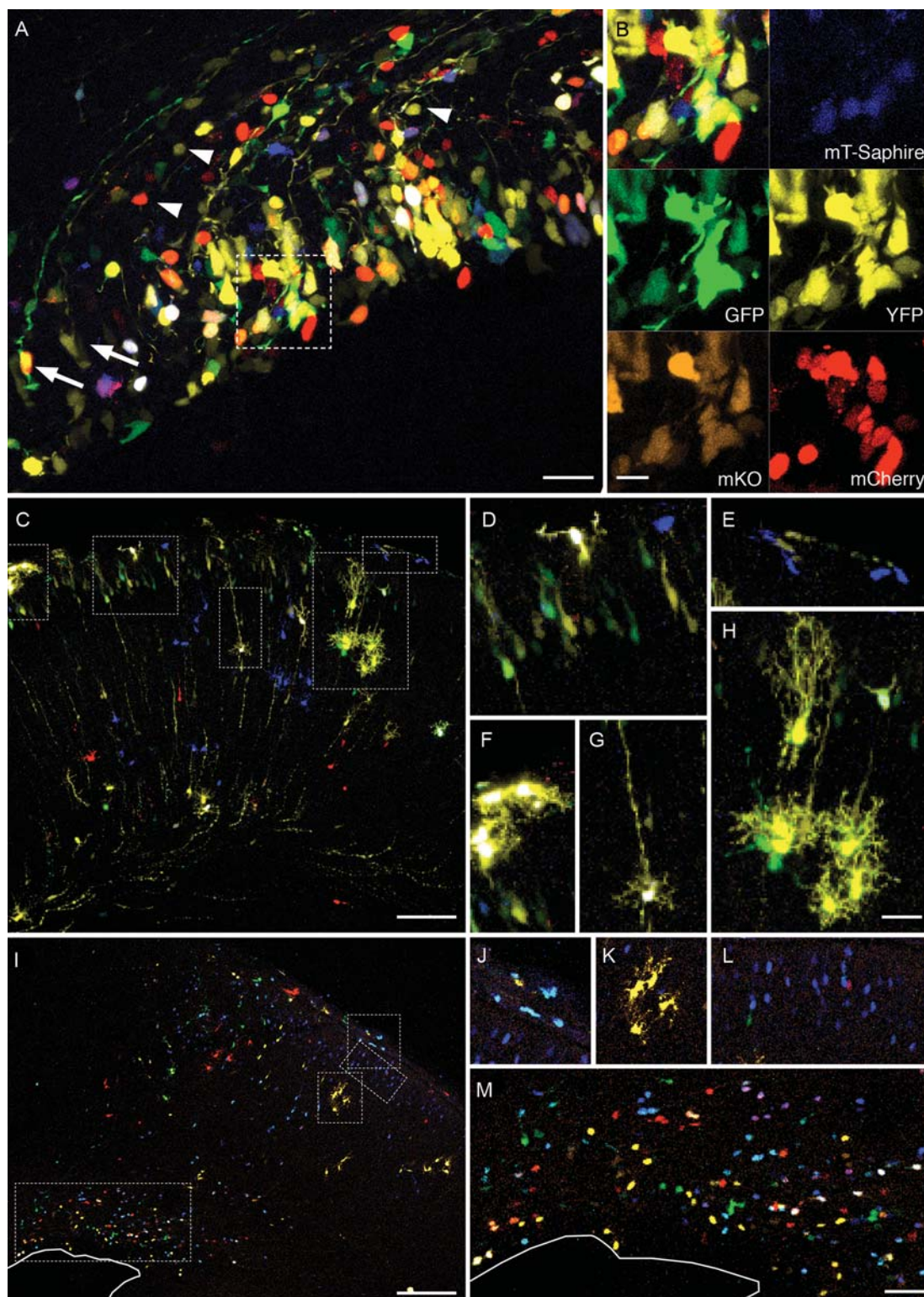
**Figure 2.** Fluorescent fingerprints acquired by progenitor cells are inherited by their progeny. E18 (*A–H*) and P0 (*I–M*) coronal sections following in utero electroporation at E14. (*A*) The large diversity of colors delineates the electroporation site, where each cell acquires a single color combination in the cytoplasm, nucleus, or both. (*B*) A higher magnification of the boxed area in *A* showing the merged and individual channels of small clonal groups containing cells that share the same fluorescent marks. Decomposition in 5 of 6 separate channels clearly revealed the specificity of each fluorescent mark in most cells. (*C*) Phenotype and pattern of dispersion throughout the developing cortical thickness of transfected cells. (*D–H*) Higher-power photomicrographs of the boxed areas in *C* showing the different cell types across the cortical mantle. (*D*) Pyramidal-like cells mostly packed in layers 2/3 displaying faint fluorescence. (*E* and *F*) Astrocyte clones confined to the pial brain surface. (*G*) Radial glia transforming into astrocytes. (*H*) Cell cluster with the same fluorescent mark formed by immature astrocytes/progenitors resembling protoplasmic astrocytes. (*I*) Phenotype and pattern of dispersion throughout the P0 cortical thickness of transfected cells at E14. (*J*) Cell clone limited to the pial cortical surface. (*K*) Clonal group formed by immature astrocytes/progenitors resembling protoplasmic astrocytes at P0. (*L*) Pyramidal-like cells mostly packed in layers 2/3 displaying faint fluorescence at P0. (*M*) Proliferative P0 region targeted by electroporation at E14. Scale bars in *A* = 25 μm; *B* = 10 μm; *C* = 100 μm; and *D–H* (shown in *H*) = 25 μm; *I* = 200 μm; *J–M* = 50 μm (shown in *M*).
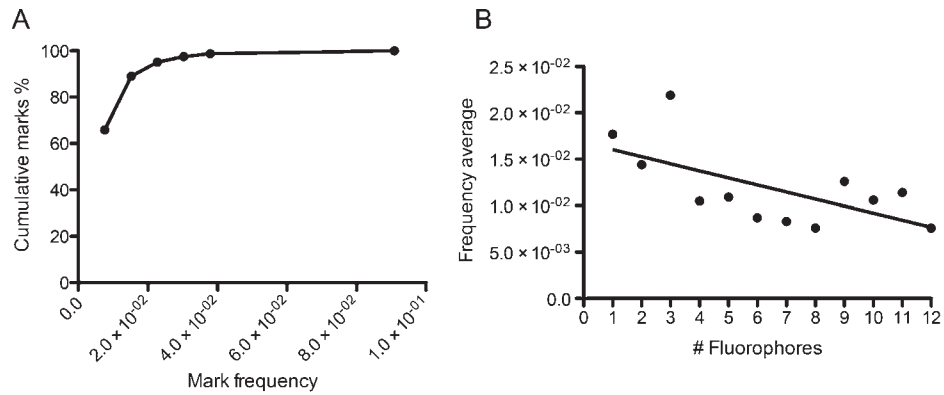
**Figure 3.** Star Track efficacy as a method for clonal analyses. (*A*) This graph corresponding to the cumulative percentage of marks in relation to their occurrence frequency. (*B*) The correlation between the number of fluorophores composing each mark and the average of occurrence frequency.
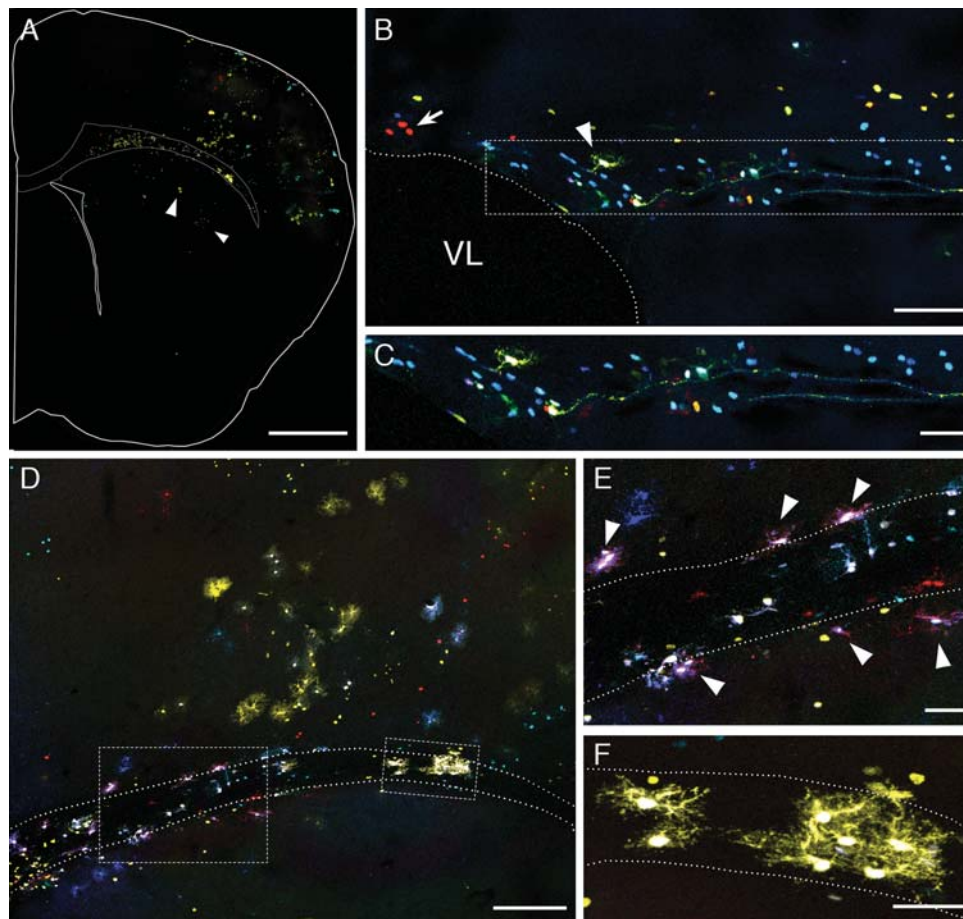


**Figure 4.** Clones widespread throughout the brain. Clonal dispersion in adult mice after in utero electroporation of the plasmid mixture into the dorsolateral LV wall at E14. (*A*) A low magnification of the coronal section illustrating cell dispersion. Note that some cells in the striatum form small clones (arrowheads). (*B*) Progenitor labeling in the ventricular wall. Small round cells cluster together (arrow), while there are scattered astrocytes with poorly refined processes (arrowhead). (*C*) A higher-power photomicrograph of the boxed area in *B* showing some cells attached to the wall of the ventricle with a large radial process extending along the subventricular zone and corpus callosum. (*D*) Clonal cell dispersion from the corpus callosum to the cortex. Groups of astrocytes parallel to the axon tracts. Channels splitting are showed in Supplementary Figure S1. (*E*) A higher-power photomicrograph of the boxed area in *D* showing fibrous astrocyte clones flanking the corpus callosum (arrowheads). (*F*) A higher-power photomicrograph of the boxed area in *D* showing fibrous astrocytes inside the corpus callosum. Scale bars in *A* = 1 mm; *B* = 100 μm; *C*, *E*, *F* = 50 μm; and *D* = 200 μm.

2003) spanning from the ventricle across the corpus callosum, to turn into the cortex perpendicular to the ventricular surface (Fig. 2*A*,*C*). In addition, a few cells invaded the striatum to establish small clones (arrowheads, Fig. 4*A*). At the ventricular wall, the labeled progenitors mostly remained as small round cells that occasionally clustered together (Fig. 4*B*,

arrow), and scattered astrocytes with poorly refined processes were also evident (Fig. 4*B*, arrowhead, and 4*C*). Some cells attached to the wall of the ventricle had a long radial process extending along the subventricular zone and corpus callosum (Fig. 4*B*, amplified in Fig. 4*C*), which resemble those described as neural stem cells (Merkle et al. 2004). This pattern of clonal cell dispersion was consistent with cell migration from the corpus callosum to the cortex and it perfectly matched the shifting of radial glia processes (Fig. 4*A*,*D*). In addition, the presence in the white matter of fibrous astrocytes, one of the two classic subtypes, led us to analyze the composition of clones in the corpus callosum (Fig. 4*D*–*F*). Clonally related cells were defined by the spatial location and color mark composition (Supplementary Fig. S1*A*). Cell clones composed of 5–20 astrocytes were evident in the corpus callosum arranged parallel to the axon trajectories (Fig. 4*D* and channels splitting in Supplementary Fig. S2), either flanking (Fig. 4*D*,*E*) or residing within the corpus callosum (Fig. 4*D*,*F*). These groups were exclusively formed by fibrous astrocytes, indicating that fibrous and protoplasmic types are generated from separate in vivo lineages. Throughout the rest of the cortex (Fig. 5*A*), cell progenitors fanned out towards the cortical surface forming specific colored astrocyte clones (Supplementary Fig. S1*B*). Cortical clones, with a typical protoplasmic morphology, contained the largest number of cells (up to 50; Fig. 5*A*,*B*,*D*,*E*). These groups were usually ellipsoid, and they formed extensive domains occupying several cortical layers (Fig. 5*A*,*B*). In addition, many clones of fewer cells (up to 10) were confined to the pia mater (Fig. 5*A*,*C*,*F* and channels splitting in Supplementary Fig. S3). These pial clones contained astrocytes that exhibited a fibroblast-like morphology and that were arranged as tangentially oriented sheets (Fig. 5*A*,*C*,*F*). Furthermore, radial clonal dispositions were constantly evident, resembling cellular columns frequently associated with a single blood vessel (Fig. 5*G*–*J*). It is intriguing how a unique clone surrounds a specific vessel in spite of the high density of vessels in this region as revealed by the Tomato lectin staining (Fig. 5*J*).

### Pial Astrocytes are Very Homogeneous Clones

Astrocytes accumulated densely at the cortical pial surface, where protoplasmic type coexisted with pial fibroblast-like astrocytes (Fig. 6*A*–*J*, channels splitting in Supplementary Figs S3 and S4). Given the prominence of clones exclusively confined to the pial surface (Fig. 6*A*,*B*,*D*–*H*, color mark segmentation and channel splitting of Fig. 6E in Supplementary Figs S1*C* and S5, respectively), we asked whether these represent a separate lineage, distinct from the classical subtypes of astrocytes. First, we reasoned that a common non-specific progenitor for pial and protoplasmic astrocytes would produce clones containing similar proportions of each. Thus, most of the clones adjacent to the pia mater would be predicted to contain both types of astrocytes. However, a detailed examination revealed that 87.3% of the clones contained only a specific type (Fig. 6*K*). Among these, 36.6% were exclusively protoplasmic (Fig. 6*I*–*J*) while the remaining 50.7% (Fig. 6*K*) contained only fibroblast-like astrocytes (Fig. 6*D*,*G*–*H*), that in spite of its atypical morphology expressed not just the GFAP promoter but also the protein (Fig. 6*L*–*M*). Summarizing, together these results indicate the existence of specialized

progenitor cells that produce a large heterogeneous astroglial cortical population.

### Discussion

Our data reveal that gliogenesis is an extremely specialized process that involves the emergence of clonal domains and heterogeneity among astrocyte cortical populations. In summary, this study provides novel insights into astrocyte generation, demonstrating the effectiveness of Star Track for in vivo cell lineage tracing.

### *Star Track Constitutes a Huge Step Forward for in Vivo Clonal Analysis*

To date, the tools available for cell lineage analysis have mainly relied on the use of either defective retroviral injection that targets a single progenitor, thereafter analyzing its progeny, or on recombination strategies based on the use of a few reporters, such as Flybow (Hadjieconomou et al. 2011; Hampel et al. 2011), RGB marking (Weber et al. 2011), or mosaic analysis with double markers system (Zong et al. 2005). However, there are many limitations associated with these methods such as errors in clone assignation (Petit et al. 2005; Costa et al. 2009; Weber et al. 2011). In spite of others approaches, like association of retrovirus to PCR-amplifiable sequence tags, attempted to overcome this limitation (Walsh and Cepko 1992), both time consumption and amplification reaction failures have limited its practice. Here, our Star Track overcomes this and many others limitations with the following advantages: 1) It prevents splitting and lumping errors, since cells are identified by a complex fingerprint and not just simply through a reporter; 2) it is less prone to epigenetic silencing because the cells retain a complex combination of fluorescent marks, even when 1 or 2 copies are silenced (such cells would be lost using other approaches); 3) it targets both dividing and resting cells, which allows the analysis of all progenitors and thus, it avoids the bias introduced when using retroviral labeling; 4) while retroviruses target just one of the daughter cells of a dividing progenitor, Star Track targets both daughter cells and it is therefore able to track the whole lineage. Moreover, qualitative criteria for Star Track analysis showed a cohort of different marks that mostly appear in lower frequencies compared with retroviral PCR-amplifiable tags (Walsh and Cepko 1992). This implies a higher efficacy of clonal resolution, which is enormously expanded including quantitative criteria, since many of marks with an exact fluorophore composition are different when applying quantitative criteria. On the other hand, compared with Cre/LoxP-based fate mapping technologies, which produces a permanent tracing in the whole lineage, Star Track allows direct lineage tracing of a specific cell population such as astrocytes. Together, Star Track reveals as a novel, easier, and reliable method that add new interesting chances within the *in vivo* clonal analysis toolbox.

### *Sibling Cells Maintain Spatial Relationships: Astrocyte Clonal Domains*

Analyzing the clonal dispersion of astrocytes in the cortex revealed several important issues. Protoplasmic astrocyte clones establish spatially restricted domains that contain up to 40 cells, occasionally arranged as a columnar clone
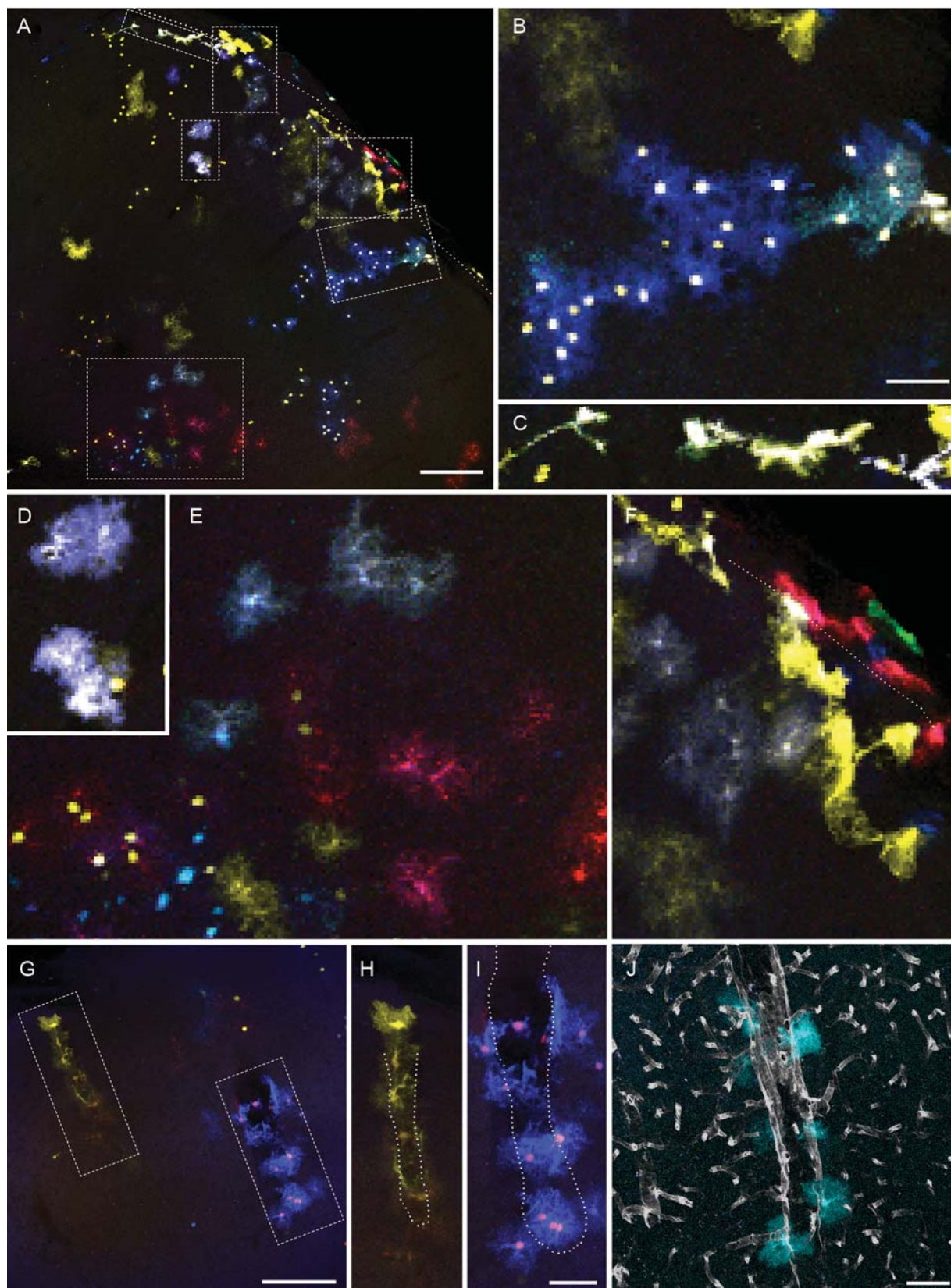
**Figure 5.** Cell progenitors fan out towards the cortical surface forming astrocyte clusters. Adult progenitor fate after co-electroporation of the plasmid mixture into the LV at E14. (A) Astrocyte clones clustered in a radial and tangential orientation, as reflected by the specific color labeling (channels splitting in Supplementary Fig. S3). Clones defined by rectangles are shown at a higher magnification in (B–F). In B, a radially orientated clone formed an extensive domain occupying several cortical layers. As depicted in C and F, tangential clones of few cells that exhibit a fibroblast-like morphology are confined to the pia mater. (G) The cortical area containing 2 different clones arranged along a single blood vessel. (H–I) A higher magnification of the boxed region in G. Dotted line demarcates the blood vessel. (J) Clone surrounding a blood vessel on a section stained by Tomato lectin, which reveals the high density of blood vessels in the cortex. Scale bars in A = 200 μm; B–F (shown in B), H–I (shown in I) = 50 μm; G = 150 μm; J = 50 μm.

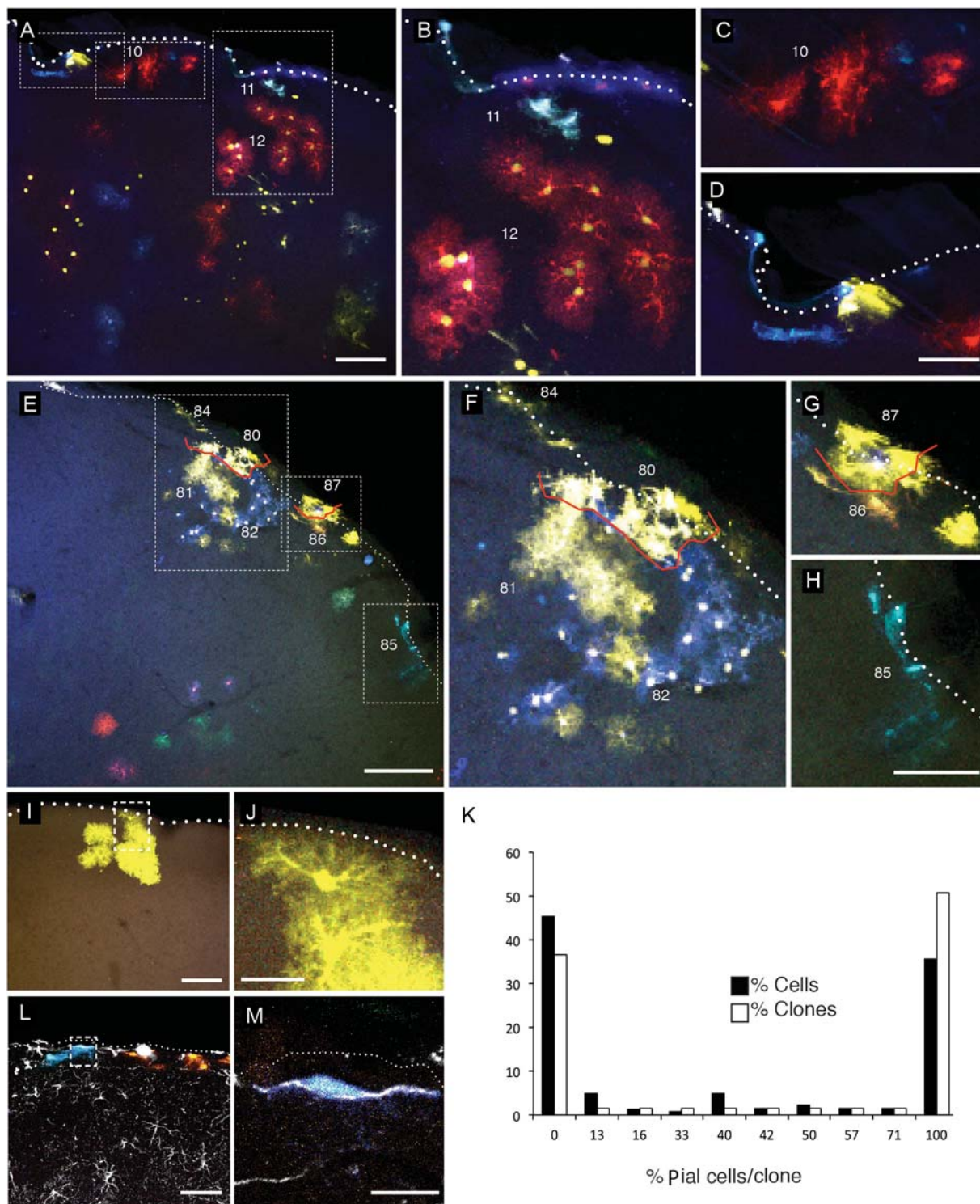**Figure 6.** Pial astrocytes represent highly homogeneous clones in the adult cortex. (*A–J*) Coronal sections showing the clonal disposition of astrocytes throughout the cortical extension. *A*, *E*, *I* show a high density of astrocytes at the pial surface. In the region adjacent to the pial surface, protoplasmic astrocytes cohabit with pial fibroblast-like astrocytes. The clones of these astrocytes are typically composed of only cell type, either protoplasmic or fibroblast-like astrocytes. Channels splitting of *A* and *E* is showed in Supplementary Figures S3 and S4, respectively. Clones defined by the rectangles in *A*, *E* and *I* are shown at a higher magnification in *B–D*, *F–H*, and *J*, respectively. Note the presence of protoplasmic astrocytes, even in contact with the pial surface, as in *I–J*. (*K*) A graph showing the percentage of clones (empty bars) or cells (filled bars) containing a particular proportion of pial astrocytes in clones <100 μm from the surface of the brain: 36.6% of clones were exclusively composed of protoplasmic astrocytes (0% pial cells/clone), while 50.7% corresponded to pial clones (100% pial cells/clone). Thus, 87.3% of clones were homogeneous, exclusively formed by only 1 type of astrocyte. Dotted line in *A–J* delineates the surface of the brain. (*L–M*) Labeled pial astrocytes also expressed GFAP. The red line distinguishes apparently similar but different clones. Scale bars in *A*, *I* = 100 μm; *B–D* (shown in *D*) = 50 μm; *E* = 200 μm; *F–H* (shown in *H*) = 100 μm; *J* = 25 μm; *L* = 50 μm; *M* = 10 μm.

spread along a single blood vessel. While the importance of these clonal domains remains unclear, it is plausible that astrocytes generated from the same progenitor share molecular mechanisms that distinguish them from other clones. In fact, the expression of factors such as Gli1 (Garcia et al. 2010), which resembles the clonal pattern described here, makes plausible the existence of molecular mechanisms that are clone-dependent. Increasing evidence for progenitor heterogeneity at the single-cell level might explain this interclonal heterogeneity, whereby sibling cells would retain their identity (Kawaguchi et al. 2008). This may have important functional consequences, as experienced by excitatory neurons that preferentially establish synapses between sisters cells (Yu et al. 2009). Clonal domains might also explain why neighboring astrocytes spontaneously exhibit synchronized calcium oscillations, forming coordinated cell groups (Sasaki et al. 2011). Furthermore, clonal domains might also influence other astrocytes activities, such as uptake from blood vessels (Fig. 5G–I), neural repair, or the modulation of synaptic transmission. Future studies will be critical to uncover how relevant these clonal domains are in terms of astrocyte function.

### Cell Lineage Determines the Heterogeneity of Astrocytes: A Novel Astrocyte Subtype in the Cortex

Classical descriptions from the late 19th century highlighted the existence of 2 different groups of astrocytes based on their location: the gray (protoplasmic) and white matter (fibrous) astrocytes (Andriezen 1893; Kölliker 1889). These first descriptions of astrocyte heterogeneity led to the traditional classification of astrocytes still used nowadays, and they were followed by many more modern reports, showing astrocytes to be an extremely diverse cell population. In this respect, we have witnessed the existence of pial astrocytes located at the pial surface that are characterized by a particular morphology similar to the lamellar described previously (Holen 2011). This suggests that pial astrocytes are functionally specialized to fulfill an active role in this protective sheet. In addition, the clones in this region appear to be mainly homogeneous, composed of either protoplasmic or pial astrocytes. In this respect, although Zerlin et al. (2004, Table 1) reported the restriction of certain astrocyte clones to the pial surface, these cells have been paid a little attention. We demonstrate that the pial and protoplasmic astrocytes are derived from partially separate lineage branches. Thus, as pial astrocytes have a different developmental origin, morphology, location, and probably a distinct function, they should definitively be classified as a new astrocyte subtype (Holen 2011). Like pial astrocytes, we also find clones exclusively composed of white matter (fibrous) astrocytes in the corpus callosum, similar to those described by Grove et al. (1993). The presence of these homogeneous clones clearly demonstrated that fibrous astrocytes derive from a lineage that can be partially distinguished from that of the pial and protoplasmic astrocytes as previously suggested by Miller and Raff (1984) and Luskin et al. (1988).

These results support the role of development, particularly highlighting clonal relationships, as important factors in the production of astrocyte heterogeneity in the adult brain. To date, 3 glia subtypes derived from spatially restricted domains have been identified in the chick embryo spinal cord, the

only clear evidence for a direct link between heterogeneity and development (Hochstim et al. 2008). However, since that analysis was carried out over embryonic stages, it might not be reconcilable with the presence of completely mature astrocytes, even though gliogenesis occurs earlier in chick than in rodents (Kálmán et al. 1998). Therefore, Star Track provides the first in vivo evidence bridging developmental diversification and astrocyte heterogeneity in the adult rodent brain.

### The Application of Star Track Beyond the Astrocyte Lineage

We have shown here that Star Track is a powerful novel method to study astrocyte diversity and function by analyzing their lineages in the rodent cortex. The differentiation of glia into specific lineages has novel implications for cell replacement therapies, and it creates additional opportunities to study the molecular mechanisms underlying lineage specification, early embryonic developmental pathways, and the pathological basis of genetic disorders.

However, due to its applicability in other cell types, Star Track will provide a new vision of cell lineages. Future versions will incorporate new fluorescent proteins that can be expressed in different subcellular compartments under the control of distinct promoters, enormously amplifying the potential of this method. Since there is no maximum number of plasmids that can be electroporated, this method constitutes a milestone for the complete "sequencing" of cell lineages in the brain.

### Supplementary Material

Supplementary material can be found at: http://www.cercor.oxford journals.org/.

### References

Andriezen WL. 1893. The neuroglia elements in the human brain. BMJ. 2:227–230.

Besnard F, Brenner M, Nakatani Y, Chao R, Purohit HJ, Freese E. 1991. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. J Biol Chem. 266:18877–18883.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A. 1994. GFAP promoter directs astrocyte-specific expression in transgenic mice. J Neurosci. 74:1030–l037.

Cajal SR. 1913. Contribución al conocimiento de la neuroglia del cerebro humano. Trab Lab Invest Biol. 11:255–315.

Cameron RS, Rakic P. 1991. Glial cell lineage in the cerebral cortex: a review and synthesis. Glia. 4:124–37.

Cary LCC, Goebel M, Corsaro BG, Wang HG, Rosen E, Fraser MJ. 1989. Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology. 172:156–169.

Costa MR, Bucholz O, Schroeder T, Götz M. 2009. Late origin of glia-restricted progenitors in the developing mouse cerebral cortex. Cereb Cortex. 19:135–143.

Elias L, Kriegstein A. 2007. Organotypic slice culture of E18 rat brains. J Vis Exp. 6:235.

Freeman MR. 2010. Specification and morphogenesis of astrocytes. Science. 330:774–778.

Garcia ADR, Petrova R, Eng L, Joyner AL. 2010. Sonic hedgehog regulates discrete populations of astrocytes in the adult mouse forebrain. J Neurosci. 30:13597–13608.

García-Marqués J, De Carlos JA, Greer CA, López-Mascaraque L. 2010. Different astroglia permissivity controls the migration of olfactory bulb interneuron precursors. Glia. 58:218–230.

Grove EA, Williams BP, Li DQ, Hajihosseini M, Friedrich A, Price J. 1993. Multiple restricted lineages in the embryonic rat cerebral cortex. Development. 117:553–561.

Hadjieconomou D, Rotkopf S, Alexandre C, Bell DM, Dickson BJ, Salecker I. 2011. Flybow: genetic multicolor cell labeling for neural circuit analysis in Drosophila melanogaster. Nat Methods. 8:260–266.

Hampel S, Chung P, McKellar CE, Hall D, Looger LL, Simpson JH. 2011. Drosophila Brainbow: a recombinase-based fluorescence labeling technique to subdivide neural expression patterns. Nat Methods. 8:253–259.

Hewett JA. 2009. Determinants of regional and local diversity within the astroglial lineage of the normal central nervous system. J Neurochem. 110:1717–1736.

Hochstim C, Deneen B, Lukaszewicz A, Zhou Q, Anderson DJ. 2008. Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. Cell. 133:510–522.

Holen T. 2011. The ultrastructure of lamellar stack astrocytes. Glia. 59:1075–1083.

Kálmán M, Székely D, Csillag A. 1998. Distribution of glial fibrillary acidic protein and vimentin-immunopositive elements in the developing chicken brain from hatch to adulthood. Anat Embryol. (Berl) 198:213–235.

Kawaguchi A, Ikawa T, Kasukawa T, Ueda HR, Kurimoto K, Saitou M, Matsuzaki F. 2008. Single-cell gene profiling defines differential progenitor subclasses in mammalian neurogenesis. Development. 135:3113–3124.

Kölliker A. 1889. Handbuch der Gewebelehre des Menschen. Leipzig: Wilhelm Engelmann.

Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW. 2007. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature. 450:56–62.

Luskin MB, Pearlman AL, Sanes JR. 1988. Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. Neuron. 1:635–647.

Marshall CA, Suzuki SO, Goldman JE. 2003. Gliogenic and neurogenic progenitors of the subventricular zone: who are they, where did they come from, and where are they going? Glia. 43:52–61.

Matyash V, Kettenmann H. 2010. Heterogeneity in astrocyte morphology and physiology. Brain Res Rev. 63:2–10.

Merkle FT, Tramontin AD, Garcia-Verdugo JM, Alvarez-Buylla A. 2004. Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci U S A. 101:17525–17532.

Miller R, Raff M. 1984. Fibrous and protoplasmic astrocytes are distinct classes of glial cells. J Neurosci. 4:585–592.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. 2001. Neurons derived from radial glial cells establish radial units in neocortex. Nature. 409:714–720.

Parpura V, Haydon PG. 2009. Astrocytes in (Patho)Physiology of the Nervous System. NY: Springer Science + Business Media.

Petit AC, Legué E, Nicolas JF. 2005. Methods in clonal analysis and applications. Reprod Nutr Dev. 45:321–339.

Price J, Thurlow L. 1988. Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. Development. 104:473–482.

Rowitch DH, Kriegstein AR. 2010. Developmental genetics of vertebrate glial–cell specification. Nature. 468:214–222.

Sasaki T, Kuga N, Namiki S, Matsuki N, Ikegaya Y. 2011. Locally synchronized astrocytes. Cereb Cortex. 21:1889–1900.

Walsh C, Cepko C. 1992. Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. Science. 255:434–440.

Weber K, Thomaschewski M, Warlich M, Volz T, Cornils K, Niebuhr B, Täger M, Lütgehetmann M, Pollok JM, Stocking C, Dandri M, Benten D, Fehse B. 2011. RGB marking facilitates multicolor clonal cell tracking. Nat Med. 17:504–508.

Yu Y-C, Bultje RS, Wang X, Shi SH. 2009. Specific synapses develop preferentially among sister excitatory neurons in the neocortex. Nature. 458:501–504.

Zerlin M, Milosevic A, Goldman JE. 2004. Glial progenitors of the neonatal subventricular zone differentiate asynchronously, leading to spatial dispersion of glial clones and to the persistence of immature glia in the adult mammalian CNS. Dev Biol. 270:200–213.

Zhang Y, Barres BA. 2010. Astrocyte heterogeneity: an underappreciated topic in neurobiology. Curr Opin Neurobiol. 20:588–594.

Zong H, Espinosa JS, Su HH, Muzumdar MD, Luo L. 2005. Mosaic analysis with double markers in mice. Cell. 121:479–492.